DISCOVERY IN CELL BIOLOGY

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FOR CELL BIOLOGY

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JOSEPH G. GALL, KEITH R. PORTER
AND PHILIP SIEKEVITZ
EDITORS

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Preface I

RICHARD McINTOSH
for the Editorial Board of
The Journal of Cell Biology

A few years ago, the Editorial Board of *The Journal of Cell Biology* recognized that the Journal's upcoming twenty-fifth anniversary was an occasion to be celebrated with appropriate retrospection. We also realized, however, that because the membership of the board is continuously evolving, we were not the people with the perspective to produce an interesting and accurate historical account of either the Journal or the field. A committee consisting of Richard McIntosh, Thomas Pollard, and David Sabatini was formed to define more clearly what should be done. We proposed, and the Editorial Board approved, that three senior scientists who had been important in the formation and early evolution of the Journal should serve as editors for a publication that would cover the recent 25 years of each of the major fields of cell biology in which the Journal had made a contribution. The hope was for a review in the sense that current knowledge would be placed in the context of recent developments. We believed such an approach would be useful both as an account of how our various fields developed and as a teaching tool: students, we thought, should be exposed more to the histories of their chosen specialties. Finally, we were confident that this account would be a major contribution because of the material it covered and because of the people who would do the writing and editing. We hope you will agree that this has been accomplished.

Preface II

PHILIP SIEKEVITZ
for the Editors of
Discovery in Cell Biology

1980 marked the twenty-fifth year of *The Journal of Cell Biology.* During this quarter-century, cell biology came of age. It is a recognized discipline: academic departments bear its name, students obtain degrees in it, more and more journals recognize its existence as a separate field of biological study. It was not always so, for the Journal was actually founded in 1955 as *The Journal of Biophysical and Biochemical Cytology.* Only later, in 1962, was its name changed to the one it bears today. In reality, this change made little difference, for the objectives of the original journal were to publish those papers in which the newer disciplines of biochemistry and biophysics would bear upon the much older discipline of cytology. The scope of the Journal, the merging of the new with the old, was exemplified by the first Editorial Board: Richard Bear, Stanley Bennett, Albert Lehninger, George Palade, Keith Porter, Francis O. Schmitt, Franz Schrader, Arnold Seligman. During the next 10 years such cytologists, biochemists, and biophysicists as Paul Doty, Daniel Mazia, Bernard Davis, Don Fawcett, Hugh Huxley, Hans Ris, Stanley Holt, Sanford Palay, Humberto Fernández-Morán, Erik Zeuthen, Rollin Hotchkiss, and Philip Siekevitz became editors.

During the past quarter-century, a veritable revolution took place in the biology of the cell. Twenty-five years ago, the cell as a structure was just being defined; the coincidence of structure with function was just being thought about; the isolation of subcellular structures was being elevated to a reproducible technique; the subcellular localization of proteins was first being performed; mitochondria were being fully recognized, intracellular membranes less so; and ribosomes were as yet virtually unknown. When one compares the knowledge then with knowledge now, it hardly seems possible that in so short a time we have come to view the cellular world as familiar terrain. We now know its geography and inhabitants relatively well; we know how it evolved, and even know something about its governance. Here we relate some history of the acquisition of our knowledge, for we are currently on a stepping-stone or plateau from which to view the past and survey the future. The future promises a challenge of even more difficult tasks: determining how all that we see and observe in the cell is organized and how the function-structure relationships are regulated. At the moment, however, the editors feel that a history is important, for besides showing the continuity of the endeavor, it also shows the pitfalls and the accomplishments, the mistakes and the correct guesses, all the bases upon which to build the experiments of the future. Above all, we think that the histories contained in these chapters indicate how truly communal is the endeavor we call science; how so many cooperated to produce what we today call cell biology.

It is fitting that at this point we honor *The Journal of Cell Biology,* for, from the very first issue, it has been in the forefront of delivering the fruits of this new discipline to the other biological sciences. We expect that it will go on for additional units of 25 years and that at each anniversary another milestone will have been reached in our knowledge of the cell.

DISCOVERY IN CELL BIOLOGY

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continued

Introduction: Recollections on the Beginnings of *The Journal of Cell Biology*

KEITH R. PORTER and H. STANLEY BENNETT

"In fame of learning, the flight will be slow without some feathers of ostentation."
Francis Bacon, *ESSAYS*, "Of Vain-glory," 1625

If, by cell biology, one means an integrative and interdisciplinary approach, utilizing techniques and concepts of anatomy, physiology, biochemistry, biophysics, genetics, zoology, botany, virology, and microbiology to seek comprehensions of the nature of living cells, one finds that neither the approach nor the term are new, both dating back more than a century. Integrative approaches are clearly embodied in Henle's *Allgemeine Anatomie* of 1841, in Kölliker's *Handbuch der Gewebelehre des Menschen* (1852), in Kühne's *Untersuchungen über das Protoplasma und die Contractilität*, published in 1864, and in Carnoy's *La Biologie Cellulaire, Étude Comparée de la Cellule dans les Deux Règnes*, of 1884. In Carnoy's introduction, he informs us that the first laboratory of cell biology was established at the Catholic University of Louvain, Belgium, in 1876, and that "Depuis trois ans, près de deux cents étudiants, Belges et étrangers, se pressent autour de nous, avides de science et ardents au travail." With such an honorable and diligent history, it is surprising that the term "cell biology" was still available for the journal title in 1962.

Some of the conversations which led ultimately to this Journal took place in the summer of 1951, when Porter, then at The Rockefeller Institute for Medical Research, went to Seattle to collaborate with Bennett in electron microscopy of thin sections of muscle. The endoplasmic reticulum had been recognized in culture cells and noted in sectioned material sufficiently to support the suspicion that it was a system generally present in all eukaryotic cells. The time seemed ripe to seek the endoplasmic reticulum of striated muscle cells. The anatomy department in Seattle had a brand-new, well-functioning RCA EMU-2 electron microscope, a recent gift from The Rockefeller Foundation. George E. Palade had found that buffered solutions of osmium tetroxide preserved very adequately the morphology of cells in tissues. S. B. Newman, E. Borysko, and M. Swerdlow had shown that tissues embedded in polymerized acrylic resins could be sectioned, and Daniel Pease and Richard Baker had demonstrated that sections thin enough for electron microscopy could be cut with a manually operated, simply modified microtome designed originally for light-microscope sections. For what they say about cell biology of these early years, selected events of that summer are recalled as follows:

We prepared and examined many sections of chicken heart muscle. We even, in a whimsical mood, took a piece of cooked ham and prepared sections from it for electron microscopy, but without happy issue. We found internal membranes in the chicken muscle, thought of them as representatives of the endoplasmic reticulum of striated muscle fibers, and considered designating this system of membranes the "sarcoplasmic reticulum." In choosing micrographs for our illustrations, we pridefully thought them to be of superior quality, although by today's standards the sections were thick, the preservation imperfect, and the resolution modest. When it came time to select a journal for this masterpiece of electron microscopy, we lamented the shortcomings of the halftones in the journals of the day and wished for something better. We decided to try a Wistar journal, perhaps swayed by Bennett's distant kinship with Isaac Wistar. In any event, our manuscript went to the *American Journal of Anatomy*, accompanied by an appeal for very special attention to the quality of the halftone engravings.

The preparation of our manuscript occupied more time than we had anticipated. We were familiar with the entire, though meager, literature dealing with electron microscopy of muscle when we started the study. However, this proved to be insufficient, for we soon discovered an extensive series of papers based on light-microscope observations, extending back over half a century, dealing with the sarcoplasmic reticulum and mitochondria of muscle as seen by Kölliker, Retzius, Cajal, Veratti, and others. We learned that we were not the first to discover the sarcoplasmic reticulum, nor were we the first to use the term. Retzius, in 1881, had even suggested that the sarcoplasmic reticulum was admirably disposed to conduct an excitatory impulse from the surface membrane of a muscle

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fiber to the myofibrils in the interior. Surprisingly, intelligent speculation is not a 20th-century invention. Our paper did establish that the sarcoplasmic reticulum is membranous, though, at that time, we did not recognize the distinction between the sarcoplasmic reticulum and the transverse "T tubules," nor did Retzius, Veratti, or others before us.

When the muscle paper appeared in the *American Journal of Anatomy* (93:61, 1953), we were disappointed in the quality of the halftones representing our electron micrographs, and talk of other outlets of publication was revived.

Soon after Porter's return to New York in the early autumn of 1951, Don W. Fawcett joined his laboratory at The Rockefeller Institute and undertook with Porter an electron-microscope study of the structure of cilia. The study prospered and described the "9+2" arrangement of pairs of tubule-like structures seen in cross sections of cilia and also of the filamentous nature of ciliary rootlets and the associated cytoplasmic matrix. These were observations that foreshadowed recognition of the "cytoskeleton," which now figures prominently in the cell biology literature. In spite of such important ingredients, the manuscript was rejected by the editors of *The Journal of Experimental Medicine* in the fall of 1953 on the grounds that the study involved little that was experimental, that it was not medical, and, moreover, that the editors did not wish to publish morphological papers. Yet this paper reported the discovery of the ingredients of a system for motility not based on actin and myosin, and provided essential background for an understanding of Kartegener's syndrome. Normal things have to be discovered and described before their pathology can be recognized.

Vincent Dole, a member of the Board of Editors of *The Journal of Experimental Medicine,* agreed to convey the bad news to Porter. Returning to his laboratory from his visit with Dole, Porter encountered Herbert Gasser, then Director of The Rockefeller Institute. Gasser stopped him with remarks to the effect that "I hear the J.E.M. is not willing to accept your paper with Fawcett." After commenting that such an action was ridiculous, Gasser queried, "Porter, why don't you start a new journal?" adding that The Institute could undertake to cover the costs. Since Porter and Fawcett could not wait until a new journal was started, they submitted their paper to a Wistar journal. It appeared in the *Journal of Morphology* (94:221, 1954), with halftones that were as disappointing in quality as had been those published a year earlier in Bennett's and Porter's paper in the *American Journal of Anatomy.*

This experience with the J.E.M., amplified by similar encounters Palade had had, made us realize that we were faced with two kinds of publication problems: one related to the quality of reproduction of electron micrographs; the other related to the editorial policies of existing journals, whose boards of editors could not always recognize the significance of new discoveries or of concepts appearing in manuscripts parading before their eyes. Unwittingly, then, or possibly wittingly, the editors of the J.E.M., played a prominent role in starting the new journal.

The incident relating to Fawcett's and Porter's article came near the end of Gasser's term as Director. Detlev Bronk was appointed his successor. Additional conversations on the journal problem took place in late 1953 and early 1954, especially between Palade and Porter. The Rockefeller conspirators realized that the problem should be presented to Bronk in a persuasive manner. Therefore, upon Porter's suggestion, Bennett, in early 1954, wrote a letter to Bronk that presented forcefully the need for a journal with editorial policies and technical capabilities appropriate for the developing field of cell biology, and asked if The Rockefeller Institute would consider adding a journal of this character to its distinguished list, then headed by *The Journal of Experimental Medicine.* We do not know if Bronk received other letters of this character, but whatever the case, his reply was encouraging. As a consequence, he assembled a group of interested persons to meet with him at lunch in Atlantic City during the April, 1954, meetings of the Federation of American Societies for Experimental Biology. In addition to Dr. Bronk and the authors of these "Recollections," the following persons attended the lunch and participated in the discussions: Francis O. Schmitt and Richard S. Bear of the Biology Department, Massachusetts Institute of Technology; Albert L. Lehninger of the Biochemistry Department, The Johns Hopkins University School of Medicine; and George E. Palade of The Rockefeller Institute for Medical Research. From the beginning of the conversations around the lunch table, Bronk seemed to have concluded that a new journal was needed and should be sponsored by The Rockefeller Institute. Most of the ensuing conversation related then to the name of the proposed journal and the types of manuscripts it should attract. All agreed that it should be interdisciplinary. Bennett proposed that it be called *Journal of Cytology.* This was quickly and enthusiastically rejected as too old-fashioned and restrictive to reflect the desired interdisciplinary nature of the new journal. No one proposed the name *The Journal of Cell Biology.* At times, disagreements about the title seemed sharp. Realizing that we had not agreed on a title, Bronk threateningly said that if that issue stood in the way, it might be that a new journal was not needed, and left the table. Thereafter the tone of the meeting became more harmonious, and after further conversation, the qualifying adjectives "biophysical" and "biochemical" were suggested, to specify and to dignify the general topic, "cytology," and to convey to all that the context of cytology, as used by the new journal, was not restricted to studies of chromosomes. By the end of the lunch, all agreed that *The Journal of Biophysical and Biochemical Cytology* was the most appropriate name that the assembly could think of. It was then recommended that most of those gathered around the luncheon table be appointed by Dr. Bronk to the founding Board of Editors of the new journal and a few persons not present were suggested as additional members.

The first issue of *The Journal of Biophysical and Biochemical Cytology* appeared less than a year after the memorable luncheon. It was dated January 25, 1955, and carried, along with other papers, the first full description of ribosomes (Palade, "A Small Particulate Component of the Cytoplasm," pp. 59–68); the first full paper distinguishing clearly between "rough" and "smooth" endoplasmic reticulum (S. Palay and Palade, "The Fine Structure of Neurons," pp. 69–88), and the first full description of synaptic vesicles and the inter-membranous synaptic spacing (E. D. P. DeRobertis and Bennett, "Some Features of the Submicroscopic Morphology of Synapses in Frog and Earthworm," pp. 47–58). Thus, the new journal got off to a good start, with Porter functioning as the first Managing Editor.

These recollections would not be complete without nostalgic comment on the spirit of goodwill, friendship, and cooperation which dominated the personal relations between those closely associated with developments of the field as presented in the Journal. Besides the authors of these "Recollections," this congenial and collegial group included Palade, Fawcett, M. J.

Moses, W. Bernhard, De Robertis, M. H. Burgos, Hugh Huxley, E. Yamada, K. Hama, Palay, Pease, and others. A new world was opening for exploration; a new information gusher had been uncorked. Excitement of discovery and community of purpose brought us together. The friendships and mutual respect engendered in those exciting days have endured and have fortified the field of cell biology.

This same spirit of cooperation was immediately expressed in the organization and participation of an international group in the first Conference on Tissue Fine Structure.* It was held in January, 1956, at Arden House in Harriman, New York, and was attended by about 100 enthusiasts, the great majority of whom presented papers. These were assembled and published in 1956 as a supplement to Volume 2 of the Journal. It was the kind of volume to attract attention and to record forever the beginnings of modern cell biology. The journal profited from immediate recognition and a pronounced increase in subscribers.

The growth of interest in and use of the electron microscope, supplemented by the applications of cell fractionation, was rapid, and by 1962 the number of manuscripts submitted to *The Journal of Biophysical and Biochemical Cytology* greatly exceeded the number it could accept and publish. The result was an increasing interest in the publication of additional journals with purposes similar to those of the JBBC. It seemed probable that one of these would preempt what was obviously a most appropriate name and one that Carnoy evidently coined. Hence the editors acted quickly and changed the name to *The Journal of Cell Biology,* under which title it will doubtless survive and prosper for many decades.

* The conference was organized under the auspices of the Morphology and Genetics Study Section, Division of Research Grants, NIH, Ernest M. Allen, Chief.

I. Information Storage and Retrieval

Chromosome Structure and the C-Value Paradox

JOSEPH G. GALL

From the time of their discovery in the middle of the nineteenth century, chromosomes have held a particular fascination for cell biologists. The past 25 years—the period covered by this volume—has been no exception. During this time a host of new approaches to problems of chromosome and gene organization has been introduced, beginning in the early 1950s with spectrophotometric analysis of nuclear staining and the application of autoradiography to questions of DNA and RNA synthesis. Many biochemical approaches were worked out, based either on subcellular fractionation or micromethods, and specific molecular probes, such as *in situ* nucleic acid hybridization and fluorescent antibodies, made it possible to study the sites of specific macromolecules. Most recently, the extraordinarily powerful combination of molecular cloning, restriction enzyme analysis, and nucleic acid sequencing has opened up an entirely new field of cytogenetic analysis.

Despite the enormous advances made during this period in understanding chromosome structure, there remains one disconcerting feature often called the "C-value paradox." The paradox is the fact that organisms at the same general level of morphological complexity, which presumably have the same genetic requirements, nevertheless often have genomes whose DNA contents differ by orders of magnitude. Fortunately, many questions about chromosomes can be approached without considering this problem. This is true, for instance, of nucleosome structure. Just now, when recombinant DNA methods make it so easy to study sequence organization of individual genes, the C-value paradox is receiving more or less benign neglect. But at some point or another the paradox intrudes upon almost all questions of chromosome organization, particularly when comparisons are made between organisms with widely varying DNA contents. For instance, the fact that a complex higher eukaryote such as *Drosophila* has a minute genome makes it impossible to argue that larger amounts of DNA are essential to carry out sequence-specific roles. In an organism with 100 times as much DNA as *Drosophila,* such as a salamander or a bean plant, most of the DNA could be unessential for coding or regulatory functions.

This review will focus on a few selected aspects of chromosome organization, in particular the question of uninemy, the nature of heterochromatin, chromatin compaction, and sequence organization of repetitive genes. An attempt is made to put findings from the past 25 years into context, but in no sense have I tried to review everything new about chromosomes in this period. The C-value paradox will provide a partial link in the story. Several times during this period it seemed that the paradox could be explained away, but each time the explanation was invalidated by new information. The unexpectedly large and variable amount of DNA in eukaryotic genomes remains a major complicating feature in understanding chromosome organization.

DNA Constancy

The staining characteristics of chromosomes, especially their coloration by a variety of synthetic dyes as well as by such natural products as hematoxylin and carmine, originally inspired the words "chromosome" and "chromatin." Flemming (1) defined chromatin as "the substance in the cell nucleus which takes up the color during nuclear staining," and in a remarkably accurate conjecture he suggested that chromatin might be the same as the recently discovered "nuclein" of Miescher (2). Even after the existence of DNA in certain nuclei, chiefly those of sperm and thymus, became well accepted, the lack of a specific stain hampered progress at the chromosome level. Fortunately such a stain was found by Feulgen and Rossenbeck in 1924 (3), who modified the familiar Schiff test for aldehydes into a simple and reliable histochemical procedure for DNA. The Feulgen reaction was quickly adopted by chromosome cytologists, who were able for the first time to verify the existence of DNA in both plant and animal chromosomes and to show that cytochemically demonstrable DNA was, with few exceptions, absent from the cytoplasm. Another quarter century passed, however, before Pollister and Ris (4) demonstrated that the amount of Feulgen stain could be used to estimate the DNA content of a nucleus. Their study and many others that soon followed it, helped convert nuclear cytology from an observational and descriptive subject to an experimental science with a developing theoretical framework.

Several generalizations about nuclear DNA emerged from the quantitative Feulgen studies (5). In particular, it was recognized that each species of animal and plant could be characterized by the amount of DNA in its nuclei. In most cases measurements were made on nondividing diploid nuclei, but in some instances the DNA contents of haploid gametes, either sperm or microspore nuclei, were measured and found to be half the diploid amount. From a large number of such measurements the idea of DNA constancy was established: the nonreplicating haploid chromosome complement of a species is characterized by a constant amount of DNA, called the C-value for that species. Feulgen dye measurements confirmed the fact, recently discovered by the new technique of autora-

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diography (6), that DNA replication occurred during interphase of the mitotic cycle.

A striking feature of the quantitative measurements was the extreme variation in C-value for different organisms, ranging from a low of 0.18 pg in *Drosophila melanogaster* through intermediate values of 3–4 pg in various mammals including man, to highs of 50–100 pg in salamanders and some monocot plants. Even within groups of closely related organisms variations were seen, a factor of two between species in the same genus being common (7, 8). The wide range of C-values at first did not trouble chromosome cytologists. They were accustomed to the fact that some organisms had large chromosomes associated with correspondingly large nuclei and cells, whereas others had only small ones, and it was not surprising that those size differences were reflected in DNA contents. More importantly, the contemporary model of chromosome organization provided an explanation for the size differences. According to this model, chromosomes were multistranded cables consisting of two, four, eight, or more identical subunits (9). It was not difficult to suppose that related organisms with different C-values simply had different numbers of subunits in their chromosomes. Support for this concept was provided by the fact that related organisms often had similar or identical karyotypes despite large differences in absolute chromosome size or DNA content (10).

This comfortable picture was called into question by experiments that suggested that chromosomes were, in a sense, much simpler: they consisted of a single gigantic DNA molecule. This so-called unineme model was slow to take hold, and many attempts were made to reconcile the new data with a multistranded chromosome. By the mid-1960s, however, it was clear that uninemy was here to stay and that the C-values posed a number of unresolved problems. If organisms with widely different C-values did not differ in the number of identical strands per chromosome, did they contain different numbers of genes? Why was there no clear correlation between morphological complexity and C-value? Why were even the lowest C-values so large? For instance, *Drosophila* with the lowest C-value outside the fungi had enough DNA to code for well over 100,000 proteins, and mammalian genomes were nearly 20 times larger. Before considering these questions in more detail, let us look briefly at the evidence for uninemy.

Uninemy

Earlier arguments about chromosome strandedness were sometimes confused by failure to define the problem explicitly. With the clear view of hindsight to guide us, the question is easy to state. How many DNA molecules are there in one chromatid? Historically the first convincing evidence came from the ingenious experiments of Taylor and his colleagues, who followed the distribution of tritium-labeled thymidine though successive chromosome replications (11). They showed that both chromatids of a chromosome were equally labeled at the first mitosis after administration of the isotope, but only one of the two chromatids was labeled at the second mitosis (or more precisely, because of sister chromatid exchanges, only one chromatid was labeled at a given point along the length of the chromosome). This distribution of label was called semiconservative to distinguish it from conservative (one labeled and one unlabeled chromatid at the first division) or dispersive (all chromatids labeled at all divisions). Taylor's demonstration of semi-conservative distribution of label during chromosome replication was published at about the same time as Meselson and Stahl's similar experiment showing the semiconservative distribution of density label during DNA replication in *Escherichia coli* (12). Both experiments implied that the unit under consideration—the chromatid or the DNA molecule—consisted of two subunits, that separated but remained intact during replication. In a second but less well-known set of experiments, Taylor demonstrated that the two subunits of the chromatid differed in such a way that rejoining after breakage was restricted (13). Because the two strands of the DNA double helix differed in polarity (5′ → 3′) the simplest interpretation was that the two subunits of a chromatid corresponded to those strands. By incorporating bromodeoxyuridine into chromosomes and staining with Giemsa (14), it is now possible to reproduce Taylor's results without the need for autoradiography (Fig. 1). The staining procedure is particularly valuable for studying multiple sister chromatid exchanges.

Evidence of a quite different sort came from observations on lampbrush chromosomes of Amphibian oocytes. Morphological analysis had shown that the lateral loops of these chromosomes occurred in pairs corresponding to the two sister chromatids (15, 16). Although the bulk of each loop consisted of a matrix of ribonucleoprotein, DNase digestion experiments carried out by Callan and Macgregor (17) established that the continuity of the loops was maintained by DNA. Extending these observations Gall (18) demonstrated that loop digestion followed two-hit kinetics, which suggested that a loop and hence a chromatid had one DNA molecule as its structural axis (Fig. 2). In the same experiments the interchromomeric fiber, which presumably corresponded to a pair of chromatids or two DNA molecules, followed four-hit kinetics. Shortly afterward Miller (19, 20) published his extraordinary electron micrographs of lampbrush loops showing that the bulk of a loop consisted of long fibrils extending laterally from a very delicate axis. Because these fibrils showed a gradient of lengths and because it was known from cytochemical studies that loops were actively synthesizing RNA, the simplest interpretation

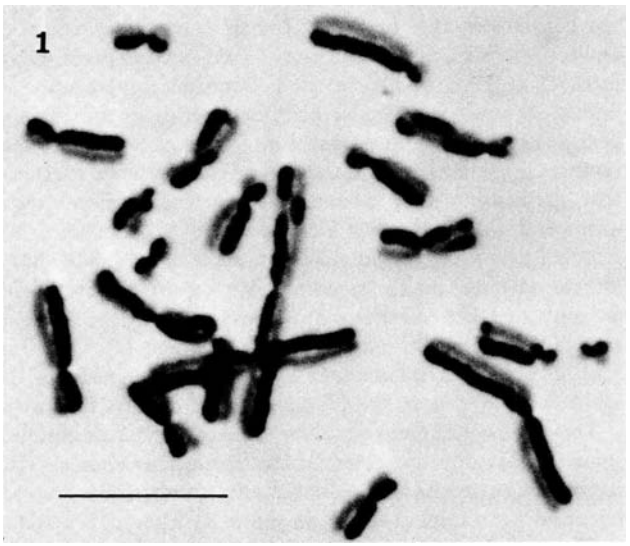

FIGURE 1 Semiconservative replication and sister-chromatid exchanges visualized in Chinese hamster chromosomes by the BrdU-Giemsa technique. From Wolff and Perry (14). Using [^{3}H]thymidine Taylor et al. (11) were the first to demonstrate semiconservative replication and provide experimental evidence in favor of a unineme model of the chromatid. Bar, 10 μm.

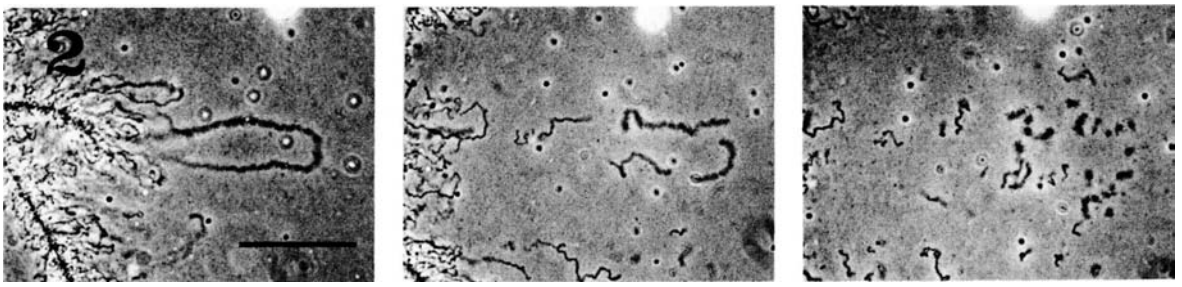

FIGURE 2 Successive stages in the digestion of a newt lampbrush chromosome loop (=chromatid) by pancreatic DNase I. Analysis of the kinetics of digestion indicates that the DNA axis of the loop consists of a single Watson-Crick double helix (18). Bar, 50 μm.

was that a loop consisted of numerous RNA transcripts still attached to the DNA segment that served as their template (see Fig. 7 in article by O.L. Miller, this volume).

The observations on lampbrush chromosomes as well as Taylor's experiments strongly suggested that a chromatid was not laterally redundant with the respect to its DNA molecules. Neither set of data proved conclusively that a single uninterrupted DNA molecule ran from one end of the chromatid to the other. In order to prove this, one needed to isolate DNA molecules long enough to contain all the DNA of a single chromatid. This has now been done for the yeast *Saccharomyces* and for several species of *Drosophila.* The key to such experiments was the development by Zimm and co-workers (21) of a suitably sensitive method for determining molecular weights in the range of 10^8–10^{11} daltons. In their procedure one measures the rate at which experimentally stretched DNA molecules resume a random coil configuration (viscoelastic recoil). Kavenoff and Zimm (22) showed that molecules as large as 4×10^{10} daltons could be isolated from *D. melanogaster* tissue culture cells. Estimates based on the relative sizes of the chromosomes and the C-value determined by Feulgen photometry showed that the total DNA of the two longest chromosomes (numbers 2 and 3) could be contained in such molecules. Viscoelastic measurements on two other *Drosophila* species, *D. virilis* and *D. americana* were likewise consistent with their karyotypes and DNA contents. More recently viscoelastic measurements have been carried out on the DNA of *Saccharomyces cerevisiae* with the same conclusion (23). In the case of yeast the C-value is so low (about 10^{10} daltons) and the chromosome number so high ($n = 17$) that the average chromosome contains only one-fourth as much DNA as *E. coli.* By contrast chromosomes 2 and 3 of *D. melanogaster* each contain a DNA molecule 2 cm in length, some 20 times the length of the *E. coli* chromosome. A determined proponent of multistranded chromosomes could argue that the minute chromosomes of yeast and *Drosophila* are just the ones expected to be unineme. Viscoelastic measurements on organisms with higher C-values pose severe technical problems, and for the moment such direct evidence for uninemy is not available for mammals, amphibians, and monocot plants.

A final argument for uninemy derives from studies on reassociation of DNA. As shown originally by Britten and Kohne (24) a large fraction of the DNA in the genome of higher eukaryotes reassociates with single-copy kinetics. That is, it reassociates at the rate predicted from the known C-value, and the assumption that sequences are present only once per genome. This finding is a strong argument against chromosome models that postulate that each chromatid consists of multiple identical subunits.

It should be noted that the good correlation that exists between C-value and genome complexity, as determined by DNA reassociation kinetics, also effectively rules out any other model of chromosome structure that postulates that all or a majority of sequences are present in multiple copies. Callan (25) proposed a model in which each "master" gene of an organism was accompanied by a number of "slave" genes in tandem array. Variations in C-value between organisms were explained as variations in the number of slave copies. The master-slave model, in addition to other ingenious features, offered a way to reconcile unineme chromosome structure with variable DNA contents, and for a while seemed to offer a solution to the C-value paradox. In its simplest form, however, it is incompatible with the fact that the largest fraction of DNA in most organisms is not present in multiple copies.

Of the five tests of uninemy discussed here—distribution of label during replication, DNase kinetics, electron microscopy of transcription, viscoelastic measurements, and reassociation kinetics—one or more have been applied to a large number of different animals and plants. No single organism has been looked at by all five methods, but eukaryotes spanning the entire range of C-values from yeast on up have been examined. If high C-values were due to multistranded chromosomes, then the salamanders and monocots should have provided the evidence.

Chromosome Organization: Euchromatin and Heterochromatin

Early in this century, cytologists recognized that certain chromosomes remained condensed during interphase and prophase when other chromosomes were either indistinguishable as such or were exceedingly long and thin. Such heterochromosomes, as they were called, proved in many cases to be sex chromosomes. In 1929 Heitz (26) showed that differential condensation was not limited to sex chromosomes, but often characterized part of an otherwise normal chromosome. He suggested the terms heterochromatin and euchromatin to describe the unusually condensed and the more typical segments, respectively. He showed that regions next to centromeres, at the ends of chromosomes, and adjacent to the nucleolus tended to be heterochromatic, and numerous subsequent studies have confirmed his observations in many plants and animals. Over the years a bewildering array of characteristics has been ascribed to heterochromatin. Chief among these is genetic "inactivity." In some cases, as in *Drosophila,* this means absence of detectable mutants in most of the Y and in the cytologically heterochromatic regions near the centromeres of the other chromosomes (27). In other cases, most clearly demonstrated by the mammalian X chromosome (28) and the paternal set of chromosomes in mealy bugs (29), inactivity means suppression

of function in an otherwise normal chromosome or set of chromosomes. Other characteristics include late replication during the S-period (30), differential replication (31, 32), absence of meiotic recombination (27), effects on euchromatic regions brought into proximity with heterochromatin (33), and even elimination of heterochromatin from certain cells (34, 35). Some order was brought into the discussion of heterochromatin by Brown and Nur (29), who recognized two broad categories that they called *facultative* and *constitutive* heterochromatin. They defined these as heterochromatin present in only one homologue or in both homologues. This rather unusual definition distinguished heterochromatin as a state of an otherwise normal chromosome (facultative) from heterochromatin as a permanent condition (constitutive).

The distinction between facultative and constitutive heterochromatin took on added significance with the demonstration by *in situ* nucleic acid hybridization that mouse satellite DNA was located in the constitutive heterochromatin adjacent to the centromeres in all the chromosomes except the Y (36) (Fig. 3). Mouse satellite DNA had been discovered as a minor component in CsCl buoyant density gradients. Extensive physical and chemical studies (24, 37, 38) showed it to consist of a 240-base-pair sequence serially repeated about one million times in the mouse genome. Because the satellite constituted about 8–10% of the DNA and the centromeric heterochromatin a similar fraction of the total chromosome length, it seemed probable that the constitutive heterochromatin of the mouse consisted largely if not exclusively of satellite DNA. Shortly thereafter the satellite DNAs of *D. melanogaster* and *D. virilis* were also shown to correspond with constitutive heterochromatin. In *D. virilis* over 40% of the genome consists of three related simple sequence satellites, correlated with very prominent heterochromatic regions in each of the mitotic chromosomes (39). For many years it had been known that the heterochromatic regions were not replicated proportionately during the formation of the giant polytene chromosomes (31). The satellites were not demonstrable by CsCl gradient centrifugation in DNA extracted from salivary glands of larvae, and *in situ* hybridization showed that their absolute amount in the giant polytene nuclei was not detectably different from that in diploid nuclei (32). It was clear, therefore, that the unusual replicative behavior of heterochromatin was correlated in this case with an unusual type of DNA. Simple sequence DNAs from a large number of organisms including man have now been localized by *in situ* hybridization to constitutive heterochromatin. So strong is the correlation that one can be fairly sure that an organism with cytologically prominent constitutive heterochromatin will have simple sequence DNA readily detectable by buoyant density analysis or reassociation kinetics.

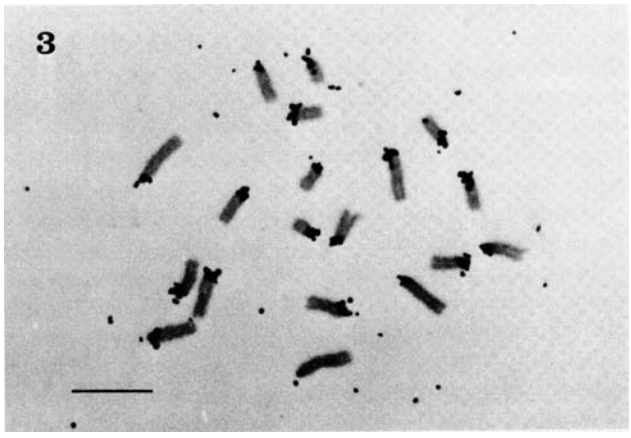

FIGURE 3 Mouse chromosomes hybridized *in situ* with [^{3}H]RNA complementary to mouse satellite DNA. Hybridization is limited to the constitutive heterochromatin adjacent to the terminal centromeres. From Pardue and Gall (36). Bar, 10 μm.

In the *in situ* hybridization experiments on mouse chromosomes Pardue and Gall (36) noted that constitutive heterochromatin was differentially stained by the Giemsa stain. By simple omission of the hybridization step from the *in situ* procedure the C-banding technique was born. Although not specific in a chemical sense, C-banding permits a useful rapid screening for regions of constitutive heterochromatin.

The simple chemical structure of the DNA in constitutive heterochromatin provides an adquate explanation for the lack of structural genes and mutations in these regions. Why this type of DNA should be so prominently associated with centromere and telomere regions is not at all clear. An answer to the unusual distribution of simple sequence DNA will probably not come before the overall significance of these sequences is discovered. Several hypotheses have enjoyed a certain amount of popularity. For example, it has been suggested that simple sequences are involved in chromosome pairing or crossing over at meiosis (40), that they provide a reservoir of sequences to be converted by mutation into more typical DNA (24), or that simply by their bulk they provide a mechanism for increasing nuclear and cell size (41). Another view stresses that these sequences have no essential cellular function, but do have special replicative properties that give them a selective advantage (42, 43).

In some cases simple sequence DNA accounts for much or all of the difference in C-value between related species. For instance the genomes of *D. melanogaster* and *D. virilis* contain 0.18 and 0.36 pg, respectively. In *D. virilis*, as already mentioned, the simple sequence satellites constitute more than 40% of the DNA compared with about 18% for *D. melanogaster*. The euchromatic portions of the genome, or more specifically the single copy DNA of the two species, are not strikingly different in amount. Such comparisons are of limited validity when dealing with major C-value differences, because in general the fraction of single copy DNA does *not* correlate with C-value. That is, the proportion of single copy to repetitive DNA varies greatly in organisms with both low and high C-values. One cannot postulate a "basic" single copy genome for eukaryotes, which is simply augmented by repetitive sequences in organisms with high C-value.

Whole chromosomes or parts of chromosomes that change from the normal mitotic cycle of condensation-decondensation to a more or less permanently condensed state are said to be facultatively heterochromatic. In the best studied cases such as the mammalian X chromosome (28) and the paternal set of chromosomes in mealy bugs (29), the switch in morphological state is correlated with suppression of gene activity. The situation in mammals was first suggested by the discovery of a condensed mass of chromatin, the Barr body, in cells of females (44). Later the Barr body was shown to correspond with only one of the two X chromosomes, the other X behaving like the autosomes (45). Genetic studies being conducted at the same time suggested that X-linked genes in the female did not follow the usual dominant-recessive rules, but instead both alleles were expressed in different patches of tissue. This was particularly well shown by coat color genes, but it was also demon-

strated for biochemical markers. The genetic and cytological features taken together indicated that inactivation of one X chromosome occurred early in development in each somatic cell of a normal diploid female, so that the adult soma is a mosaic of clones, each clone expressing the genes of only one X chromosome. The situation in mealy bugs is similar in principle, but in this case a whole set of chromosomes is inactivated in male somatic cells. This is usually the paternal set so that males express only genes inherited from their mother.

The mechanism by which functionally active euchromatin is converted to condensed, inactive heterochromatin is completely obscure. It has been suggested several times that methylation of DNA might be a primary event in inactivation (46, 47). Despite the attractive nature of such an hypothesis, including analogy to the restiction-modification systems of bacteria, the available evidence is scanty. Now that restriction enzymes are available whose specificity depends on the state of methylation of nucleotides at the recognition site, it is possible to examine methylation of particular genes and to test the methylation hypothesis critically (48, 49). Whatever the mechanisms may be by which regions become heterochromatic, those mechanisms may shed light on the process of gene activation during development. Nearly all models of embryonic development and cell differentiation rely on the concept of differential gene activation and inactivation. It is possible to imagine that activation or inactivation of individual genes or blocks of genes during development might proceed by mechanisms similar to those involved in facultative heterochromatinization.

Nucleosomes and Chromosome Fine Structure

The DNA molecule contained in a chromatid is several thousand times longer than the chromosome seen at metaphase of mitosis. For instance, the X chromosome of *D. melanogaster* is about 1.8 μm long at metaphase, but contains 1 cm of DNA. How this compaction is achieved, and what happens when the chromatid partially unwinds during interphase or in the formation of giant polytene and lampbrush chromosomes are structural problems yet to be resolved in detail. The first order of compaction, that which converts the extended DNA molecule into a beaded string of nucleosomes, is now well understood from a structural standpoint.

Early attempts to examine chromosome structure by electron microscopy were notably unsuccessful. Thin sections, which revealed exquisite detail in the organization of mitochondria, the endoplasmic reticulum, flagella, and many other cytoplasmic structures, showed only an indistinct fibrillar and granular arrangement of the nuclear contents. Just as light microscopical studies proceeded very slowly until squash methods were introduced, so electron microscopy of chromosomes had to await methods for unraveling chromosomes for whole mount observations. It was no more possible to deduce the structure of an interphase nucleus by sectioning it that it would be to do the same with a ball of string. Even so, the first attempts to spread chromatin using surface tension forces at an air-water interface were not overly informative (50–52). Such studies did establish that nuclei and chromosomes of many organisms consisted of irregular fibers some 200–300 Å in diameter, but little internal

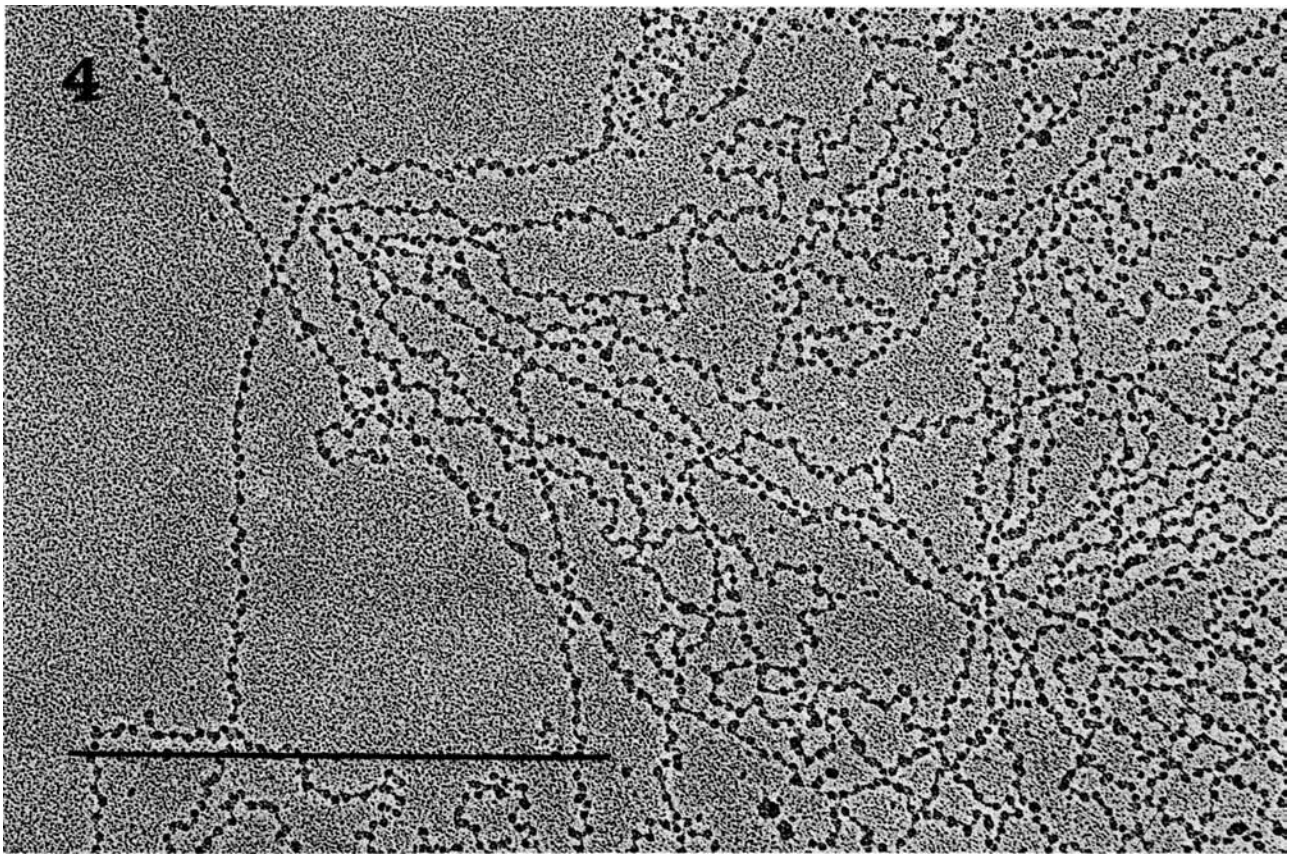

FIGURE 4 Electron micrograph of chromatin spread under low ionic conditions on a hydrophilic substrate ("Miller spread"). Nucleosomes are the most prominent feature of such transcriptionally inactive regions. From McKnight and Miller (67). Bar, 1 μm.

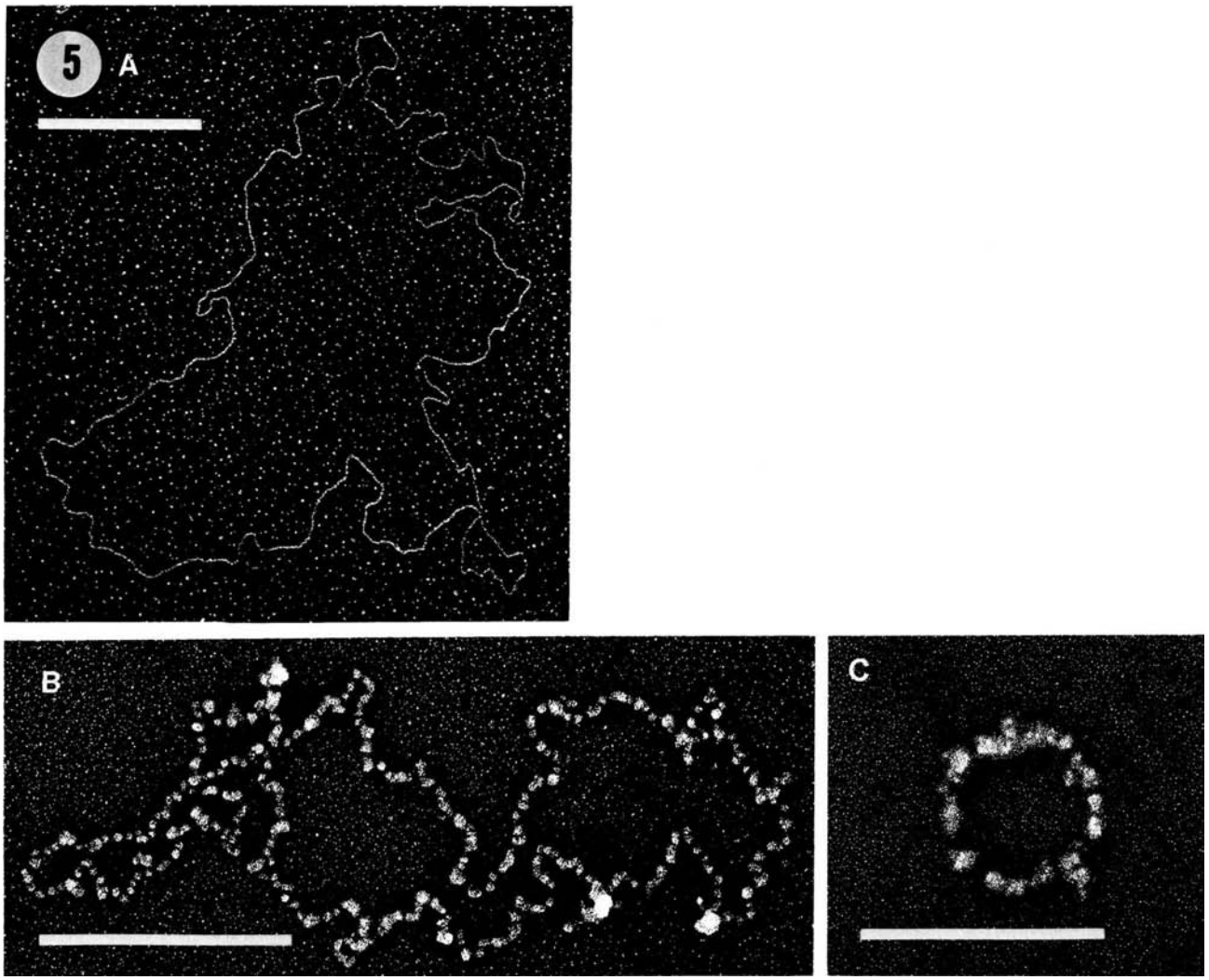

FIGURE 5 Three stages in packaging of DNA, illustrated by extrachromosomal rDNA molecules from the oocyte of the water beetle, *Dytiscus*. (*A*) free DNA molecule; (*B*) DNA and histones in a beaded nucleosome condition, probably lacking histone H1; (*C*) supercoiled state or "200 Å fiber." In each case the length of DNA is the same. From Scheer and Zentgraf (135). Bar, 1 μm (*A*); 0.5 μm, (*B* and *C*).

detail was evident. The situation changed dramatically when Miller (19, 20) introduced the simple expedient of centrifuging chromosomes and chromatin preparations from hypotonic solutions onto hydrophilic substrates (Figs. 4 and 5). Under these conditions the delicate chromatin fibrils were beautifully displayed in an extended condition, and it became possible to examine regions of transcriptional activity, because the nascent ribonucleoprotein molecules remained attached. Olins and Olins (53) first called attention to the regularly beaded structure of chromatin prepared in this fashion. They designated the beads *ν*-bodies and suggested that they constituted a new structural feature characteristic of chromatin from many different sources. The *ν*-bodies, as described by Olins and Olins, were about 70 Å in diameter and were connected by a thinner fiber of irregular length. Combined biochemical and electron microscopical studies by Chambon and his co-workers (54) on adenovirus-2 chromatin demonstrated that each bead was associated with about 200 base-pairs of DNA. Chambon called the beads nucleosomes, the name now generally used.

At about the same time biochemical experiments also suggested a repeating structure for chromatin. Hewish and Burgoyne (55) noticed that DNA isolated from rat liver nuclei, which had been allowed to self-digest, was cut into a series of fragments having lengths of about 200, 400, 600, etc. nucleotides. The effect was traced to an endogenous nuclease activated by Ca^{++} or Mg^{++}. Exactly comparable digestion of isolated chromatin was obtained with micrococcal nuclease so long as the chromatin was isolated with minimal shearing (56). The nuclease digestion studies demonstrated that chromatin, as opposed to free DNA, was organized in some manner that made the DNA preferentially susceptible to enzymatic attack at regularly repeated intervals.

The key to the enzymatic susceptibility clearly had to lie in the association of DNA with histone. Kornberg (57) proposed a model of nucleosome structure in which 200 base-pairs of DNA were wrapped around a histone octamer consisting of two each of the most highly conserved histones, H2A, H2B, H3, and H4. Kornberg's model made use of the long known fact that DNA and histone occur in approximately equal amounts by weight and that the four conserved histones are present in equimolar amounts. It was also based on his own studies, which showed strong binding in solution between the pairs H2A-H2B and H3-H4 (58). Subsequent studies have done much to clarify the specific arrangement of DNA and histone

in nucleosomes, but they have left the basic model intact (59–61). Particularly important has been the realization that the histone octamer is closely associated with about 140 base pairs of DNA to form a "core" nucleosome, the remaining DNA being less tightly associated with the octamer and indeed varying in length from one organism to the next and apparently even between tissues of the same organism. Histone H1 is associated with this variable linker region between successive nucleosomes.

The behavior of nucleosomes during DNA replication has been studied by Weintraub and co-workers making use of density labeling of the proteins (62). They have shown that "old" histone octamers remain intact during replication and that "new" octamers consist entirely of proteins synthesized during the time of replication. The exact distribution of old and new octamers has not yet been determined, although it is known that successive octamers on a replicated chromatid tend to be all old or all new over short distances. Permanent changes between two daughter chromatids could arise if the new octamers associated with one of the strands differed from the old in some respect. In this connection it is of considerable interest that the histones of early and late sea urchin embryos are coded for by different structural genes (63).

Transcriptionally active genes differ from bulk chromatin with respect to their nuclease sensitivity, as originally found for the globin gene in erythropoietic tissue. Weintraub and Groudine (64) showed that the β-globin gene in a transcriptionally active tissue was more susceptible to nuclease digestion than bulk chromatin, whereas it was not so in a transcriptionally inactive tissue such as liver. Similar findings have been reported for the chick ovalbumin gene (65) and several other highly active genes. Because these studies involve digestion of total chromatin followed by hybridization with specific probes, they are difficult to relate to the behavior of individual nucleosomes during transcription. Electron microscopic studies of active genes (66, 67) often show widely spaced transcripts between which the chromatin appears to have a normal nucleosome structure, an exception being the ribosomal RNA genes that always have closely spaced transcripts. Nevertheless, detailed analysis of specific genes using a combination of DNase 1 digestion and blot hybridization (68, 69) suggests that the whole region of active transcription is altered in a highly specific manner and that sites of preferential cutting are exposed.

The coiling of DNA around the nucleosome core results in a six- to sevenfold reduction in length relative to fully extended DNA (200 base-pairs represent 680 Å of DNA, whereas nucleosomes are approximately 100 Å in diam.). Clearly, therefore, there must be higher orders of coiling or folding to account for the known dimensions of chromosomes. Because a fiber of approximately 200–300 Å diameter occurs as the next most complex feature seen by electron microscopy (Fig. 5), several hypotheses have been suggested to account for its structure. These models are concerned with the way in which arrays of nucleosomes may be packed into helical supercoils (70, 71) or superbeads (72). Until detailed X-ray data become available it will be hard to choose among these models.

Even the 200- to 300-Å fiber is considerably longer than a metaphase chromatid. Electron micrographs of chromosomes spread at a water-air interface clearly show the 200- to 300-Å fibers projecting as short loops all over the chromosome surface; they can also be seen in sections of isolated chromosomes. If the histones are removed from isolated metaphase chromosomes and the chromosomes are then centrifuged onto an electron microscope grid for examination, extremely long loops of DNA project from an irregular "scaffold" whose dimensions are similar to the original intact chromosome (73). The nature of the scaffold is unclear, and it may, in fact, be some type of precipitation artifact. Nevertheless the striking morphology of such histone-depleted chromosomes suggests that the basic 200- to 300-Å fiber of metaphase chromosomes may be thrown into numerous loops reminiscent of the loops of lampbrush chromosomes. Whether these loops are permanent features of the chromosome, dividing it into specific domains, or represent a less ordered arrangement simply for purposes of packaging, is an important unanswered question.

Specific Sequence Organization

During the past 10 years an enormous amount of information has been collected about the organization of specific gene sequences. The earlier studies concerned rDNA, 5S DNA, and the genes coding for the histones, because these sequences are the most abundant and could be isolated by relatively simple physical methods, particularly centrifugation (74). Within the past few years, however, methods for gene cloning, the availability of numerous restriction enzymes, and rapid methods for DNA sequencing have made it possible to obtain detailed information on almost any desired gene. As a result molecular taxonomy has become a boom industry. From the plethora of information now available generalizations are beginning to emerge. For instance, serially repeated genes are generally separated by spacer regions that are not transcribed; specific sequences are found at the 5′- and 3′-ends of coding regions and probably represent promoter and terminator signals; many coding regions are interrupted by so-called intervening sequences or introns, which are transcribed but later removed from the mature messenger RNA. I will not attempt to cover these details of sequence organization, which have been well summarized in recent reviews (75, 76). Instead I will concentrate on a limited set of properties having to do with overall chromosome organization, especially the number of repeats of a given sequence in the genome, the chromosomal distribution of these sequences, and the total amount of DNA involved (as coding sequences, spacers, and introns). The general conclusion that I would like to emphasize is that the number and arrangement of sequences are often closely related to the life history and evolution of an organism, in much the same way that chromosome numbers, special sex-determining mechanisms, and the presence of chromosomal rearrangements reflect evolutionary and developmental strategies (10). This general conclusion will delight those who enjoy variety for its own sake. At the same time it puts an added burden on the molecular biologist who must decide which features of a particular gene family are of general significance and which apply only to the specific case.

These generalizations are most easily illustrated by the genes coding for 18S and 28S ribosomal RNA. In almost all eukaryotic organisms studied so far, these genes are present in serially repeated copies consisting of alternating transcribed and nontranscribed regions. The transcribed region contains the sequences for the 18S, 5.8S, and 28S RNA, read in that order, along with a short region preceding the 18S gene and short stretches between the genes, which are subsequently removed. The nontranscribed spacer region can be very short or much longer than the coding segment, depending upon the

organism, and often shows variability even within an organism. In at least some cases it has an internal repeating unit so that it resembles simple sequence DNA (77).

The localization of rDNA at the nucleolus organizer was first shown for *Drosophila* and *Xenopus* by a combination of cytogenetic and biochemical data (78, 79). Subsequently the position of rDNA has frequently been demonstrated by *in situ* hybridization (see review in reference 76). Many organisms have a single organizer, although multiple sites are not uncommon (humans have five, for instance). Although many organisms have an organizer near the centromere or at an interstitial position on a chromosome arm, a surprising number have their nucleolus organizer near the end of the short arm of one chromosome (80). The significance of this generalization is unknown.

Most organisms have from a few dozen to a few hundred rDNA repeats. For instance, yeast has 140 copies, *D. melanogaster* has about 200 copies, and humans between 150 and 200. Very high numbers up to 5,000 or more have been reported for salamanders and some plants (see review in reference 76). In assessing the percent of the genome devoted to rDNA, one must know the lengths of the nontranscribed spacer and intervening sequences when they occur. The lengths of spacers are quite variable. They are very short in *Bombyx* and *Sciara*, for instance, so that most of the repeat length is accounted for by the coding region. At the other extreme very long repeats have been described in the mouse and humans (40 kb), the cricket *Acheta* (35 kb), and the water beetle *Dytiscus* (29 kb). Intervening sequences have been described in several eukaryotic 28S gene sequences, where they range in size from 407 bases in *Tetrahymena pigmentosa* (81), about 5 kb in *D. melanogaster* (82), to 9.8 kb in *D. virilis*, (83) all organisms with relatively low C-values. There is no simple relationship between number of rDNA copies, total rDNA (including spacers and intervening sequences), and C-values. One might have predicted that the number or size of rDNA repeats would go up in proportion to C-value. Although it is true that the highest values are found in high C-value organisms, there is, if anything, a tendency for low C-value organisms to devote a larger percentage of their genome to rDNA (for instance the numbers are 5% for yeast, 1% for *D. melanogaster*, and 0.2% for *Xenopus laevis*). The long nontranscribed spacers do not belong to organisms with especially large genomes, and the longest known rDNA intron (*D. virilis*) is in an organism with a low C-value.

One of the most striking features of rDNA is the fact that copies may exist as free, extrachromosomal molecules in addition to the more typical, chromosomally integrated repeats. The most extreme case of this phenomenon, termed amplification, is found in oogonia and oocytes of many animals (84, 85). In *Xenopus* oocytes there are about 2×10^6 rDNA repeats organized in approximately 1,000 extrachromosomal nucleoli located around the periphery of the giant oocyte nucleus. These amplified genes have been the object of intense investigation (reviewed in reference 86). It is known that they arise from chromosomal copies during the earliest oogonial stages (probably as single repeats), that they replicate extrachromosomally by a rolling circle mechanism primarily during the pachytene stage of meiosis I, and that they engage in intense ribosomal RNA synthesis during vitellogenesis. The overall biological significance of amplification is reasonably clear. The oocyte is a single cell, which grows to a size many thousand times larger than a somatic cell and which accumulates ribosomes for protein synthesis during embryogenesis. The 4C oocyte nucleus of *Xenopus* contains only 2,000 integrated rDNA copies, which, if transcribing at maximal rate, would require many years to produce the 4 μg of rRNA contained in a mature oocyte. Many giant cells, which are faced with a similar problem (such as silk gland cells in *Bombyx*) become polyploid, thereby increasing the total number of rDNA sequences along with the whole genome. Such an avenue would not be open to an oocyte without a complete restructuring of the meiotic phenomena. In a sense, then, by amplification the oocyte manages to polyploidize its rDNA while leaving the rest of the genome intact at the 4C level. This general conclusion was reached in 1942 by Painter and Taylor (87) long before the nucleolar DNA of the oocyte was recognized as coding for rRNA.

rDNA amplification is found in oocytes of many animals, both vertebrate and invertebrate, but it is not universal. For instance, the oocyte nucleus of *Drosophila* shows no sign of amplification, yet the oocyte is large and well supplied with ribosomal RNA. Here, as in many insects, rRNA comes from polyploid nurse cells whose cytoplasm is physically continuous with the oocyte cytoplasm (88). In still other organisms with small oocytes neither amplification nor nurse cells occur, the chromosomal copies of rDNA being adequate for the number of rRNA molecules needed.

rDNA amplification is also found in the macronucleus of the ciliated protozoan *Tetrahymena* (89, 90). Its occurrence here is correlated with the well-known nuclear dualism of ciliates, which have a transcriptionally inactive diploid micronucleus (the germinal nucleus) and a transcriptionally active polyploid macronucleus (the somatic nucleus). There is a single chromosomally integrated rDNA copy in the micronucleus, but several thousand amplified extrachromosomal copies in the macronucleus (91). The significance of amplification in this case seems to be much the same as in oocytes—the large, rapidly growing cell could not synthesize enough rRNA from the rDNA copies present in the chromosomes.

Amplification of genes other than rDNA is known in two cases. The first involves cultured cells resistant to the folate analog methotrexate in which the nomally single copy gene for dihydrofolate reductase may be present in several hundred copies (92). The amplified genes are responsible for greatly increased production of dihydrofolate reductase, permitting the cells to function in the presence of the drug. The second is the recently discovered amplification of chorion protein genes in the ovary of *Drosophila* (93). This is an unusually interesting case because it is the first example of a protein-coding gene that amplifies during normal cell differentiation. Cells that produce massive amounts of a single protein, for example silk fibroin or egg albumin, ordinarily do so without amplification of the corresponding gene (94). Large amounts of protein can be synthesized because the mRNA is stable and because the tissue is active for many hours or days. Spradling and Mahowald argue that *Drosophila* oogenesis proceeds so fast that only multiple gene copies can produce the required number of chorion mRNA molecules (93). If their argument is correct, one should find other cases of amplification (or multiple chromosomal copies) of structural genes in extremely rapidly developing systems.

The existence of amplified genes in diverse organisms and cell types underscores the conclusion that the number of gene copies is often understandable only after considering the life history of the organism and the specific features of the cell type in which the genes are transcribed. The same general conclusion is illustrated in a dramatic fashion by the genes coding for

5S RNA. 5S RNA is a small molecule, 120 nucleotides in length, present as a single copy in the larger ribosomal subunit. In two organisms (yeast and *Dictyostelium*) the 5S coding sequence is located between the 17S and 25S genes (95, 96), but in all other investigated cases it occurs in tandemly repeated units unlinked to the other ribosomal RNA sequences. As with the 18S and 28S genes, highly conserved 5S coding regions alternate with spacers that may be internally repetitive and variable in length (97, 98). The cytological location of the 5S genes varies from organism to organism. In *Xenopus* they occur in clusters near the tips of the long arms of all the chromosomes (99); in the newt *Notophthalmus* they are found in the pericentromeric heterochromatin of four chromosomes and at one interstitial site (100); in *Drosophila* (101) and maize (102) they occur at a single site. Thus there is no obvious pattern to their location.

Their numbers are equally variable. *Drosophila* has about 160 copies (103) whereas *X. laevis* and *X. borealis* have about 24,000 and 9,000, respectively (104). As just discussed, the nurse cells of *Drosophila* make amplification of the genes for 18S and 28S rRNA unnecessary. The nurse cells probably produce 5S RNA as well. On the other hand, *Xenopus* lacks nurse cells, and one would suppose that the oocyte would amplify the 5S genes just as it does the 18S and 28S sequences. This is not the case, however (85); instead, the large number of 5S genes is maintained in the chromosomes primarily for use during oocyte development. This remarkable conclusion grew out of the discovery that oocyte and somatic 5S RNA sequences differ by a few nucleotides (105, 106). When the 5S genes were isolated by centrifugation from bulk genomic DNA, they were found to consist largely of oocyte-type sequences (107). Only after other minor sets of 5S genes had been isolated and characterized were the somatic genes finally discovered in both *X. laevis* and *X. borealis* (108). They consist of several hundred repeats with an entirely different spacer from the major oocyte species. The sequence data make it clear that thousands of 5S genes are carried as extra baggage in somatic cells to be expressed only in oocytes. During oogenesis 5S RNA is synthesized at a high rate in previtellogenic oocytes, well before the maximal rise in 18S and 28S rRNA synthesis (109). Thus even though each ribosome will eventually contain one 5S molecule for each 18S and 28S molecule, the genes are unlinked, and their transcription is temporally uncoordinated.

The formation of ribosomes during oogenesis is an important developmental event requiring synthesis of large amounts of 18S, 28S, and 5S ribosomal RNA. As just discussed, it is now clear that different organisms utilize quite different mechanisms to deal with the problem. In some cases genes are amplified extrachromosomally, in others the gene product is supplied by polyploid nurse cells, and in still others a special set of oocyte genes is maintained in the chromosomes. Surprisingly a single organism may utilize two different mechanisms, as in the case of *Xenopus*, which amplifies the 18S and 28S genes, but carries special oocyte 5S genes, even though the mature ribosome must contain equimolar quantities of each RNA. Another such case occurs in the beetle *Dytiscus*, which amplifies the 18S and 28S genes in oogonia and oocytes, but which also has polyploid nurse cells that supply RNA to the oocyte (110).

The genes coding for histones have been studied extensively in three species of sea urchin and in *Drosophila* (reviewed in reference 63). Earlier investigations by ultracentrifugation demonstrated that the genes were repetitive and probably clustered (111). With the advent of molecular cloning it was possible to obtain a restriction enzyme fragment 7 kb in length from the sea urchin *Strongylocentrotus purpuratus* that contained one coding region for each of the five histones in the order H1, H4, H2B, H3, H2A (112). Each coding region was separated from the next adjacent one by a spacer. Altogether there are several hundred serial repeats of this five-membered unit. A similar organization including the same gene order was demonstrated in two other species of sea urchin, *Lytechinus pictus* and *Psammechinus miliaris*. In *Drosophila* there are fewer gene copies, about 110 in all, but here too there is a repeating unit containing one each of the five genes (113). A notable difference in organization between the three sea urchins and *Drosophila* is that the coding regions are all in one strand in the sea urchins, whereas two coding regions are on one strand and three on the other in *Drosophila*. The *Drosophila* genes, therefore, cannot be transcribed as a single polycistronic messenger. Studies on histones during sea urchin development have shown the remarkable fact that histones produced at different stages may have different primary amino acid sequences (114). For instance, histone H1 from cleavage stages differs from its counterpart during gastrulation. The mRNAs for the two species are different and must be coded for by separate genes. Even in the case of histone H4, which has the same amino acid sequence at different stages, the messenger RNAs are distinct. The genes that have been cloned are in every case those which code for the earliest histone, suggesting but not proving that the later variants are coded for by relatively rare genes. If this turns out to be true, the analogy with the 5S genes would be close. That is, the organism may maintain a family of similar, repeated genes for use during a critical stage in its life history when unusually rapid synthesis is necessary. As in the case of the ribosomal RNA genes the mechanism used by the sea urchins might not represent a unique solution to the problem. For instance, *Xenopus*, which has much the same need for histones during embryogenesis, has only 20–50 gene copies (115). Adamson and Woodland (116) suggest that *Xenopus* synthesizes and stores histones and histone mRNAs during oogenesis, a protracted period lasting several months, and that the increase in histone synthesis in cleavage stages is dependent on stored mRNA. It appears that the difference in number of histone gene copies between the sea urchins and *Xenopus* may be correlated with different solutions to a developmental problem, although more information is needed before this conclusion is firm.

Genes coding for various proteins have now been cloned by recombinant DNA methods and their structure examined in detail; the number of new proteins analyzed is increasing at a rapid rate, and only a few general comments can be made here. In most cases the genes are ones which code for abundant or superabundant proteins, a fact that may have some bearing on the structures discovered. Although some of the genes may be present in only one copy in the genome, most of the examples studied consist of a small family of closely related sequences, for instance α- and β-globin (117–119), actin (120), ovalbumin (121), and vitellogenin (122). A few, such as the chorion protein genes in the silk moth, *Antheraea* consist of a family of sequences coding for a large number of similar but not identical polypeptides (123). Among the most surprising features is the widespread occurrence of intervening sequences (or introns) separating the coding sequence into two or more segments (discussed in reference 124). The number of intervening sequences per gene varies considerably, there being two in mam-

malian β-globin, seven in the ovalbumin gene of the chick, and an incredible 33 in the vitellogenin gene of *Xenopus*. There is good evidence in the case of the hemoglobin genes that the intervening sequences are ancient from an evolutionary standpoint. This is shown by the fact that two intervening sequences occur at approximately the same places in the β-globin genes of several species, as well as in the δ- and γ-variants and in α-globin (119). Either the intervening sequences were present in their current locations in the progenitor gene from which these related genes were derived or transpositions occur preferentially to these sites.

Intervening sequences obviously add to the total DNA content of organisms that possess them. Because of limited data it is not yet possible to relate the C-values of organisms with the number and length of their intervening sequences. In yeast very short intervening sequences have been described in tRNA genes (125), but until now only one (304 bp) in a protein-coding gene, that which codes for actin (126). In *Dictyostelium*, which has a very small genome (C = 0.05 pg), two small introns have recently been found in a gene coding for an mRNA of unknown function.[1] In *D. melanogaster* (C = 0.18 pg) some of the rDNA repeats have introns (82), and an intron has been described in a gene coding for actin (120). From the limited information available, one gets the impression that organisms with small C-values may have fewer introns than those with larger ones, but this may be caused in part by spotty sampling. The C-value of the chicken (C = 1.2 pg) and *Xenopus* (C = 3.2 pg), which contain such remarkably discontinuous genes, are small to moderate by comparison with many other eukaryotes. Organisms with very high C-values have not been examined for intervening sequences in protein-coding genes. *Notophthalmus* (C = 45 pg) has an average sized rDNA repeat (about 15 kb) and a very short 5S repeat (231 bp) with no evidence for introns.[2] I feel that major differences in C-value will probably not be directly ascribable to differences in the number and sizes of introns.

Chromomeres, Bands, and Loops

Perhaps the most obvious feature of chromosomes at the light microscopical level is that they are neither uniform nor regularly periodic. Instead they possess aperiodic discontinuities represented by chromomeres (especially in meiotic prophase), by bands in polytene chromosomes of insects and other organisms, and by loops in the lampbrush chromosomes of oocytes. The number of bands has been counted carefully in the salivary gland chromosomes of *D. melanogaster*. The best estimate, based on the studies of C. B. Bridges and P. N. Bridges is 5059 (127). The number of chromomeres in a lampbrush chromosome set varies with age of the oocyte, but the number counted during the maximal lampbrush stage is similar to the number of polytene bands, e.g., about 5,000 for *Triturus* and 3,000–6,000 for *Plethodon* (128, 129). The number of loop pairs is somewhat higher, because there is often more than one pair of loops per chromomere. It is a striking fact that the number of bands in *Drosophila* and the number of chromomeres in the salamanders are very nearly the same, even though the DNA contents of the two organisms differ by more than 100-fold. Does this mean that there are domains of chromosome structure, whose number remains relatively constant during chromosome evolution, but whose size varies with C-value? This question will be easier to answer once a clearer picture is obtained of a band and interband in *Drosophila* and other Diptera. Fortunately that time is not far away. Already from genetic analysis of Judd and Young (130) and others, we know that a band contains no more than a few complementation groups, even if the simple correlation of one band = one complementation group is an overstatement. The amount of DNA in a band, i.e., per chromatid, averages 20 kb with a range of perhaps 10-fold between the faintest and most prominent bands (the average is obtained by dividing the amount of euchromatic DNA, about 10^5 kb, by the number of bands). The smaller bands simply do not have enough DNA to contain many structural genes along with whatever control regions, spacers, and the like must be present. In the case of the histone genes it is known from *in situ* hybridization that the repeated sequences extend over several bands (131). Even in the case of an extraordinarily large puff, the Balbiani ring 2 of *Chironomus tentans*, there may be only one transcription unit (132). Because by hybridization techniques it is possible to select overlapping clones from a clone library of *Drosophila* ("walking" along the chromosome), there will soon be available several sets of clones that extend over more than one band's worth of DNA. From these it should be possible in principle to evaluate the number of structural genes and transcription units per band. In the case of the lampbrush chromosomes, it is reasonably certain from morphology both at the light microscopical and electron microscopical levels, that a loop often consists of a single transcriptional unit. On the other hand, there are clear cases where the morphology suggests two or more transcription units (133). *In situ* hybridization experiments demonstrate that the RNA transcripts over a long segment of a loop may hybridize with a specific DNA probe, once again consistent with the notion that a loop contains one or a small number of transcription units (134). The missing information in the case of the lampbrush chromosomes, in order to make a comparison with the bands of the polytene chromosomes, is how many structural genes or complementation groups may reasonably be present in one loop and its associated chromomere.

A unified model of eukaryotic chromosome structure might begin with the postulate that higher organisms have a more or less constant number of chromosome units or domains, roughly equal to the number of bands in *Drosophila* polytene chromosomes or loops in *Triturus* lampbrush chromosomes. Each of these domains would contain one or a small number of structural genes and a correspondingly small number of transcription units. As the DNA content of the organism went up or down during evolution, the number of units would remain the same while the amount of DNA per unit varied enormously. Thus both *Drosophila* and *Triturus* would have the same 5,000 or so chromosome domains, but the domains in *Triturus* would contain on average more than 100 times as much DNA as those in *Drosophila*. Just how the extra DNA might be organized is open to conjecture. My preference is to suppose that much of it may occur as spacers between the active transcription units. The extreme form of this model postulates that active gene regions are similar in number and organization throughout the range of eukaryotic organisms, but that they are more widely spaced in organisms with high C-values. A corollary of this model is that changes in DNA content occur more or less uniformly along the length of the chromosome to account for the common observation that related organisms with different C-values may have very similar karyotypes (10). In order to test these speculations at the molecular level, it will

[1] Kimmel, A. R., and R. A. Firtel. Personal communication.

[2] Kay, B., and J. G. Gall. Unpublished observation.

be necessary to compare the organization of structural genes in organisms with a wide range of C-values. Obviously this model of chromosome structure does not "explain" the C-value paradox. It does, however, stress that the number of active genes and transcription units need not be correlated with the total amount of DNA. From a structural standpoint it focuses attention on the organization of the individual chromosome domains, and it could be critically tested by showing that the spacing of active genes varies more or less linearly with C-value. It has been pointed out several times that DNA content is positively correlated with nuclear and cell size and inversely with rate of mitosis and rate of embryonic development (41). If these correlations are more than fortuitous, it would be useful to look for ways in which the DNA content of the chromosome domains might regulate the timing of mitosis and the rate at which the embryonic program is read.

An adequate model of chromosome structure must ultimately encompass not only the organization of individual genes but also the ways in which these genes are regulated during cell function and especially during embryonic development. In this light the study of chromosome organization has only just begun, and we can confidently predict major changes in our outlook during the next 25 years.

Many of the topics discussed in this review are treated more extensively in the Cold Spring Harbor Symposium, Volume 38 (1974) on Chromosome Structure and Function, and Volume 42 (1978) on Chromatin.

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The Nucleolus, Chromosomes, and Visualization of Genetic Activity

OSCAR L. MILLER, JR.

The Nucleolus and Ribosomal RNA Genes

By the time of Montgomery's classic paper on the nucleolus in 1898 (1), there were already 700 or so articles with observations on this nuclear organelle, beginning with a study by Fontana in 1781 entitled "Venom of Vipers." The early cytological emphasis on the nucleolus undoubtedly was due to the high visibility of the organelle in interphase nuclei of most cell types; however, the fact that nucleoli are directly involved with chromosomal activity was not demonstrated until Heitz (2) and McClintock (3) showed that nucleoli form during telophase at specific chromosome regions called "nucleolar organizers" (NOs) by McClintock. In 1940, Caspersson and Schultz (4), using ultraviolet (UV) absorption spectra, concluded that both nucleoli and cytoplasm of cells are generally rich in ribonucleic acid (RNA). Brachet (5) came to the same conclusion independently after discovering that RNase treatment of amphibian oocytes removed the basophilic components of both cytoplasm and nucleoli. Following these early observations, Caspersson and co-workers produced convincing evidence that a positive relationship exists between nucleolar size and levels of RNA and protein synthesis in cells (6).

In the early 1950s, Estable and Sotelo (7) used a silver-staining technique on a variety of cells, and suggested that during interphase, nucleoli contain a threadlike structure, termed the "nucleolonema," which associates with all of the chromosomes during mitosis and regroups at the NO during telophase, after which the nucleolus acts as a collecting site to accumulate additional material. Although the assignment of a hereditary continuity to the nucleolonema independent of chromosomes turned out to be incorrect, this provocative concept spurred interest by many investigators regarding the function of the nucleolus in cell metabolism. A comprehensive 1955 review by Vincent (8) provides a nice overview of earlier research on nucleoli.

Evidence for a Direct Relationship Between Cytoplasmic and Nucleolar RNA

Starting with Claude's "microsome" fraction in 1941 (9), techniques continued to be developed which allowed separation and biochemical analysis of different cellular fractions (reviewed in [10]). Cytoplasmic microsomal fractions were found to be rich in RNA and active in protein synthesis. When small ribonucleoprotein (RNP) particles were isolated from microsomal fractions treated with detergents (11), the RNP particles were found to contain essentially all of the RNA components and to be highly active in protein synthesis. Similar RNP particles already had been found in bacteria, and these were shown to contain two stable RNA molecules with sedimentation constants of 16S and 23S, which are complexed with a large number of proteins. Further studies using eukaryotic cells demonstrated that microsomal particles also contain two stable RNAs, but with somewhat higher S values of 18 and 28 (12). Porter (13), Sjöstrand and Hanzon (14), Palade (15), and Palade and Siekevitz (16), by using electron microscopy (EM), were the first to observe cytoplasmic granules in fixed cells and to correlate the morphology and chemistry of these granules. A similarity between such "Palade granules" with regard to size and composition and a granular component of the nucleolus was first noted by Porter (13) and later by Gall (17) and Swift (18). The first good indication that the nucleolus probably is involved in the production of the stable RNA components of the granular cytoplasmic "ribosomes" (a term introduced by Roberts in 1958 [19]) was provided by Woods and Taylor in 1959 (20). By use of autoradiography (ARG), these investigators showed that ^{3}H-labeled cytidine first appears in nucleolar RNA of *Vicia faba* root tips, then in cytoplasmic ribosomal RNA (rRNA), and that pulse-labeled nucleolar RNA moves from the nucleolus to the cytoplasm in the presence of unlabeled medium. More rigorous proof of this relationship was given by Perry and co-workers (21, 22), who demonstrated that selective UV microbeam irradiation of HeLa cell nucleoli prevented the appearance of about two-thirds of newly synthesized RNA into the cytoplasm, relative to control cells. Additional evidence for a nucleolar origin of rRNA came from Edström and colleagues (23), who used ingenious microdissection and microelectrophoretic techniques in the analysis of starfish oocyte RNA. They showed that the base composition of nucleolar RNA, but not other nuclear RNA, is essentially the same as that of cytoplasmic RNA, the large majority of which is rRNA. Similar results were subsequently obtained by Edström and Beermann, (24) using *Chironomus* salivary glands, and by Edström and Gall (25), using amphibian oocytes. Complementing these studies on RNA, Birnstiel and co-workers (26) showed by amino acid analyses that the nucleolar

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proteins of pea seedlings are very similar to those of isolated cytoplasmic ribosomes.

Evidence for a Large Precursor to 18S and 28S Cytoplasmic Ribosomal RNA

Strong evidence that the 18S and 28S rRNAs are derived from larger nucleolar molecules was first provided by Perry (27). He used parallel ARG and sedimentation studies on control and actinomycin-D-treated mouse L cells, and found that rapidly labeled nucleolar RNA contains heterogeneous fast-sedimenting components, some of which sediment faster than the heaviest cytoplasmic rRNA. Scheerer et al. (28) next reported that the largest rRNA precursor molecule (pre-rRNA) in HeLa cells sediments at 45S and is cleaved to an intermediate 35S molecule in the derivation of the rRNAs. Precise details of the conversion of HeLa pre-rRNA into 18S and 28S rRNA were later given by Weinberg et al. (29), using very clean nucleolar fractions and acrylamide gel electrophoresis methods developed by Loening to accommodate RNA molecules as large as pre-rRNA. It was found that a single pre-rRNA molecule gives rise, through two intermediate RNA cleavage pathways, to one molecule each of 18S and 28S rRNA. In that the existence of the high molecular weights (mol wt) of pre-rRNA based on apparent sedimentation constants continued to be questioned, Granboulan and Scheerer (30), using Kleinschmidt's protein film technique to visualize RNA molecules by EM, showed that there is a good correlation between the molecular-weight estimates by the two methods, and that the conversion of 45S pre-rRNA to rRNAs is the result of changes in lengths rather than configurations.

Evidence for Redundancy of 18S and 28S rRNA Cistrons

The first evidence that genomes of eukaryotic cells contain highly multiple sequences coding for cytoplasmic 18S and 28S rRNAs was provided by Chipchase and Birnstiel (31) who estimated from rRNA/DNA hybridization results that 0.3% of total pea-seedling DNA contains sequences homologous to rRNA. Also reported was the fact that nucleolar RNA competed with rRNA for such sequences. X-irradiation experiments done earlier by McClintock (3) and later by Beermann (32), showing that translocations involving partial NOs could function equally as well as intact NOs by morphological and growth criteria, had demonstrated that functional redundancy existed in NOs. This redundancy had now been given a molecular basis. Soon after the hybridization data from peas was obtained, McConkey and Hopkins (33) used similar methods to estimate that an average HeLa cell contains 400 28S rRNA cistrons and, more importantly, showed that rRNA sequences are enriched in nucleolar fractions.

Evidence for Localization of 18S and 28S rRNA Cistrons to NOs

The first highly suggestive evidence that all 18S and 28S rRNA cistrons are localized at the NO site of a specific chromosome was provided by Brown and Gurdon (34), using the Mendelian, anucleolate deletion mutant of *Xenopus laevis* first described by Elsdale et al. (35). In homozygous anucleolate tadpoles, the mutation prevents formation of normal nucleoli. These investigators showed that there also is no synthesis of 18S or 28S rRNA or of higher molecular weight precursors, whereas 4S RNA and rapidly labeled heterogeneous nuclear RNA (hnRNA) are synthesized. These results indicated that the cistrons for 18S and 28S rRNA are under coordinate control and are located at a single chromosomal site, the NO. More definitive evidence that the rRNA cistrons are localized within NOs was soon provided by Ritossa and co-workers (36, 37), who used cytogenetically derived *Drosophila* stocks carrying from 1 to 4 NOs, and Birnstiel and colleagues (38, 39), who compared normal (2-NO) tadpoles with heterozygous (1-NO) and homozygous (0-NO) anucleolate *Xenopus* mutants. Both groups demonstrated by rRNA/DNA hybridization that the number of rRNA cistrons present in the various stocks is precisely correlated with the number of NOs present.

Isolation of Ribosomal DNA and the Arrangement of the 18S and 28S rRNA Cistrons

Birnstiel and co-workers (38, 39) predicted from the high guanosine-cytosine (G-C) content of rRNA that its complementary DNA sequences should have a higher G-C content (~63%) than that of total *Xenopus* DNA (~40%), and that this DNA should separate from bulk DNA in CsCl gradients because of the difference in buoyant density. These investigators showed that about 0.2% of the *Xenopus* genome separates on CsCl gradients as a high-density satellite that contains essentially all of the genomic DNA complementary to 18S and 28S rRNA. This marked the first isolation in pure form of DNA sequences of known function.

The question of whether 18S and 28S rRNA sequences are present in the NO in homogeneous contiguous blocks of one or the other or are strictly alternating, was then approached independently by Brown and Weber (40) and Birnstiel et al. (41). Their experiments were carried out by shearing high-density satellite DNA (rDNA) to progressively lower molecular weights, challenging the DNA with 18S and 28S rRNA, then determining the buoyant density of the hybrid molecules. Because of the difference in the G-C content of the two rRNAs, it could be determined that linkage between the two sequences was not disrupted until a DNA with close to 1.5×10^6 daltons was reached. When DNA with a molecular weight 0.5×10^6 daltons or lower was used, essentially no linkage between the two rRNA sequences was present. These results forced the conclusion that the two cistrons are strictly alternating and that their products are contained together within the 40S pre-rRNA molecule of amphibia.

5S rRNA

Using HeLa cells, Knight and Darnell (42) showed that, in addition to the 28S rRNA, there is one 5S RNA molecule per large ribosomal subunit. That this 5S rRNA becomes associated with nascent ribosomal particles in the nucleolus that contain the 32S precursor to 28S rRNA was demonstrated by Warner and Soeiro (43). Brown and Weber (44) showed by RNA/DNA hybridization that 5S rRNA genes (5S DNA) in *Xenopus* are not linked with rDNA, and Pardue et al. (45) subsequently demonstrated by recently innovated in situ hybridization techniques that the some 20,000 or so 5S rRNA genes are distributed among the ends of the long arms of probably all of the 18 chromosomes of *X. laevis*. A much more localized site was found by Prensky et al. (46) for the approximately 160 5S rRNA genes of *D. melanogaster*, in which the genes can be assigned to bands 56e–f on chromosome 2R. On the other hand, linkage between 5S DNA and rDNA was reported by Cockburn et al. (47) and Maizels (48) for *Dictyostelium discoideum* and by Maxam et al. (49) for *Saccharomyces cerevisiae*. In both of these primitive eukaryotes, the 5S genes

are present with their own promoters in the spacers between pre-rRNA genes. Because of this arrangement, it was proposed that these two primitive eukaryotes may represent an intermediate divergence from the bacterial organization in which 5S cistrons share promoters with the other rRNA cistrons (48).

A dual 5S rRNA system was reported by Wegnez et al. (50) and Ford and Southern (51) for *X. laevis*, in that somatic cells synthesize one type of 5S RNA whereas oocytes synthesize both the somatic type and several oocyte-specific types which differ slightly from one another in nucleotide sequence. The mechanism by which such differential regulation of oocyte-type 5S RNA synthesis is controlled remains obscure. The nucleotide sequence of the major oocyte 5S DNA (average repeat length, 720 base pairs [bp]) has been determined (52–54). The repeat unit consists of two regions: a G-C-rich region that contains both the 5S gene and a "pseudogene" sequence homologous to much of the 5S gene, and an A-T-rich region. The G-C-rich region is constant in size within families of 5S DNA repeats, whereas the A-T-rich region, which is composed of repeating, closely related 15-bp sequences, can vary considerably in length. The pseudogene is not transcribed, and may have arisen by gene duplication followed by mutational inactivation of one gene (52). The 5S DNA repeat unit of *D. melanogaster*, on the other hand, contains no pseudogene sequence and exhibits only slight heterogeneity in length of the A-T-rich spacer segment (55, 56).

Whereas 5S RNA is present in a 1:1 ratio with 28S rRNA in ribosomes, numerous studies, beginning with that of Perry and Kelley (57), have shown that 5S RNA synthesis is not coordinate with pre-rRNA production (see [58] for other references).

Amplification of Nucleolar Genes in Amphibia and Insects

Although chromosomal NOs are inherited as Mendelian units and there is only one to a few such loci, depending on the organism, rDNA has been shown to be preferentially amplified extrachromosomally in oocytes and oogonia of many animals, both invertebrate and vertebrate, and in the vegetative nuclei of some primitive eukaryotes (see review by Tobler) (59). The early cytological studies of this phenomenon, which, in many cases, results in the formation of highly multiple extrachromosomal nucleoli, were elegantly reviewed by Gall (60), and only a few of the early works pertinent to this chapter will be mentioned.

King (61), using a safranin-gentian-violet double-staining procedure, concluded that extrachromosomal chromatin becomes associated with the multiple nucleoli of *Bufo* oocytes after pachytene. Bauer (62) used the recently introduced Feulgen stain for DNA, and demonstrated that "Giardina's body" in *Dytiscus* oocytes, as well as extrachromosomal bodies in oocytes of several other insect species, contains DNA. Brachet (5) next used this specific stain to show the presence of DNA in the multiple nucleoli of *Rana* oocytes. His work was followed quickly by a more extensive study of *Bufo* oocytes by Painter and Taylor (63), who independently confirmed Brachet's observations and concluded that the extrachromosomal nucleoli are involved in the production of cytoplasmic RNA and that the extrachromosomal chromatin granules probably are equivalent to the NOs of somatic cells. After a significant interim, Kezer (64) and Miller (65, 66), in examining the circular nucleoli found in certain salamander oocytes, independently showed by enzymatic digestion experiments that the circular continuity of such nucleoli is maintained by DNA (Fig. 1). Considering evidence then becoming available regarding the function of somatic cell NOs in rRNA synthesis, these authors also concluded that extrachromosomal nucleoli probably are involved in rRNA synthesis. Similar conclusions regarding the probable role of extrachromosomal DNA in insect oocytes soon followed (see discussion in Gall [60]). Proof that the amplified DNA of amphibian oocytes is rDNA was independently shown by rRNA/DNA hybridization by Gall (67), using young *Xenopus* ovaries, and Brown and Dawid (68), using isolated oocyte nuclei of four amphibia. Macgregor (69) demonstrated by microspectrophotometry that the amount of extrachromosomal DNA per *X. laevis* oocyte is about 30 pg, or five times the total diploid genome. Evidence for amplified rDNA in insect oocytes was soon presented for *Dytiscid* water beetles by Gall et al. (70) and for the cricket *Acheta* by Lima-de-Faria et al. (71). Gall and Rochaix (72) subsequently demonstrated that much, if not all, of the amplified rDNA of *Dystiscid* beetles is present in circular form (Fig. 2).

The process of amplification in *Xenopus* oocytes begins before meiosis and is completed by the end of pachytene (73, 74). Brown and Blackler (75) presented evidence from reciprocal crosses between *X. laevis* and *X. borealis* (*mulleri*), in which only *X. laevis* rDNA is amplified in the oocytes, that rDNA amplification apparently proceeds by a chromosome

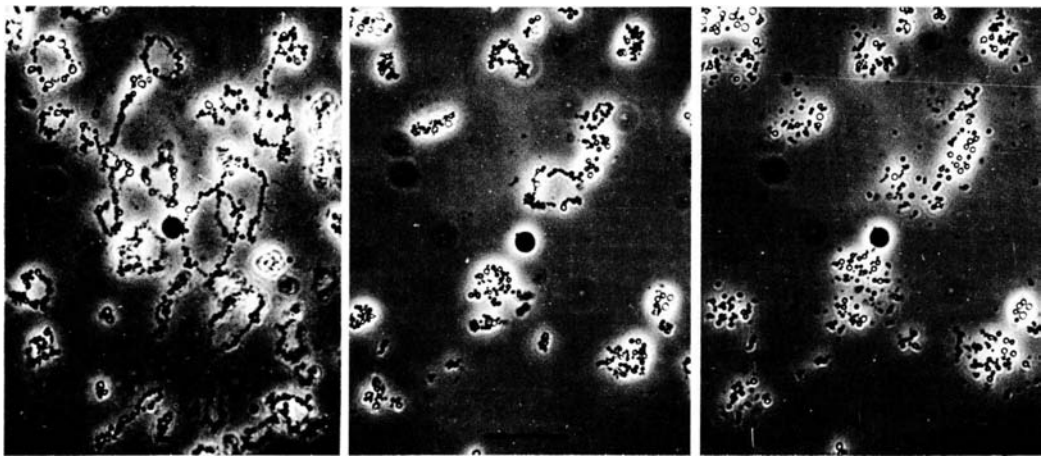

FIGURE 1 Phase contrast micrographs of circular nucleolar cores from a *Triturus pyrogaster* oocyte in the process of being cleaved by the action of pancreatic DNase, from Miller (66). Bar, 50 μm. × 250. All of the remaining figures are derived from electron micrographs.

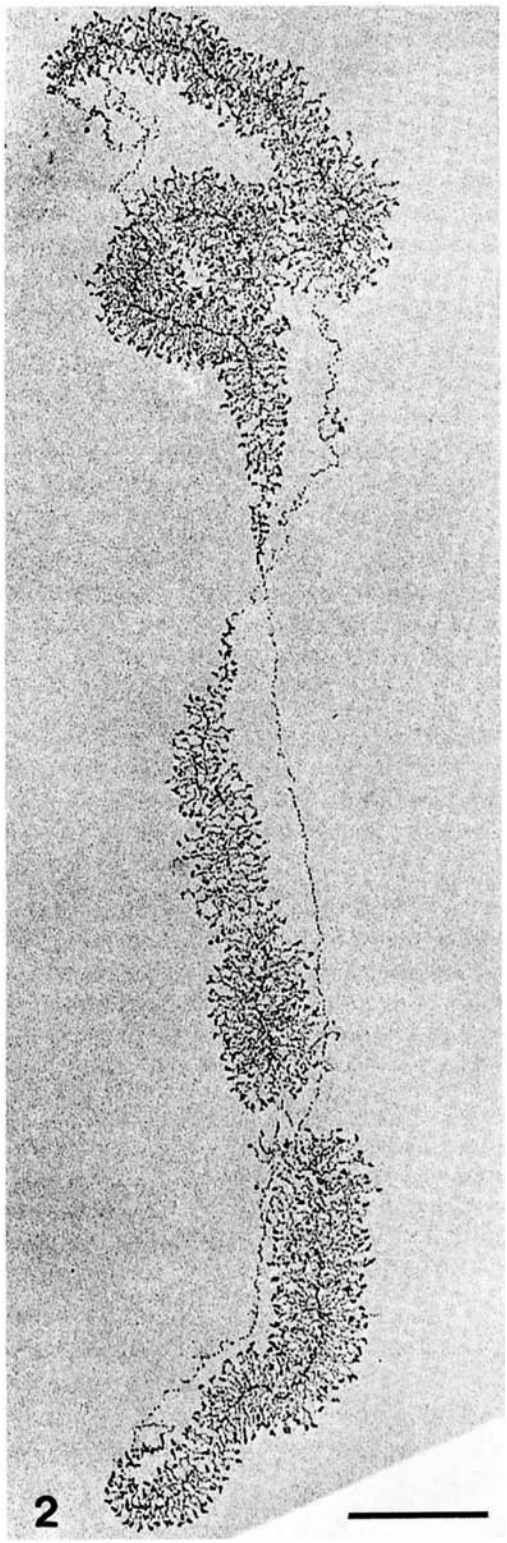

FIGURE 2 A circular rDNA molecule isolated from a *Dytiscus* oocyte, showing transcriptional gradients of active rRNA genes separated by inactive spacer segments, from Trendelenburg (192). Circularity of such molecules was first demonstrated by Gall and Rochaix (72) by visualization of deproteinized rDNA molecules spread in a surface film. Bar, 1 μm. × 18,000.

copy mechanism rather than by germ-line transmission of episomal rDNA. Subsequent studies by Hourcade et al. (76) and Rochaix et al. (77) provided evidence that, after the presumptive chromosome copy event(s), the amplification process of *Xenopus* proceeds extrachromosomally by a rolling-circle mechanism (Fig. 3). To date, however, no definitive information regarding the molecular aspects of the initial events in rDNA amplification is known for either amphibia or insects.

Ultrastructural Visualization of Nucleolar Function in Higher Eukaryotes

Excluding vacuoles, nucleoli typically consist of two major ultrastructural components, one coarsely fibrous and one granular. The spatial relationships of the two components vary considerably depending on cell type, ranging from seemingly random interspersion to strict compartmentalization into a central or excentric fibrous core surrounded by a granular cortex (Fig. 4; for further examples, see Busch and Smetana [78]). In an early EM study of polytene chromosomes, Beermann and Bahr (79) clearly showed that the central core region of the nucleolus is directly connected with the NO of the chromosome. Subsequently, EM-ARG studies by Granboulan and Granboulan (80), using tissue culture cells, and by Karasaki (81), using amphibian embryos, demonstrated that initial incorporation of RNA precursors occurs in the fibrous nucleolar component, and both concluded that the newly synthesized RNA appearing later in the granular component is derived from the fibrillar one. Similar results were obtained later by Macgregor (82) for amphibian oocyte nucleoli, the fibrillar core regions of which were already known to contain DNA.

By using newly devised spreading techniques for EM preparations, Miller and Beatty (83, 84) were able to visualize clearly the structure of dispersed core and cortex components of amphibian oocyte nucleoli. Analyses of EM-ARG and enzymatic digestion, combined with biochemical data from other sources, allowed the conclusion that the cores consist of single, circular deoxyribonucleoprotein (DNP) molecules of varying lengths that contain highly active, repetitive rRNA genes, each of which is separated from its neighboring genes by apparently inactive "spacer" segments of variable length (Fig. 5). The granular nucleolar component, which presumably

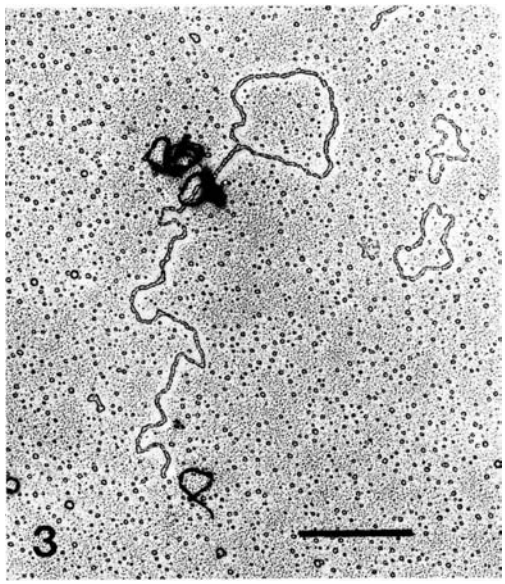

FIGURE 3 An extrachromosomal rDNA molecule isolated from a young *X. laevis* ovary, courtesy of A. H. Bakken (unpublished material). The silver grains indicate incorporation of [^{3}H]thymidine in the "tail" extending from a small rolling circle. Bar, 1 μm. × 14,250.

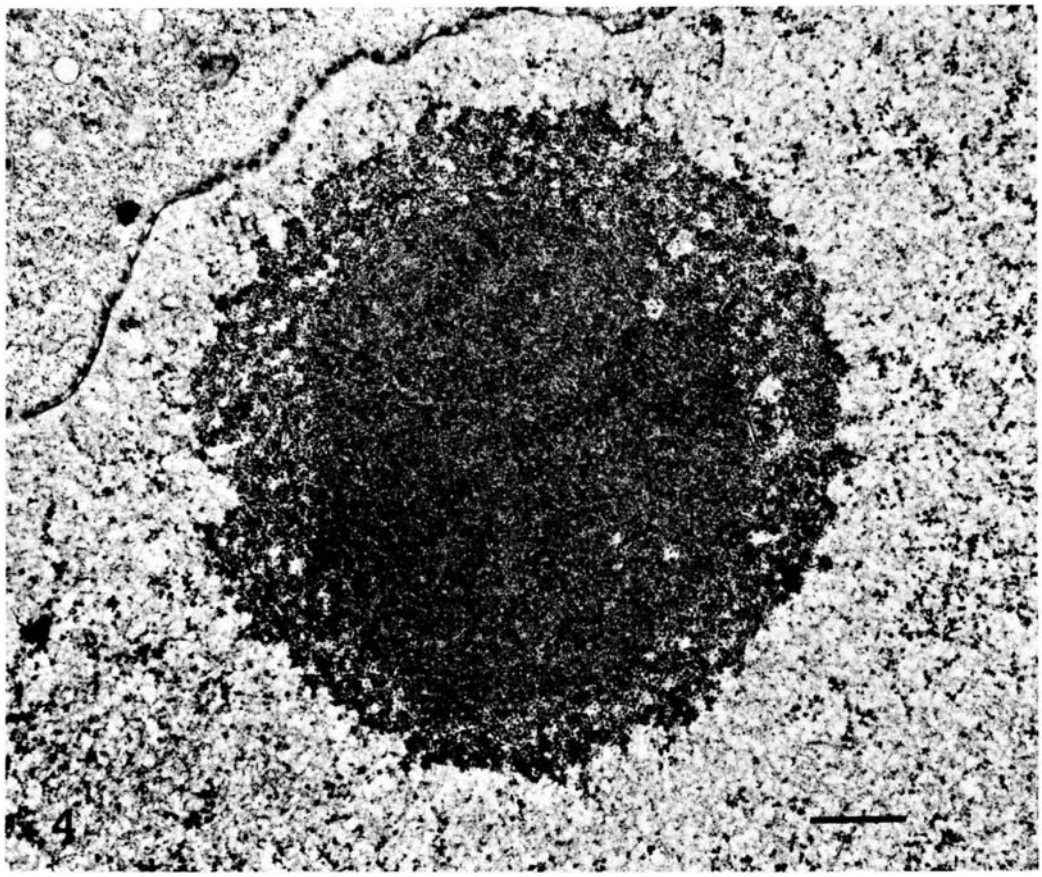

FIGURE 4 Thin section of an extrachromosomal nucleolus of *Notophthalmus* (*Triturus*) *viridescens* showing the bipartite structure of fibrous core and granular cortex typical of nucleoli in many cell types, from O. L. Miller, Jr. (66). Bar, 1 μm. × 12,500.

contains the 30S RNA precursor to 28S rRNA, was found to consist of small granules fairly widely spaced on thin, but well-defined fibrils. The significance of the fibrillogranular network in the biogenesis of the large ribosomal subunit remains unknown.

Subsequent studies by Miller and Bakken (85) with HeLa cells, and Hamkalo et al. (86) on *Drosophila* embryos showed a basically similar organization of -spacer-gene-spacer-, with the length of the rRNA genes reflecting the different molecular weights of the pre-rRNA molecules in the three cell types. Similar techniques were used by Franke and co-workers who rapidly extended observations of active nucleolar genes to amplified rDNAs of *Acheta* (87) and *Dytiscus* (88) (Fig. 2). All of the rDNA repeats within one NO of higher eukaryotes appear to have the same transcriptional polarity, except for some infrequent observations of adjacent convergent or divergent gene polarity in amplified rDNA. Perhaps unsurprisingly, it could now be concluded that all higher eukaryotes probably have the same general morphological arrangement of active rDNA.

Chromatin spreading techniques have provided some information about regulation of rRNA genes in several different cell types. McKnight and Miller (89) found that maximal packing of RNA polymerases occurs on both newly activated and fully transcribed rRNA genes of *Drosophila* embryos, indicating that in this system the rate of transcription, rather than frequency of polymerase initiation, regulates pre-rRNA production on individual genes. On the other hand, modulation of RNA polymerase initiation appears to be involved in two other systems. Scheer et al. (90) observed that amplified rRNA genes of young oocytes of *Triturus alpestris* have reduced RNA polymerase packing ratios as compared with those of more mature oocytes, and Foe et al. (91) showed that newly activated rRNA genes of milkweed-bug embryos typically have quite low RNA polymerase densities compared with later stages. In addition, McKnight and Miller (89) found that the number of active rRNA genes increased as cellularization proceeds in *Drosophila* embryos, although no more than 50% of the rRNA genes ever appeared to be activated. A similar observation was reported earlier by Meyer and Hennig (92) for primary spermatocytes of *Drosophila hydei*.

Molecular Anatomy of rDNA Repeat Units of Higher Eukaryotes

In all cases in which rDNA of higher eukaryotes has been examined in detail, the rRNA genes have been found in tandem repeated units with each unit consisting of an rRNA gene and a nontranscribed spacer (NTS) segment. Each rRNA gene contains three cistrons coding for the 28S, 18S, and 5.8S rRNA found respectively in ribosomes. The 5.8S rRNA in ribosomes was first detected in HeLa cells by Pene et al. (93), who found it to be hydrogen-bonded to the 28S rRNA and presented evidence that the 5.8S molecule is derived from the same intermediate precursor molecule as the 28S rRNA. Subsequently, Speirs and Birnstiel (94) concluded from hybridization studies with *X. laevis* rDNA satellite that the 5.8S rDNA sequence is located between the 18S and 28S rDNA cistrons.

The question of transcriptional polarity within pre-rRNA molecules was a controversial subject for a number of years.

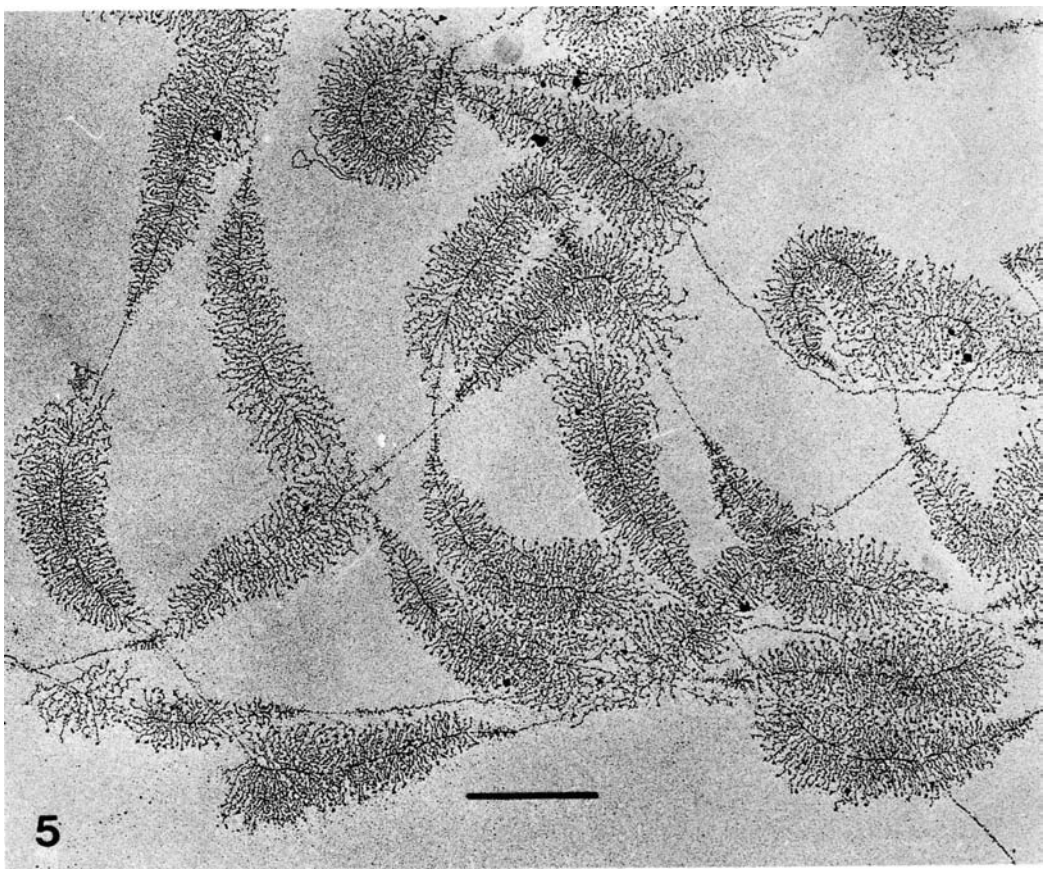

FIGURE 5 Extrachromosomal rRNA genes isolated from an oocyte of *N. viridescens*, from Miller and Beatty (83). The genes, which appear maximally loaded with RNA polymerase molecules and are separated by transcriptionally inactive DNP segments, have the same polarity and are contained in a circular rDNA molecule. Bar, 1 μm. × 16,750.

Experiments indicating an initiation-5′-18S-28S-3′-termination polarity included kinetics of rRNA labeling in *Euglena* (95), synthesis of *X. laevis* rRNA in vitro (96), and differential sensitivity of rRNAs upon inhibition of synthesis by 3′-deoxyadenosine (97) and UV irradiation (98). Results indicating an opposite polarity included identification of similar 5′-termini in 28S rRNA and pre-rRNA (99), kinetics of rRNA labeling in isolated nuclei from *Rana* (100), and secondary structure analysis of pre-rRNA and rRNAs after partial 3′-exonuclease digestion (101). More recently, results obtained by secondary structure analysis of nascent pre-rRNA compared with rRNAs and mature pre-rRNA (102), by new 3′-exonuclease experiments (103), and by restriction endonuclease analysis of repeating rDNA units with attached nascent pre-rRNA transcripts (104) have provided conclusive evidence of a 5′-18S-28S-3′-transcriptional polarity in *Xenopus*.

The average length of NTSs can be quite different, depending on the organism being examined. For example, the spacers in *Colymbetes* are about 15 kilobases (kb) long, whereas those in *Dytiscus* are about 45 kb long (72). Heterogeneity in NTS length has been detected in several organisms including mouse (105), *Drosophila* (106), and *X. laevis*, with the latter having NTS varying from about 11 kb to 22 kb or so in length (107). Reeder et al. (108) showed that the patterns of chromosomal NTS lengths of *Xenopus* are inherited in a Mendelian manner. Wellauer et al. (109) found that in some individual frogs repeat lengths rarely present in their chromosomal rDNA are amplified selectively, whereas others amplify their most abundant size classes, and that the preference for size-class amplification is inherited.

Wellauer et al. (107, 109) and Botchan et al. (110) studied the molecular basis for variable NTS length in *Xenopus* by heteroduplex mapping and restriction enzyme analysis of cloned rDNA. Their results indicated that such NTSs consisted of two conserved regions having no internal repetitions that alternate with two regions of variable length composed of short repetitive sequences. Somewhat later, Birnstiel and colleagues (111) reported the sequencing of essentially an entire cloned *Xenopus* NTS. Their data showed that this NTS is composed of four internally repetitive regions interdigitated with conserved nonrepetitive regions. High-sequence homology was found between a short segment immediately upstream from the pre-rRNA transcription initiation site and segments within the next two upstream nonrepetitive regions of the NTS. Similar high-sequence homology was demonstrated by Sollner-Webb and Reeder (112) who used a different cloned NTS. The arrangement of the high homology sequences within NTSs suggests that such sequences have been reduplicated and displaced upstream into *Xenopus* NTSs by saltation of repetitive region repeats during recent evolutionary time (111). As yet, however, there is no definitive evidence regarding the function of any portion of NTSs. Short transcription gradients occasionally are present on amplified rDNA spacers of *Xenopus* (113), and it is possible that these result from reduplicated and displaced promoters in the high homology regions which have remained functional (111, 112). It is typical, however, that no

transcription is observed on NTSs, especially with regard to chromosomal rDNA. In contrast, McKnight et al. (114) have provided preliminary evidence from chromatin spreads of *Drosophila* embryos that NTSs may contain initiation sites for chromatin replication.

Another basis for length heterogeneity of rDNA repeats has been reported for *D. melanogaster*, in which a DNA segment that is not included in pre-rRNA is present in 60% of the rRNA gene sequences (106, 115, 116). The intervening sequences occur primarily in the NO of the X chromosome, and genes containing insertions appear to be randomly interspersed with genes without insertions. The insertions are located about two-thirds of the way into the 28S cistron, and range in length from 0.5 to 6.0 kb. Chooi (117) has reported the occurrence of a few longer-than-normal transcription units in spread NOs of *D. melanogaster*, suggesting that some insert-containing genes may be transcribed. Long and Dawid (118), however, used cloned insertion sequences, and have shown that the number of nuclear RNA molecules with insertion sequences is on the order of 10–20 per nucleus and, thus, cannot make any significant contribution to the production of 28S rRNA. Sequences homologous to the rDNA inserts and comprising some 0.2% of the haploid genome of *D. melanogaster* are present in chromatin outside the NOs (119).

Amplification of rDNA in Primitive Eukaryotes

In addition to that shown for amphibia and insects, extrachromosomal amplification of rDNA has been documented for several primitive eukaryotes, including *Tetrahymena pyriformis* (120–122), *Physarum polycephalum* (123, 124), *Paramecium tetraurelia* (125), and several species of green algae (126–128). Restriction enzyme analysis and denaturation-renaturation studies showed that the free rDNA molecules of *Tetrahymena* (129, 130) and *Physarum* (123, 124) are large palindromes in which each molecule has two rRNA genes. The genes are separated by nontranscribed spacer regions and localized toward the ends of the molecules, with the 17S rRNA cistrons proximal to the 26S rRNA cistrons. Grainer and Ogle (131) showed that the rRNA genes on *Physarum* panlindromes are transcribed divergently (Fig. 6), the polarity of the smaller and larger rRNA cistrons thus agreeing with that found previously in other eukaryotes (see previous section). Campbell et al. (132) found that the 26S rRNA cistron of *Physarum* contains two intervening sequences, in a manner somewhat analogous to *Drosophila* rDNA. In this case, however, it seems likely that the intervening sequences are usually transcribed, because they occur in at least 88% of the rRNA genes, and other data indicate that all of these genes are probably active in growing plasmodia.

Yao and Gall (133) have proposed a tentative model for the origin of extrachromosomal *Tetrahymena* palindromes that involves branch migration of the single rDNA unit integrated in the germline genome to form an extrachromosomal molecule, which unfolds into a linear palindrome by semiconservative replication. Such a mechanism would explain why the two sides of the palindrome are virtually identical and why there is no heterogeneity in the rDNA of *Tetrahymena* at the time of formation of the vegetative macronucleus.

In green algae and paramecia, the rDNA was found to exist not as palindromes, but in arrays of tandem repeats similar to that found in higher eukaryotes. Although, as discussed above, the rRNA genes in such arrays typically exhibit the same transcriptional polarity, a so-far unique arrangement has been reported by Berger et al. (134) for *Acetabularia exigua* in which rDNA repeats exhibit a strictly alternating polarity.

Chromosomes and Nonnucleolar RNA Synthesis

Through the years, many of the cytological studies of nonnucleolar RNA synthesis on eukaryotic chromosomes have focused on the so-called "giant chromosomes," primarily the diplotene-stage lampbrush chromosomes of amphibian oocytes and the polytene chromosomes of dipteran flies. The basic structural organization of these chromosomes is described by Gall in this volume, so only morphological and chemical aspects involving RNA synthesis will be considered here. Visualization of synthetic activity in the lampbrush-type loops found in primary spermatocytes of *Drosophila*, in embryos, and in certain miscellaneous cell types are also discussed.

Lampbrush Chromosomes of Amphibian Oocytes

Although lampbrush chromosomes have been observed in the oocytes of many vertebrate and invertebrate animals (135) and even in green algae (136), they attain their largest dimensions in the oocytes of amphibia. Although seen previously, the first extensive study of such chromosomes was done by Rückert in 1892 (137) on sectioned shark oocytes. It was not until 1940, after the Feulgen stain was introduced, that the DNA nature of the chromomeres forming the main axis of lampbrush chromosomes of *Rana* was demonstrated (5). In 1937 (see Duryee [138] and previous articles), Duryee made an important contribution toward the study of lampbrush chromosomes by showing that the germinal vesicles of amphibian oocytes can be isolated and their lampbrush chromosomes observed in the phase-contrast microscope in what appears to be essentially an in vivo condition. After earlier studies by Dodson (139), which indicated the presence of RNA in the lateral loops of lampbrush chromosomes, Gall (140), in a careful study of the lampbrush chromosomes of the newt, clearly demonstrated the presence of RNA in the Feulgen-negative lateral loops, which were presumed to be products synthesized or organized by the

FIGURE 6 A palindromic rDNA molecule isolated from *Physarum polycephalum* showing single rRNA genes with divergent transcriptional polarity located near each end, courtesy of R. M. Grainger and R. C. Ogle (unpublished material). Bar, 1 μm. × 8,000.

Feulgen-positive chromomeres of the main axes. In this study, Gall introduced a very important optical innovation by using an inverted phase-contrast microscope and holey slides with coverslip bottoms, an arrangement which allows observation of undistorted chromosomes at the highest resolution provided by light microscopy. Although there had been several earlier EM studies, Gall (141) was the first investigator to demonstrate that lateral loops contain loosely associated granules some 300–400 Å in diameter. Both Callan and Gall (see references in [141]) had previously postulated from earlier EM studies that each lateral loop has a submicroscopic axis. That this is so was also clearly demonstrated by Gall (141), who used pepsin digestion of loop matrices after immobilizing lateral loops on support films. Soon thereafter, Lafontaine and Ris (142) observed lampbrush chromosomes of several amphibia after critical point-drying in carbon dioxide. The similar fibrillar nature of loops and chromomeres after such drying suggested to these investigators the possibility that the main axis or chromonema of each chromosome consists of a bundle of fibrils that may be continuous through chromomeres and loops, but that varies in composition within the two structures. Gall's earlier study, and subsequent studies by others, clearly showed that this was not so. Very shortly thereafter, the nature of the submicroscopic axes of lateral loops was nicely shown by Callan and Macgregor (143), who demonstrated that DNase breaks the continuity of both loops and main axes without disturbing the RNP matrix material associated with the loop fragments until the loop axes have been disintegrated.

The fact that RNA is being actively synthesized on lateral loops was demonstrated by Gall (144) and Gall and Callan (145) who autoradiographed isolated chromosomes after labeling them with tritiated RNA precursors. The association of newly synthesized protein with the RNA also was shown in the second study. Previously, Callan and Lloyd (146) had introduced the concept that the genetic information within lampbrush chromosome loops may be serially repeated along the loop axes. To avoid the problem of random mutations, it was proposed that a "master copy" would correct any sequence changes as the repeats along a loop spun out of its chromomere to be transcribed during early diplotene. This concept was reinforced by evidence from Gall's and Callan's study on RNA synthesis; they observed sequential labeling of one morphologically distinct loop and concluded that it probably was continuously being spun out of and back into its chromomere as oogenesis progressed. The so-called "Master-Slave" hypothesis was expanded upon by Callan in 1967 (147), and further evidence for loop-axis movement was provided by Snow and Callan in 1969 (148). Inherent in this concept are the assumptions that no genetic diversity exists within individual chromomeres and that RNA synthesized on such chromomeres would come from repetitive DNA sequences (see Macgregor [149] for discussion of this concept). Although this hypothesis stimulated considerable thought and research, it does not appear to be valid in view of later results which indicate that most of the template-RNA synthesized and stored during amphibian oogenesis is transcribed from unique or single-copy sequences (150, 151).

More definitive observations regarding the ultrastructural nature of the RNP molecules in loop matrices was next provided by Miller (152) and Miller and Beatty (153), who used newt oocytes and techniques designed to observe chromosomes free of nucleoplasm and to unwind the RNP fibrils attached to loop axes (Fig. 7). Their results demonstrated that the RNP fibrils of typical loops form gradients of fibrils of increasing lengths from the thin insertion end, with RNA polymerases quite closely spaced and extremely long RNA molecules being synthesized. Subsequently, the structural organization of loop RNA fixed under physiological conditions was reported by Mott and Callan (154), who found that nascent RNA transcripts and associated protein are arranged in linear arrays of 300 Å particles. Similar configurations were found in all loops, no matter what their gross morphology, but many loops had such strings of particles wound back on themselves to form dense aggregates some 2,000–3,000 Å or more wide. Malcolm and Sommerville (155) previously had isolated such particles and had shown the protein-to-RNA ratio to be at least 30:1. Scott and Sommerville (156) demonstrated by immunofluorescence techniques that some of the nonbasic proteins in lampbrush chromosomes are common to all loops, whereas others may be localized in specific groups of loops.

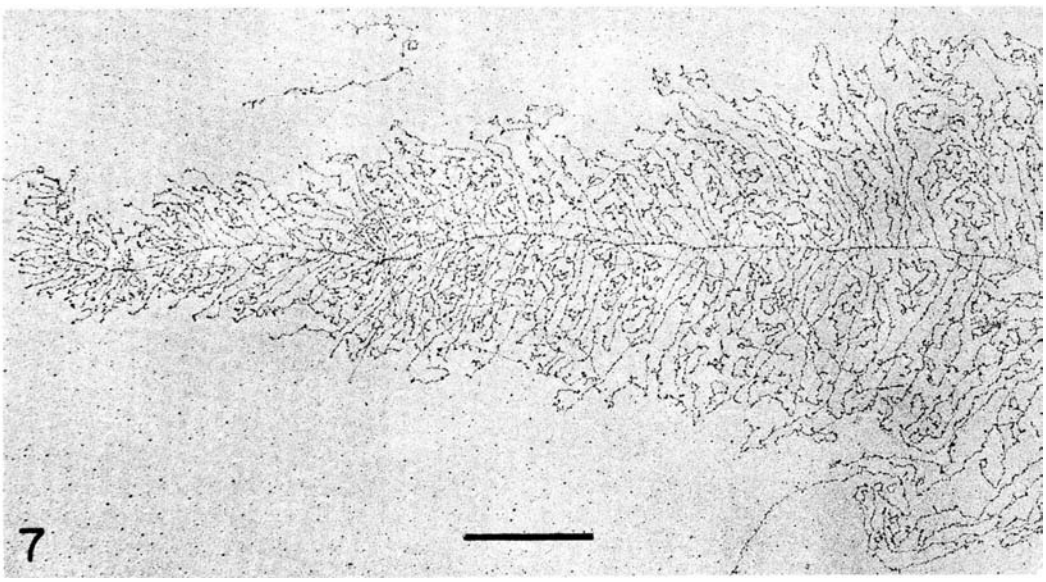

FIGURE 7 A portion of a lampbrush chromosome loop at the thin, chromomeric insertion end where RNA synthesis is initiated, from Miller et al. (193). Preparation was isolated from an oocyte of *N. viridescens*. Bar, 1 μm. × 16,500.

Scheer and co-workers (157, 158) used chromatin-spreading techniques to expand greatly observations on the arrangement of transcriptional complexes in salamander oocytes and the green algae *Acetabularia.* In addition to loops that appear to be single transcription units, as inferred from single RNP fibril gradients, loops with multiple gradients of divergent, convergent, and/or similar polarities are sometimes observed. Estimates of the sizes of nascent RNA molecules range up to some 82 kb, based on lengths of transcriptional units, and similar sizes have been determined by sedimentation and gel electrophoretic analyses (157, 159).

The functional significance of the high levels of transcriptional activity on lampbrush chromosomes is not clear. Davidson and co-workers (160) estimated that about 2.2% of lampbrush-stage RNA in *X. laevis* is template RNA that is synthesized on about 2.7% of the genomic DNA. Subsequent studies by Sommerville and Malcolm (159) demonstrated that about 4% of the chromosomal DNA of *Triturus cristatus* is transcribed during oogenesis. However, only some 0.05–0.1% of the RNA contains coding sequences; the remainder are noninformational repetitive sequences. Further studies, by Rosbash and colleagues (150, 151), show that the poly(A)-RNA molecules present in mature *X. laevis* oocytes contain some 20,000 different sequences that are transcribed almost entirely from single-copy DNA. The sedimentation profile of poly(A)-RNA from oocytes and *X. laevis* kidney-cell cultures were found to be similar. Whether loop transcription represents a relatively high activity on loci that are transcribed at much lower rates in somatic cells or rather represents transcription of larger segments of DNA than occurs in somatic cells remains to be determined.

Y Chromosome Lampbrush Loops in Drosophila Spermatocytes

The early genetic and light-microscope cytogenetic studies of Y-chromosome function in *Drosophila* spermatogenesis were reviewed in 1968 by Hess and Meyer (161). Emphasis was placed on the *D. hydei* subgroup, in which morphologically distinctive structures comparable to the loops of lampbrush chromosomes were found to be determined by a minimum of five Y-chromosome loci. The loop morphologies are species specific, and, as shown by deficiency-duplication studies, the loci are involved in postmeiotic sperm differentiation. After labeling with [^{3}H]uridine, ARG demonstrates that RNA synthesis occurs on each of the loci, with some loci showing polarized labeling. A microspreading method for dispersing contents of primary spermatocyte nuclei as a surface film was used by Meyer and Hennig (162) and Hennig et al. (163) to observe structural aspects of these loci by EM. It was estimated that RNP molecules considerably longer than 10 μm are synthesized on some loops. Hennig (164) has more recently reviewed the state of knowledge about Y-chromosome loops, and has suggested that optional points for RNA polymerase initiations along a loop could account for the polarized incorporation that takes place on some of the loops after pulse-labeling with RNA precursors.

Polytene Chromosomes of Dipteran Flies

The occurrence, structure, and synthetic activities of polytene chromosomes have been the subject of a number of reviews (e.g., 165–167). The composition and function of "puffs," which form by the unfolding of usually one chromosomal band and appear in the polytene chromosomes of many larval tissues of *Dipteran* flies, have received the most attention. This is especially true of the very large puffs, or Balbiani rings (BRs), found in the salivary glands of *Chironomus* species. Early light-microscope ARG by Pelling (168) and Rudkin and Woods (169) showed that such puffs are highly active in RNA synthesis. The early EM study by Beerman and Bahr (79) demonstrated that BRs consist of numerous branching filaments ~100 Å thick, with granules ~300 Å in diameter apparently attached to their ends. This study was extended later by Stevens and Swift (170), who provided EM evidence that the RNP products of BRs move into the cytoplasm through the pores of the nuclear envelope.

Because of the high lateral redundancy of polytene chromosomes, Swift (171) and, later, Gorovsky and Woodward (172), were able to show that there is no difference in the amount of histone in inactive and puffed loci. That nonhistone proteins become associated with RNA in puffs was demonstrated by Helmsing and Berendes (173), who also showed that some nonhistone protein will move into induced puffs even in the absence of RNA synthesis.

Grossbach (174) presented evidence that the BRs of *Chironomus* probably contain the genes for several secretory polypeptides. Because of this, and the fact that BRs and their associated RNAs can be isolated by microdissection techniques, the BRs, especially BR2, of *C. tentans* have been the subject of intensive investigation, and much of this work has been reviewed recently by Case and Daneholt (175). The primary transcripts of both BR1 and BR2 have sedimentation constants of 75S and are estimated to contain 37 kb. The 75S molecules of BR2 have been shown to be present in cytoplasmic polysomes and, thus, probably to code for one or more of the salivary secretion polypeptides. Recently, Lamb and Daneholt (176) were successful in employing chromatin-spreading techniques to visualize transcription units of chromosome 4 of *C. tentans* which contains the BRs. Highly active transcription units with a mean length of 7.7 μm are most often observed, and are presumed to be the units forming BR1 and BR2 which form the most conspicuous puffs.

Visualization of Nonnucleolar Transcription in Other Cell Types

After the observations on lampbrush chromosomes, the first clear visualization of the morphology of nonnucleolar or presumptive heterogeneous nuclear RNA (hnRNA) synthesis was reported by Miller and Bakken (85) for HeLa cells. RNP molecules were found to be attached to the genome at irregular intervals and widely spaced, indicating that the initiation of transcription occurs infrequently on active loci in this undifferentiated tissue-culture cell. Miller and co-workers (86) next dispersed chromatin from 4- to 6-hour *Drosophila* embryos and found well-defined RNP fibril gradients, presumably reflecting the genetic activity involved in differentiation events that occur during that embryonic period. More precise quantitative studies of hnRNA synthesis in insect embryos were done by Laird and co-workers for *Drosophila* and *Oncopeltus* (91, 177, 178) and McKnight and Miller (89) for *Drosophila.* The latter authors compared transcription during the syncytial stage and early cellular blastoderm, and found that, whereas there is only a low level of template activity with a few short, dense, RNP fibril gradients present in the syncytial stage, a large new class of much longer gradients with generally intermediate polymerase densities appears at cellular blastoderm, again presum-

ably reflecting genetic activity involved in differentiation events. In all of the embryonic studies, a large variation in length and RNA-polymerase density was found among hnRNA transcription units. Estimates of the average size of hnRNA molecules synthesized on such units range from 10 to 18 kb. Similar studies subsequently were done by Busby and Bakken (179) on sea-urchin embryos. These investigators found that a large majority of active transcriptional units exhibited only a single nascent RNP fibril, and concluded that the polymerase density on single, versus multiple, fiber loci is caused by polymerase initiation frequency.

In their initial study of hnRNA synthesis in *Drosophila*. McKnight and Miller (89) noted that homologous, nascent, fiber arrays often could be identified on sister chromatids after chromatin replication in late S or G2 stage of early cellular blastoderm. Such arrays appeared to offer a unique opportunity to compare regulation of transcription on two copies of the same genetic locus, and a number of these were analyzed in a subsequent study (180). The results showed that, although size and polymerase density vary considerably among different loci, nascent fiber frequency and distribution is essentially the same for homologous pairs, indicating that sister chromatids inherit precisely similar transcriptional potentials. In addition, it was noted that different, but immediately adjacent, genetic units can differ in polarity and fiber frequency.

The first presumptive visualization of a specific structural gene was reported by McKnight et al. (181) for the silk fibroin gene of *Bombyx mori* (Fig. 8). The long, RNP-fibril gradients observed in this study were identified as active silk fibroin genes on the basis of gene size, the presence of such gradients only in the posterior portion of the silk gland where fibroin synthesis is localized, their single-copy nature, and high RNA-polymerase density, all of which can be correlated with known biochemical parameters of silk fibroin gene activity.

In Vivo and In Vitro X. laevis Oocyte Systems for Transcription of Specific DNAs

Except in cases where, predominately, only one to a few genes are expressed in a cell type, the analysis of transcription of a single gene is difficult, because its contribution to total RNA synthesis is small. The two, recently developed transcriptional systems discussed below, when combined with the availability of purified specific genes, offer the potential of overcoming such difficulties.

In Vivo Transcription of DNA Injected into Amphibian Oocyte Nuclei

The first report of transcription of DNA after microinjection was given by Mertz and Gurdon (182), who showed that RNA homologous to Simian Virus 40, as well as to several other foreign DNAs, is synthesized in oocyte nuclei. Very soon thereafter, Brown and Gurdon (183, 184) showed that, after microinjection, accurate transcription of both genomic and cloned *Xenopus* 5S rDNA takes place, and is sensitive to the α-amanitan concentration expected for RNA polymerase-III inhibition. As much as half of the RNA synthesized by an injected oocyte can be a result of injected 5S DNA, although

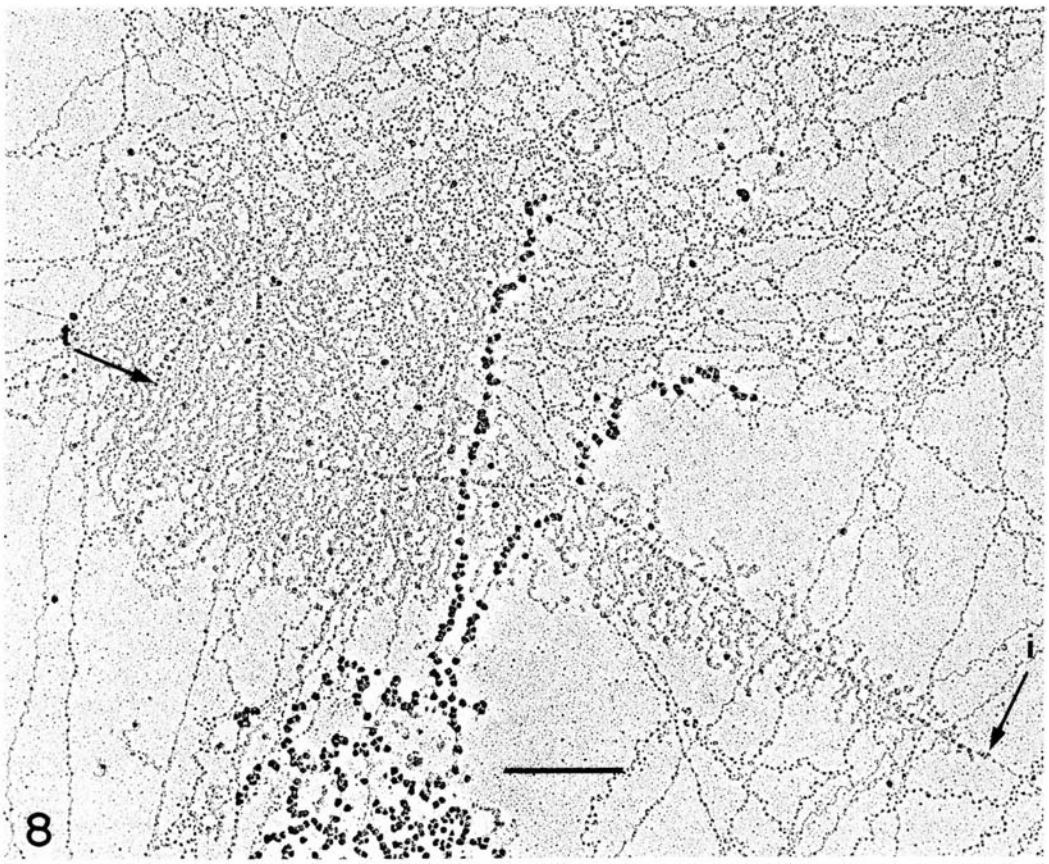

FIGURE 8 A putative silk fibroin transcription unit with arrows indicating sites of initiation (*i*) and termination (*t*) of transcription, from McKnight et al. (181). The contour length of the locus is ~5.3 μm, and ~200 RNA polymerase molecules were simultaneously transcribing the gene at time of isolation. The strings of dense granules lying across the gene are cytoplasmic polyribosomes. Bar, 0.5 μm. × 30,000.

at low inputs it can be shown that the injected DNA is transcribed only about one-fifth as efficiently as the endogenous 5S DNA. After injection, the 5S DNA becomes complexed with a near-equal mass of protein, which may be important for accurate transcription. Telford et al. (185) injected a *Xenopus* DNA segment containing the structural gene for $tRNA_1^{Met}$ and only 22 base pairs to the 5′ side of the gene. They found that mature $tRNA_1^{Met}$ was produced at a high rate from the injected fragment, and suggested the possibility that recognition between DNA and RNA polymerase III may be determined by the structural tRNA gene itself rather than 5′ sequences outside of the gene. Grosschedl and Birnstiel (186) identified three regulatory segments in the prelude sequences of a sea urchin H2A histone gene by injection of cloned specific deletion mutants, and, in view of their results, speculated that eukaryotic promoters may have to be viewed as three-dimensional, rather than linear, chromosomal structures. The first visualization of transcription of injected DNA was reported by Trendelenburg et al. (187), who used circular amplified *Dytiscus* rDNA as a source of foreign DNA. The injected rDNA becomes complexed with protein, and apparently normal, as well as abnormal, transcriptional patterns are observed (Fig. 9). A high frequency of abnormally long RNP fibrils suggests that proper termination of nascent pre-rRNA molecules may not always occur. Subsequently, Trendelenburg and Gurdon (188) injected homologous cloned rDNA and found that accurate transcription takes place, with activated genes exhibiting the typically dense gradients of endogeneous rRNA genes. However, more than 90% of the injected DNA is assembled into inactive nucleosomal chromatin configurations, indicating that transcription is not regulated by the supply of RNA polymerase I but presumably by some limiting component which switches genes maximally on.

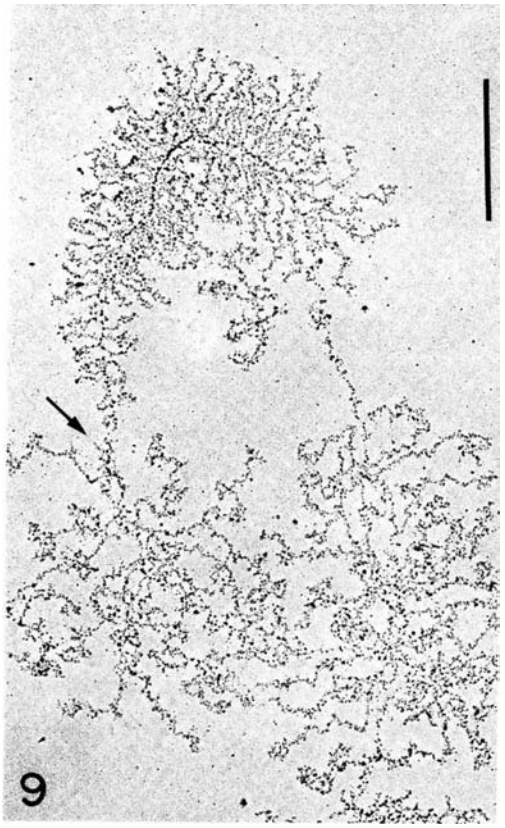

FIGURE 9 A circular, amplified rDNA molecule of *Dytiscus marginalis* isolated after microinjection into a *X. laevis* oocyte nucleus, from Trendelenburg et al. (185). The arrow indicates the initiation site of an apparently normal pre-rRNA fibril gradient. The longer fibrils in the spacer region of the molecule may possibly have arisen from lack of proper termination of nascent pre-rRNA fibrils. Bar, 1 μm. × 18,000.

In Vitro Transcription of DNA in a Nuclear Extract from Oocytes

Brown and co-workers (189) recently demonstrated that cloned 5S genes are transcribed accurately after an initial 30′ lag period when mixed with a supernatant fraction obtained from manually isolated, disrupted *X. laevis* oocyte nuclei. Although there is also significant transcription of the noncoding 5S strand, spacer, and plasmid DNA, up to 40% of the total RNA transcribed has been shown to be 5S RNA. Transcription involves RNA polymerase III, because this is the only active polymerase in this system. More recently, Brown and colleagues have shown by using deletion mutants that initiation of RNA polymerase III on 5S gene sequences can be maintained, as nucleotide pairs are sequentially removed from the 3′ end of the gene until nucleotides between 50 and 55 are reached (190). Similarly, initiation can be maintained as nucleotide pairs are removed from the 5′ end of the gene until between nucleotides 80 and 83 (as counted from the 3′ end of the gene) (191). These results demonstrate somewhat unexpectedly that the sequences responsible for proper initiation of RNA polymerase III are contained within the 33 nucleotides between nucleotides 50 and 83 of the gene itself.

Concluding Remarks

It has been possible, in a short review such as this, to list only some of the highlights of the discoveries by investigators studying the nucleolus and synthetic activities of chromosomes. Regretfully, many observations of interest have had to be omitted. I have attempted to communicate some of the excitement generated by the increase in our knowledge regarding the function of the nucleolus and structural aspects of genetic transcription. Much of the progress in these areas, as in others, has been a result of the application of new techniques that have proved to be powerful probes in our attempts to understand the molecular basis of genetic activity. Much, much more remains to be discovered, but many tools are available and others will be forthcoming. Only the continued imagination and diligence of young scientists is required for further, exciting discoveries.

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RNA Processing Comes of Age

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During the past two decades, an awareness of the importance of RNA processing has evolved as part of the quest to understand how living cells express the information encoded in their genes. As the knowledge of gene expression has expanded, we have come to realize that the old central dogma of DNA → RNA → protein is embellished with elegant and intricate design features, many of which are revealed in the processing of primary gene transcripts into the functional forms of RNA. The production of the two large structural RNA components of the ribosome (rRNAs), the synthesis of transfer RNA (tRNA), and the formation of messenger RNA (mRNA) in higher organisms all involve rather elaborate processing reactions, including nucleolytic cleavages, ligations, terminal additions, and nucleoside modifications.

A *raison d'être* for processing is readily apparent in the case of the coordinate production of rRNA components from a single transcriptional unit and the synthesis of mRNA from noncontiguous genetic elements. However, the purpose of the polyadenylate and methylated cap structures that are added to the termini of mRNA and the modification of internal nucleotides in most RNA species is less clear. These structural alterations may serve to improve the stability of the RNA and the efficiency of its function, but they might also be implicated in more subtle forms of discriminative regulation that are yet to be discovered. In any event, it is clear that RNA processing constitutes a major cellular activity and an integral part of the mechanism of gene expression.

In this essay I shall try to trace the evolution of our concepts of RNA processing in relation to the contemporary issues of cellular and molecular biology and to the introduction of key experimental tools which were critical to the development of these concepts. My idea for treating the subject in this way came from an engaging article on the nucleolus by a former colleague and source of inspiration, Jack Schultz (1). It is my intention to provide both a historical and a reasonably up-to-date overview of the subject without the burden of extensive detail. Fortunately, there are several recent reviews to which the reader can refer for a more comprehensive coverage of particular aspects of RNA processing (2–7).

Conceptual Origins

By the late 1950s, the idea that RNA plays the role of principal intermediary in information transfer between DNA and proteins was generally accepted (8). This concept became very popular and was considered part of a central dogma with which all phenomena dealing with genetic expression should be interpreted (cf. [9] for details). However, there was considerable confusion as to how RNA served this role until it was realized that there were several distinct classes of RNA, each with a different function in the overall process. The problem was being attacked in both prokaryotic and eukaryotic systems, the prokaryotes offering ease of genetic and nutritional manipulation, the eukaryotes the advantage of cellular compartmentalization and microscopic visibility. Several powerful new techniques were applied: autoradiography with tritiated nucleosides to localize the intracellular sites of RNA synthesis; multiphase extraction with phenol and detergent to obtain undegraded preparations of RNA; ultracentrifugation and sucrose gradient sedimentation to fractionate various RNA molecules and subcellular particles according to size; and electrophoresis and chromatography to measure RNA base composition.

It soon became evident that the bulk of the stable RNA in all types of cells consists of two homogeneous components, both of which are associated with the ribosome, a structure already known to be implicated in protein synthesis. The rRNAs were given names according to their sedimentation coefficients—16S and 23S for the bacterial species and 18S and 28S for the mammalian species. The other abundant RNA species identified at that time was transfer RNA, then called soluble RNA or 4S RNA because of its nonparticulate nature and small size. The biosynthesis of ribosomal RNA in *Escherichia coli* was studied by elegant isotope incorporation experiments (10), which served as models for future kinetic studies of RNA processing. However, there was not yet any evidence for precursors that were larger than the mature rRNAs or even for the existence of rRNA genes in bacterial cells. Nevertheless, by 1960 there was substantial evidence from both autoradiographic and cell fractionation studies to indicate that in higher organisms cytoplasmic RNA was derived from the nucleus, and in particular from nucleoli, which appeared to be especially active sites of synthesis (cf. [11] for references). Recalling earlier cytogenetic evidence that invoked the existence of the nucleolus organizer locus, one began to deduce that nucleoli were in fact the sites of the rRNA genes. This was conclusively demonstrated in the early to mid-1960s by a large variety of experiments which exploited both genetic and biochemical tools (12). Important in this regard was the introduction of the very powerful nucleic acid hybridization technique (13), which not

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only provided evidence for the existence of rRNA genes in both prokaryotes and eukaryotes, but allowed their enumeration, as well.

In 1962, when radioactively labeled nuclear (nucleolar) precursors of rRNA were examined by sucrose gradient sedimentation analysis, an unexpected fact was revealed (14, 15). Instead of the precursors being the same size as the mature cytoplasmic rRNA, they were substantially larger. The use of actinomycin D to block RNA synthesis (16), while allowing some processing to occur, helped overcome the difficulty of doing an effective chase experiment in mammalian cells, and provided compelling evidence that these large RNA components were indeed rRNA precursors. The kinetics of labeling of the pre-rRNA components suggested the following sequence for rRNA processing events in mammalian cells: 45S → 35S → 28S + 18S (15). Thus, although there was no precedent for it, the notion arose that primary products of genes might need some sort of "transformation" or "processing" in order to convert them into functional entities. The idea that RNA could be altered posttranscriptionally was concurrently being developed in studies which showed that the "minor nucleotides" in tRNA arise by the modification of previously synthesized polynucleotides (17).

By this time, a substantial number of convincing experiments with bacterial and bacteriophage systems had indicated the existence of messenger RNA (9), and considerable effort was being made to determine whether mRNA was also present in higher organisms. Fractions of a "DNA-like" heterogeneous RNA were extracted from nuclei (15, 18–20) and suspected of being related to mRNA, but because we knew relatively little about the properties of mRNA, there was still some uncertainty as to whether it was also subject to processing. When it became apparent that polyribosomal mRNA was indeed smaller than the heterogeneous nuclear RNA (hnRNA), one began to give serious consideration to the notion that mRNA, like rRNA, was derived from a larger primary transcript. Although this idea was spawned in the mid-1960s, it took more than a decade for it to gain universal acceptance (see reference [6] for details).

The resolution of RNA on sucrose gradients is barely adequate to distinguish the 20% average-size difference between newly made and mature tRNA. Hence, the late 1960s, when polyacrylamide gel electrophoresis came into use for RNA size analysis (21), its superior resolution made it relatively easy to discriminate the larger pre-tRNAs found in pulse-labeled mammalian RNA from mature tRNA (22). Thus, it became generally accepted that tRNAs are also processed from larger precursors in eukaryotes. Similar analyses of 5S ribosomal RNA failed to reveal any oversized precursors, and 5S rRNA was thought to be the "exception to the rule." However, recent experiments using gels of higher resolving power and nucleotide sequence analysis (23) have demonstrated that 5S rRNA transcripts are 5–10% larger than mature 5S rRNA, and, hence, that they require processing as well.

For many years it was believed that the processing of large RNA precursors into smaller, mature forms was a distinctive property of eukaryotic cells. However, studies in the early to mid-1970s with prokaryotic systems carrying mutations that block or attenuate processing (24) revealed that processing also occurs in prokaryotes, the main difference being that the processing reactions follow transcriptional events much more closely than in eukaryotes, so that full-length transcripts are less readily observed. Other types of processing reactions, such as methylations and terminal additions, also exist in prokaryotes, although they are generally less elaborate than in higher organisms.

Further Developments

RIBOSOMAL RNA: Several important technical developments helped extend our knowledge of rRNA processing. Detailed chromatographic analyses of rRNA derivatives revealed that methylations occurred on both base and ribose moieties, with the ribose methylations largely predominating (25). It was found that most of the methyl groups are added to the initial (45S) precursor and that essentially all are conserved during processing (26). This finding, together with improved cellular fractionation techniques, with which one could obtain highly purified nucleoli or nucleoplasmic fractions, and polyacrylamide gel electrophoresis, with which one could separate the various precursors and processing intermediates, led to the formulation of a fairly detailed processing pathway for rRNA (27). Additional facts were supplied by fingerprint analysis of methylated oligonucleotides (28) and by analysis of the patterns of base-paired loop structures that persist under partial denaturing conditions (29).

Valuable contributions to our understanding of rRNA processing have also come from studies of the organization of rRNA genes. Buoyant density/hybridization studies of rDNA (30, 31) and electron-microscope visualization of transcribing rRNA genes (32) provided convincing evidence for the linkage of the 18S and 28S gene elements into a single transcriptional unit, and revealed the existence of nontranscribed spacer DNA separating each transcriptional unit. The mutual reinforcement of the parallel studies with rRNA precursors and with rDNA greatly accelerated the general acceptance of a rather complicated and apparently "uneconomical" scheme for the production of ribosomal RNA.

Our current view of rRNA synthesis and processing in mammalian cells is diagrammed in Fig. 1 (2). The 18/28S transcriptional unit is of the general form: $^{5'}$spacer-18S rRNA-spacer-28S rRNA$^{3'}$. Its primary product is a 45S molecule of about 12.5 kb. Lower eukaryotes have the same general organization, except that the lengths of the various segments, especially the spacers, are shorter. The processing pathway can be described in terms of four principal events involving cleavages at or near the sites numbered 1 to 4. The first cleavage at site 1 removes the 5′-terminal leader sequence. The second

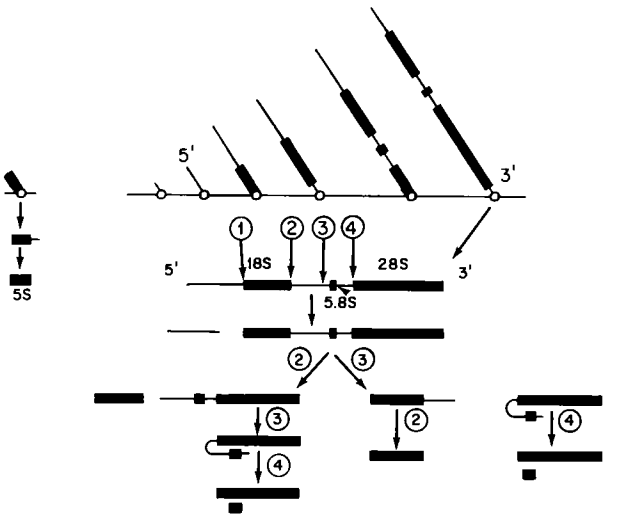

FIGURE 1 Scheme for processing of mammalian ribosomal RNA.

cleavage can be either at sites 2 or 3, depending on the species of cell and to some extent on environmental conditions; the predominant pathway apparently is determined by the conformational state of the first intermediate. The final triming near site 4 is usually the rate-limiting step in the processing pathway, thus causing a substantial accumulation of the proximal intermediate, the 32S component. This trimming involves at least two cuts in the polynucleotide backbone, one at the 5′ end of the 28S component and another at the 3′ end of the 5.8S component. The 5.8S component, 140 nucleotides in length, is a stretch in the 5′ region of the 32S component which remains bound to 28S component by base pairing after the final cleavage. In a portion of the rRNA genes of *Drosophila* and in the mitochondrial and chloroplast rRNA genes of some primitive eukaryotes, the segment encoding the large rRNA components is interrupted by an intron (33–35). When such genes are expressed, the intron sequence is probably removed by a splicing mechanism akin to that used for the excision of mRNA or tRNA introns (*vide infra*).

The 5S rRNA genes, which are situated remotely from the 18/28S genes (usually on different chromosomes), are transcribed into molecules which have the 5′ terminus of mature 5S RNA and a stretch of 8 or more extra nucleotides at the 3′ end (23). Processing involves removal of these extra nucleotides and possibly the addition of a terminal U residue.

The organization of rRNA genes in the well-studied prokaryote, *E. coli*, resembles that in eukaryotes, except that the 5S rRNA gene and one or more tRNA genes are also included in the transcription unit, the 5S gene being located on the 3′ side of the 23S gene and the tRNA genes being either in the spacer between the 16S and 23S genes or at the 3′ end beyond the 5S gene (3). This difference in gene organization is accompanied by a basic difference in transcribing enzymes. In prokaryotes all genes are transcribed by a single species of RNA polymerase, whereas in eukaryotes polymerase I is used for 18/28S genes and polymerase III for the tRNA and 5S rRNA genes. Another basic difference, alluded to earlier, is that in prokaryotes processing cleavages usually occur before the polymerase is finished transcribing the gene, whereas in eukaryotes cleavages generally occur on complete transcripts.

Several enzymes are known to be implicated in the processing of *E. coli* rRNA (3). The best known is RNAse III, which requires double-helical RNA as part of its recognition element. This enzyme is responsible for separating the precursor segments containing the 16S, 23S, and 5S components. These precursor segments are in turn acted upon by at least two additional ribonucleases. The tRNA segments are processed by a separate set of enzymes (*vide infra*). It is generally believed that some of the cleavages involved in the processing of eukaryotic rRNA also require double-helical specificity, although the isolation and characterization of the relevant nucleases has not yet been achieved (see reference [2] for details).

The development of the powerful tools of gene manipulation, e.g., the use of transducing phages and recombinant DNA methods, together with the development of techniques for rapid nucleotide sequencing, has now made it possible to describe the rRNA genes and their products at the level of nucleotide sequence. The sequence information enables one to construct plausible models of RNA secondary structure, and hence to examine possible substrate specificities for the various processing enzymes (3, 36). Such an analysis has led to the remarkable conclusion that the substrates for RNase III are a pair of hairpins with loops consisting of the entire 16S (~1,600 nucleotides) or 23S (~2,800 nucleotides) rRNA components (Fig. 2). These models also indicate additional cleavage sites for the other enzymes that may be involved in rRNA maturation. If eukaryotic rRNA precursors have an analogous structural organization, one could imagine that there may be multiple cleavages at each of the processing sites described in Fig. 1.

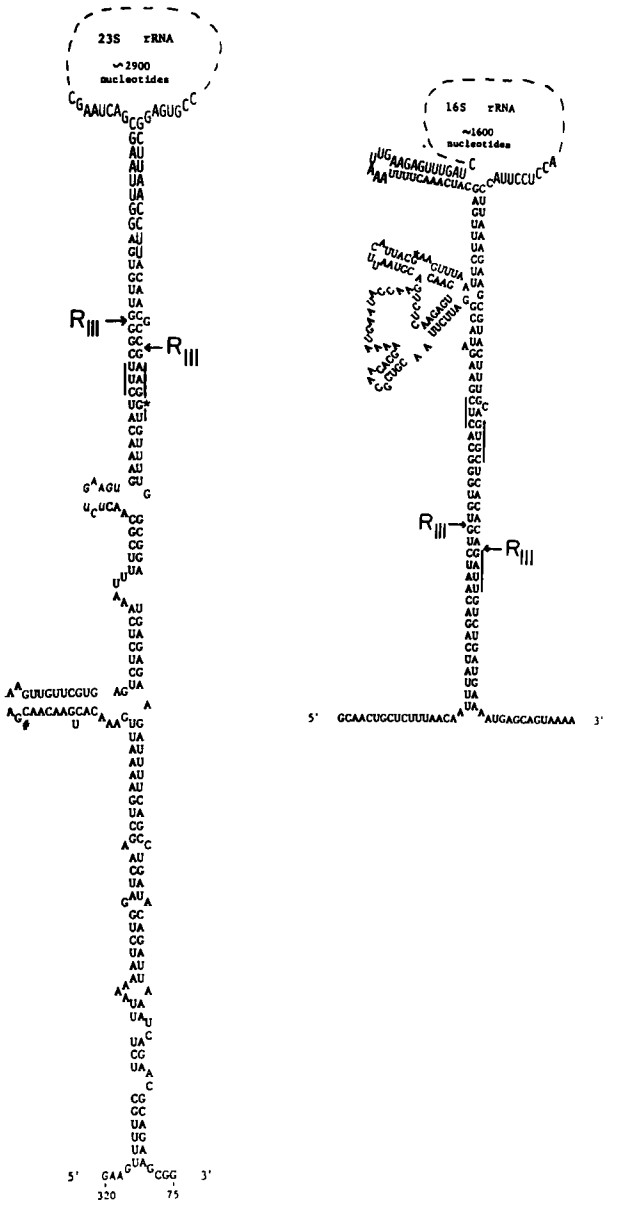

FIGURE 2 Secondary structures in the *E. coli* rRNA precursor which form cleavage sites for RNAse III (from [36]). The terminal nucleotides of the mature rRNA are in large type; the remainder are shown as dashed lines. Vertical lines show regions of homology with T7 mRNA sequences that appear at the same location relative to RNAse III cleavage sites.

TRANSFER RNA: Although tRNA precursors were initially found in mammalian cells, the major progress in elucidating the details of tRNA processing has been made with bacterial and yeast systems. The key factors in this development were the discovery of mutants blocked in the processing of tRNA and the ability to manipulate the tRNA genes (see [2] and [3] for references). Processing-defective mutants with lesions in either the tRNA genes themselves or in key tRNA

processing enzymes were used to work out the details of the pathways. In *E. coli*, the tRNA precursors have the general form $^{5'}p$-leader-(tRNA-spacer)$_n$-tRNA-trailer-$^{3'}$OH. They may be either monomeric ($n = 0$) or multimeric ($n = 1$–6), and in cases such as the rRNA transcription unit, they may be joined to other gene products. In higher organisms, most, if not all, tRNA precursors are monomeric. The 5′-leader sequence is removed by an enzyme termed RNAse P. This enzyme appears to contain an RNA molecule, as yet not well characterized, that is essential for its activity (37). The substrate recognition for RNAse P seems to reside largely in the structural features of the mature tRNA product, rather than in the sequences surrounding the cleavage point. Removal of the trailer sequence apparently requires an additional endonuclease as well as an exonuclease. Sometimes the tRNA precursors do not contain the universal CCA 3′ terminus; when this is the case, the CCA is generated by a specific terminal transferase. Nucleoside modifications can occur on intact precursor molecules as well as on cleaved products.

With the advent of recombinant DNA methodology, detailed investigations of various yeast tRNA genes were made. These studies have revealed the presence of introns in several (but not all) tRNA genes (3). Thus, the processing of yeast tRNA requires, in addition to the cleavages described above, a splicing activity to remove the introns. In vitro processing experiments have revealed that the splicing activity can be resolved into two steps: a nuclease activity, which occurs in the absence of ATP, and a ligase step, which requires ATP (38). Surprisingly, the nuclease catalyzes the cleavage of phosphodiester bonds so as to yield 3′-phosphates and 5′-hydroxyl groups. This is in marked contrast to RNAse P and RNAse III cleavages, which yield 3′ hydroxyls and 5′ phosphates. In higher eukaryotes, tRNA genes containing introns may be less common (39, 40), although the number of organisms studied in detail is too small to permit broad generalizations. Intron-containing yeast pre-tRNA can be properly processed in *Xenopus* oocytes, indicating that the tRNA processing enzymes are very similar in different species (41).

MESSENGER RNA: As mentioned above, the notion of mRNA processing began with the finding that heterogeneous nuclear RNA (hnRNA) and polyribosomal mRNA have strikingly different size distributions and yet very similar base compositions. Before the development of recent techniques that have enabled us to study the synthesis of individual species of mRNA, an investigator wishing to probe the relationship between hnRNA and mRNA had to employ methods that were suitable for complex mixtures of RNA sequences, and to exploit, whenever possible, features such as poly A that are common to a substantial fraction of the mRNA species. Nucleic acid hybridization is a technique that may readily be applied to complex mixtures of RNA, and thus enjoyed wide popularity in the study of hnRNA and mRNA, beginning in the late 1960s and extending over more than a decade (see [2, 4, 6, 42] for references). The information gained from these studies, together with concurrently acquired knowledge of the general properties of eukaryotic DNA sequences, e.g., the existence of single-copy and repetitive sequence elements (43), provided a new framework for comparing the properties of hnRNA and mRNA. It was evident that moderately repetitive, as well as unique, sequences are transcribed into hnRNA and that at least a portion of these same sequences are processed into mRNA (44). However, the biological significance of these repetitive sequence transcripts was not obvious and, in fact, still remains one of the challenging mysteries in our understanding of eukaryotic gene expression.

Around 1970, two important discoveries helped accelerate progress on the problem of mRNA processing. First was the finding that the majority of mRNA molecules and a significnt fraction of hnRNA molecules possess a 3′-terminal poly-A segment, 150–200 nucleotides long, which is constructed post-transcriptionally (see [5] for references). The poly-A tail represented an interesting new aspect of processing, but, even more importantly, it was rapidly exploited for purifying mRNA away from the bulk of the cellular RNA (45–47). Second was the discovery of reverse transcriptase (48, 49), which was later used to synthesize DNA complementary to mRNA (cDNA), thus providing a valuable probe for the study of mRNA frequency distributions and the homology relationships between mRNA and hnRNA (see [42] for references). Most cell types were observed to have a very broad distribution of mRNA abundancies, ranging from a few species present at several-thousand copies per cell to thousands of species present in a few copies per cell. About 10–20% of the hnRNA sequences are homologous to mRNA.

In the mid-1970s, it was discovered that the mRNAs of eukaryotic cells and many types of viruses contain an unusual methylated "cap" structure (Fig. 3) at their 5′ terminus and one or more internal 6-methyl adenine residues (see [7] for references). These modifications, like poly A, are added posttranscriptionally to the mRNA precursors, and then carried along through the rest of the processing stages. For a long time it was thought that the capacity to be methylated was a property confined to the structural RNAs, i.e., the RNAs that do not encode proteins. This idea persisted because the level of methylation in mRNA is almost an order of magnitude lower than in rRNA, and without a means for effectively separating these two RNA species, the mRNA methylation is entirely masked—especially if one doesn't know that it is there in the first place. However, when methods for isolating mRNA based on its unique poly-A structure came into use, one could obtain sufficiently pure preparations of mRNA so that an unambiguous identification of its methylated derivatives could be made (51, 52). The parallel development of efficient cell-free systems for the synthesis of certain viral mRNAs contributed similarly to the characterization of their modified components, and, moreover, provided an excellent means for studying the biochemistry of cap formation (53, 54).

The formation of a complete cap structure (Fig. 3) involves the participation of four to six different enzymes (see [7] for

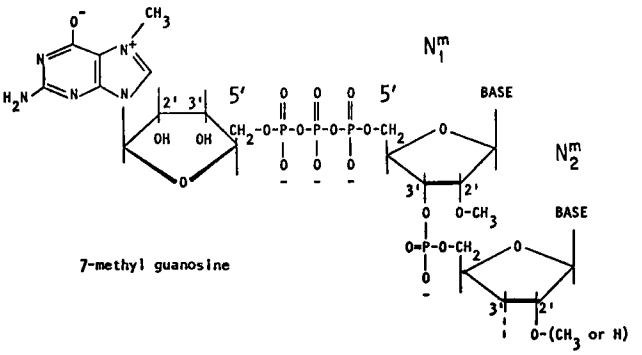

FIGURE 3 The 5′-terminal cap structure on eukaryotic messenger RNA. The 2′-*O*-methylation at position N_2 occurs in the cytoplasm on some, but not all mRNAs (50).

references). These are summarized by the following set of reactions:

(*a*) $pppN_1pN_2p.... \rightarrow ppN_1pN_2p.... + p_i$; or

(*a'*) $\underset{\alpha}{pN_1pN_2p}.... + ATP \rightarrow \underset{\alpha\beta\alpha}{ppN_1pN_2p}.... + ADP$;

(*b*) $Gppp + \underset{2\beta\alpha}{ppN_1pN_2p}.... \rightarrow GpppN_1pN_2p.... + pp_i$;

(*c*) $AdoMet + GpppN_1pN_2p.... \rightarrow {}^7mGpppN_1pN_2p.... +$ Ado-S-homocys;

(*d*) $AdoMet + {}^7mGpppN_1pN_2p.... \rightarrow {}^7mGpppN_1{}^mpN_2p....$ + Ado-S-homocys;

(*e*) If $N_1{}^m = A^m$: $AdoMet + {}^7mGpppA^mpN_2p.... \rightarrow {}^7mGppp{}^6mA^mpN_2p....$ + Ado-S-homocys;

(*f*) $AdoMet + {}^7mGpppN_1{}^mpN_2p.... \rightarrow {}^7mGpppN_1{}^mpN_2{}^mp....$ + Ado-S-homocys.

The relevant enzymes are (*a*) RNA triphosphatase; (*a'*) RNA 5′ monophosphate phosphokinase; (*b*) mRNA guanylyltransferase; (*c*) 7-methylguanosine methyltransferase; (*d*) and (*f*) 2′-*O*-methyltransferase; and (*e*) 6-methyl-(2′-*O*-methyladenosine) methyltransferase. Reactions (*a*) through (*e*) occur in the nucleus either during transcription of pre-mRNA or soon after its completion (55, 56). Reaction (*f*) occurs in the cytoplasm after the mRNA has been incorporated into polyribosomes (50).

There is presently some uncertainty about whether cap formation occurs exclusively at the sites of transcriptional initiation (via reaction [*a*]), or at internal cleavage sites as well (via reaction [*a'*], or by a variation in reaction [*b*], cf. reference [7]). Initially it was believed that transcription could initiate only with purine nucleoside triphosphates and that cap structures with pyrimidines in position N_1 (about one-fourth of the total mRNA in mammalian cells) must be formed at internal cleavage sites. Indeed, studies of the 5′ termini of hnRNA seemed to confirm this idea (55). However, the recent demonstration that transcription can also be initiated by pyrimidine nucleoside triphosphates (57) suggests that this point should be reexamined (cf. also reference [56]).

To date, all of the known cap structures on mRNAs of defined coding specificity have purines at position N_1, and for several of these mRNAs there is evidence to indicate that N_1 is also the site of transcriptional initiation. This evidence is sometimes of a negative type, i.e., failure to detect any transcripts of sequences that are located upstream from the cap site (cf. references [58, 59]), so that the possibility of extremely rapid processing of a 5′ initiator region cannot be rigorously excluded. However, in the case of adenovirus mRNA synthesis, there is strong positive evidence from both in vivo and in vitro studies to indicate that the mRNA cap sites and transcriptional initiation sites for polymerase II are, in fact, one and the same (60–62). Comparisons of nucleotide sequences in the 5′-flanking regions of genes coding for several cellular and viral mRNAs (including the adenovirus mRNAs) has revealed the existence of a 7 base pair AT-rich sequence about 28 nucleotides upstream from the cap site (63, 64). This sequence is similar, although not identical, to the so-called Pribnow box ($^{5'}$-TATAATG-$^{3'}$), which is universally part of the promoter regions of prokaryotic genes (65). Given an equivalence of initiation and cap sites (at least for some mRNAs) and the fact that the mRNA guanylyltransferase does not require a lengthy polynucleotide acceptor, it is reasonable to expect that cap formation will often occur on growing pre-mRNA chains (cf. references [55, 56]).

It is conceivable that transcription of some genes can initiate at more than one site with different relative efficiencies, as happens with rRNA genes in *E. coli* (57). This might be an explanation for the heterogeneity of cap structures on certain SV40 and polyoma mRNAs (66, 67), and for the ability of certain SV40 mutants to survive deletions at a capsite (68). However, if such imprecise initiation ever occurs with cellular mRNAs, it presumably is confined to relatively sparse mRNA species, because the various abundant mRNA species studied to date all seem to have a homogeneous 5′ cap (7).

Certainly one of the most surprising developments in the history of mRNA processing was the discovery of splicing. The initial observations were made in 1977 during investigations of adenovirus mRNA synthesis (69, 70), in which the relationship between viral mRNA and the DNA that encodes it were examined in the electron microscope using the powerful R-loop technique (71, 71a). The striking multiloop structures were correctly interpreted to mean that the mRNA was specified by several noncontiguous genetic elements. In spite of its novelty, this interpretation was readily accepted because it explained the (then) puzzling observation that mRNAs made from distinct portions of the adenovirus genome have the identical 5′-terminal capped sequence (72, 73). Moreover, it also seemed to be a possible solution to the riddle of how a large hnRNA molecule with a cap structure on one end and a poly-A tail on the other could be processed into a smaller mRNA molecule without losing either its cap or its poly A. Within a matter of months, experiments employing restriction-enzyme analysis with Southern's blotting technique (74) and R-loop or heteroduplex analyses of cloned gene fragments established the widespread occurrence of split genes and gave some idea of their organizational features. Studies of the organization of SV40 genes (75, 76) and of cellular genes like globin (77, 78), immunoglobulin (79), and ovalbumin (80, 81) indicated that the interruptions, termed intervening sequences or introns, can occur in the coding portions of the gene as well as in 5′-untranslated leader sequences.

The expression of split genes always seems to involve production of a composite RNA transcript and subsequent excision of the intron sequences. This has been established first by showing that there are large nuclear transcripts which are colinear with the complete gene (82), and second by using kinetics and pulse-chase experiments to demonstrate that the large transcripts are actually processed into mRNA (83–86). The tendency of hnRNA to aggregate because of intermolecular base-pairing (87) makes it imperative to use rigorous denaturation conditions in such studies, for example, fractionation of the hnRNA by electrophoresis on methyl-mercury-agarose gels (88). This technique coupled with a blotting procedure by which the fractionated hnRNA is covalently attached to diazotized paper (89), enables one to visualize precursors of any mRNA for which a pure sequence probe is available. In fact, by using an assortment of probes for structural and intronic sequences, one can in principle delineate the processing pathway. An example of such an analysis for immunoglobulin light chain mRNAs is shown in Figs. 4 and 5.

In a transcript containing multiple intronic sequences, there may be a preferential order of excision, but in some cases the order is not necessarily absolute (91). This is reminiscent of the alternative temporal order observed in the processing of mammalian rRNA (2). In certain viral systems like adenovirus and SV40, a given transcript can give rise to multiple mRNA species, depending on the choice of different splicing modes. In this case, processing can have a role in determining qualitatively which gene elements are utilized. Such qualitative discrimination at the processing level could provide a basis for certain types of cellular differentiation. An example of this principle has been recently invoked for early B lymphocytes,

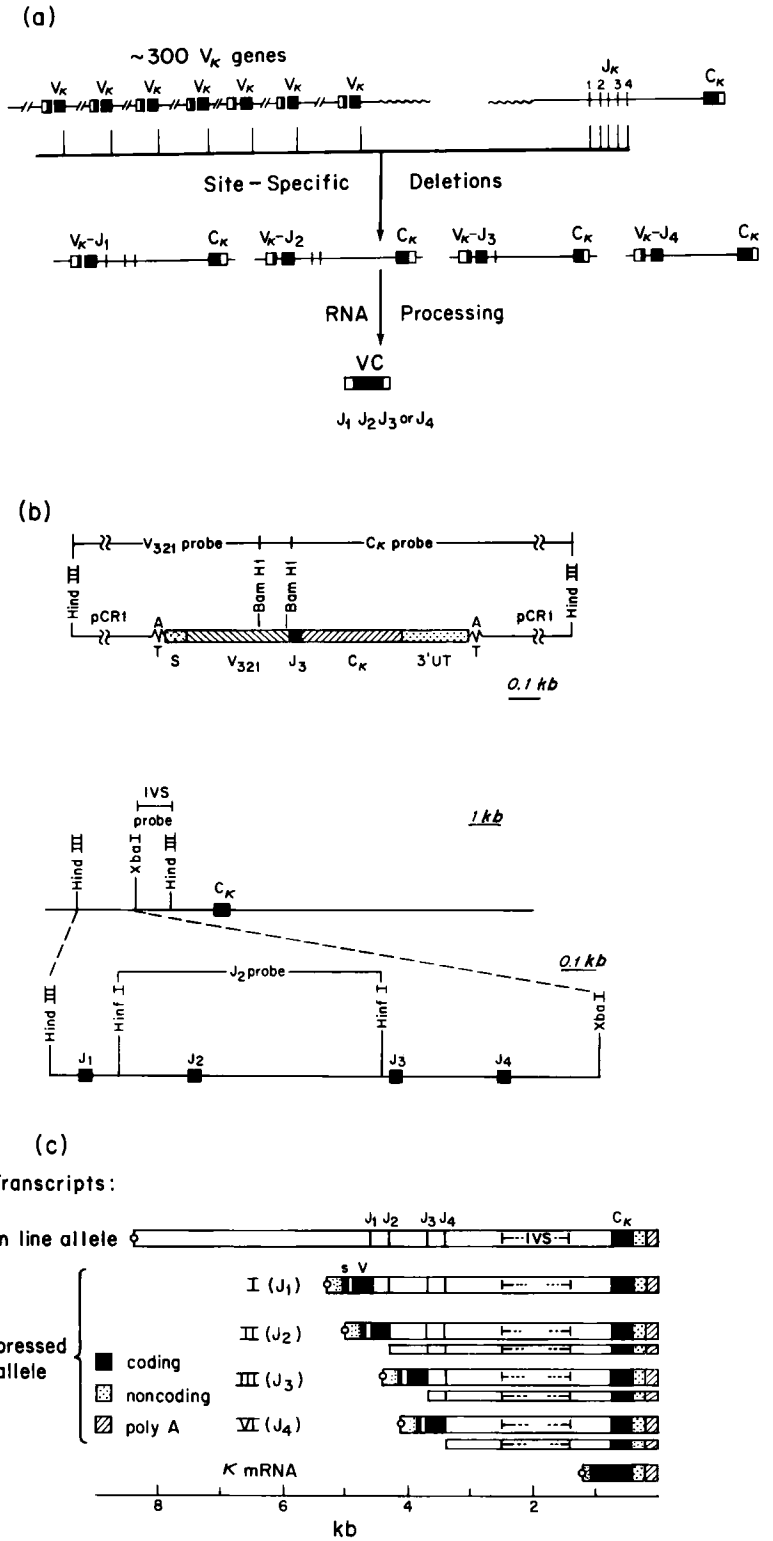

FIGURE 4 (a) Scheme for the formation and expression of a κ-chain immunoglobulin gene. In germ line DNA the several hundred genes coding for the variable part of the κ-chain (V_κ genes), and the gene coding for the constant portion (C_κ gene) are separated by an unknown distance. During B cell differentiation, a site-specific deletion occurs between one of the V_κ genes and any one of four J_κ segments located 2.4–3.9 kb upstream from the C_κ gene. This event creates a functional κ-gene the size of which depends on the particular J_κ segment being utilized. Such functional rearrangements are normally found on only one of the allelic pair of chromosomes. The intervening sequence between J_κ and C_κ (J-C intron) is transcribed and the corresponding RNA sequence excised during RNA processing. The 3′-untranslated sequence is contiguous to the C_κ sequence. The 5′-untranslated sequence and the sequence encoding the amino-terminal signal peptide are separated from the V_κ gene by a small (~0.1 kb) intron. (b) Four probes used in the analysis of κ-mRNA transcription and processing shown in Fig. 5. The V_κ and C_κ probes are obtained by restriction endonuclease digestion of a cloned cDNA sequence corresponding to the κ-mRNA produced by MOPC 321 myeloma cells. The J_2 and intervening sequence (IVS) probes are similarly obtained from a cloned fragment of germ line DNA containing the J_κ-C_κ region. (see reference [90] for details). (c) Schematic representation of the transcripts produced by various myeloma cells. The unrearranged (germ line) allele produces an 8.4 kb transcript which is not processed into any functional mRNA. The allele encoding the expressed κ-chain is transcribed into a component the size of which varies according to the J segment being used (5.3, 5.0, 4.4, and 4.1 kb, respectively, for J_1, J_2 J_3, and J_4 expressors). These precursors are processed into a common 1.2 kb κ-mRNA. In J_2, J_3, and J_4 expressors components are found which seem to arise by an asynchronous cleavage at the 5′ boundary of the J-C intron (narrow bars).

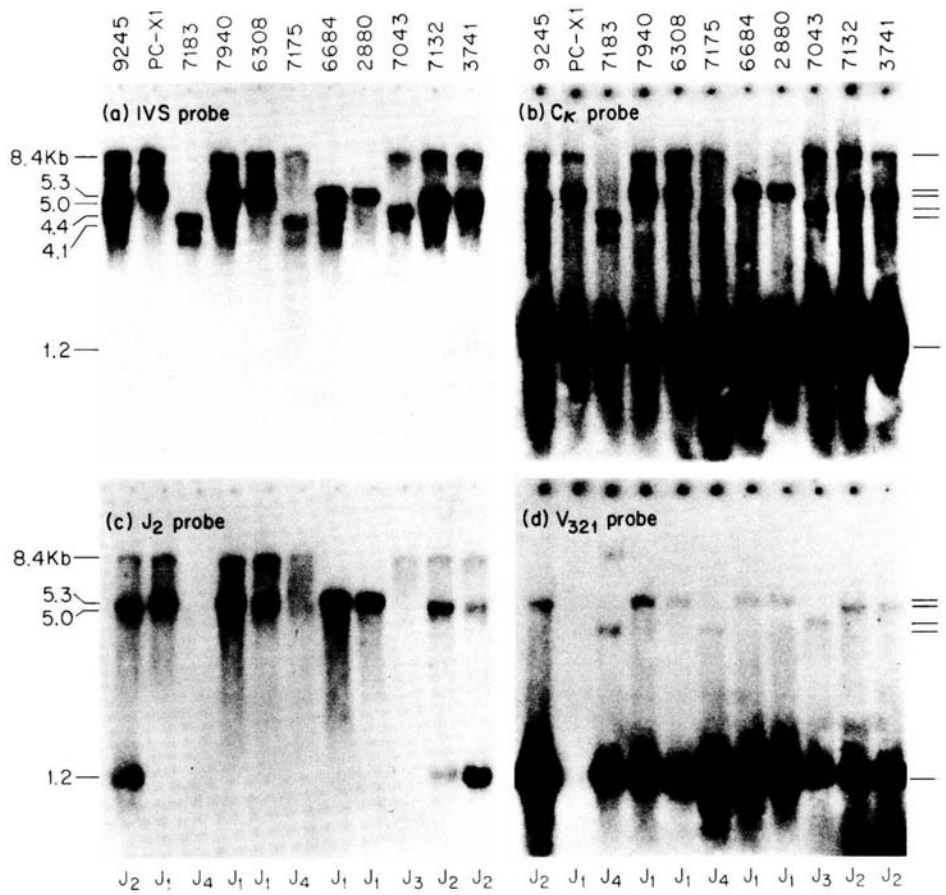

FIGURE 5 A "northern blot" of the poly A$^+$ nuclear RNA from eleven different myelomas expressing distinctive κ-chains. The poly A$^+$ nuclear RNA was electrophoresed on methylmercury hydroxide gels and blotted onto diazotized paper. The immobilized RNA was annealed with the four probes described in Fig. 4b, and the nuclear components containing the corresponding sequences were revealed by autoradiography. The tumor designation is shown at the top; the expressed J segment (from amino acid analysis of the κ-chain) is shown at the bottom. The size of the various bands (in kilobases) is at the left. The interpretation of these data is given in Fig. 4c. See reference (90) for other details.

in which two distinct mRNA species encoding the membrane-associated and secreted forms of the μ heavy chain are apparently produced from a single set of μ-gene elements by variations in the modes of splicing (92). The mRNAs are identical except for a region near the 3′ end, which in one case encodes the carboxy-terminal tailpiece of the secreted μ chain, and in the other, a hydrophobic segment that apparently anchors the membrane μ chain to the lipid bilayer. During its ontogeny the B lymphocyte shifts from producing predominantly membrane μ chain to predominantly secreted μ chain, presumably by shifting its major mode of μ-mRNA processing.

Although the enzymes involved in mRNA splicing have not yet been characterized, some clues concerning the splicing mechanism have come from comparisons of the nucleotide sequences surrounding the splice junctions and from studies of the consequences of perturbations in gene organization. A compilation of a large number of junction sequences of both cellular and viral pre-mRNAs has resulted in the consensus sequence shown in Fig. 6a (93). The doubly underlined nucleotides at the extreme ends of the intron are almost ubiquitous, being present in more than 95% of the sequences. Thus, it seems reasonable to suppose that they are essential for the splicing reactions. An interesting complementarity has been noted between the consensus sequence and a 5′-terminal sequence of one of the small nuclear RNAs (snRNAs), termed U1 (94), suggesting a model in which U1-RNA helps juxtapose

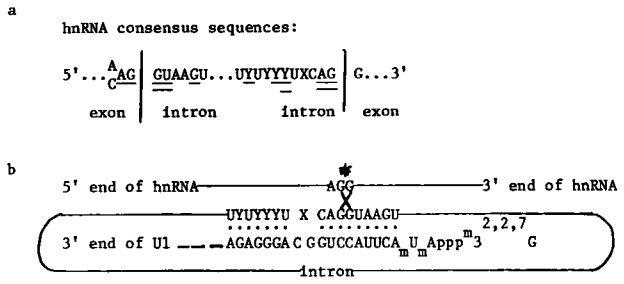

FIGURE 6 (a) A consensus sequence obtained by comparing 36-splice junction sequences (93). To appear in this sequence a base must be the most common in that position and occur with a frequency of at least 45%; bases occurring in 75% of the sequences are underlined; those present with 95% or greater frequency are underlined twice. Y indicates pyrimidines. X marks the position of a single non conserved base in the consensus sequence. Vertical lines mark the intron-exon boundaries. (b) A possible alignment of intron-exon boundaries by base pairing between the 5′-terminal portion of U1 RNA and sequences at both ends of an intron. Processing would consist of cleavage of two G-G bonds and formation of a new one (asterisk).

the two-splice junctions by appropriate base pairing interactions (Fig. 6b). A similar role has been invoked for the similarly sized VA-RNA in the splicing of adenovirus mRNA (95). If this speculation turns out to be correct, the function of at least

one small nuclear RNA will have finally been elucidated. Although the snRNAs were discovered more than 10 years ago, it has not been possible to assign them any specific cellular function. Fortunately, the uncertainty about their physiological significance did not deter studies of their structural characteristics, and by the mid-1970s the complete nucleotide sequence of two snRNA species was known (96). Indeed, their novel, highly modified 5′ termini served as a model for subsequent elucidation of the cap structures of mRNA (97, 98). In the snRNAs the ^{7}mG moiety is replaced by $^{2,2,7}m_3G$; otherwise the structures are essentially the same. The snRNAs are evolutionarily conserved, a fact which may be related to the apparent conservation of mRNA processing systems (41, 99–101).

Studies with total pre-mRNA populations (55, 56) and individual pre-mRNAs (86, 91, 102–105) indicate that cap formation internal methylation and polyadenylation usually precede the splicing out of intronic sequences. Thus, in regard to the general order of processing reactions, mRNA seems to resemble rRNA and tRNA, in that the cleavages are directed at molecules which have already been subjected to other types of post-transcriptional modification. Poly-A formation, catalyzed by a terminal transferase enzyme, consists of the sequential addition of 150–200 adenylate residues to the 3′ end. The recognition signal for the terminal transferase seems to involve the hexanucleotide AAUAAA, because this sequence is approximately 11–30 nucleotides upstream from the 3′ end of all poly A-containing mRNAs but absent from poly A-lacking mRNAs. In some cases, e.g., adenovirus late mRNAs and SV40 mRNAs, the poly-A addition site may be formed by endonucleolytic cleavage of the growing transcript rather than by termination of the RNA polymerase (106, 106a). To what extent this applies to cellular mRNAs is presently unclear.

Future Directions

It should be evident from this narrative that progress in our understanding of RNA processing, like that of other natural phenomena, is largely dependent on our ability to formulate fresh and meaningful questions and to develop the appropriate methodologies to help answer these questions. Two decades ago our concern was nuclear/cytoplasmic or genotype/phenotype relationships, and our approach was limited by the cytological and biochemical tools then available. Kinetics of incorporation of radioactively labeled RNA precursors, base-composition analyses, autoradiography, microspectrophotometry, and sedimentation analyses in the ultracentrifuge, determined the scope of our experimental protocols. Today, we seek to define gene organization and expression in terms of arrangements of and changes in nucleotide sequences, and our horizon is confined to the information obtainable from restriction analysis and various blotting procedures, from heteroduplex and R-loop analysis, from nucleotide sequencing, etc. To be sure, serendipity plays a large and unpredictable role in our progress. Who would have imagined that the discovery of 3′-terminal poly A on mRNA would provide a basis for methods of purifying mRNA and pre-mRNA, which would lead, in turn, to the uncovering of many new structural and functional features of these molecules? Or that the reverse transcriptase enzyme found to be associated with RNA tumor viruses would become a key tool in the recombinant DNA cloning of mRNA sequences? Yet, such developments are not really rare, and we can be sure that many of today's discoveries will be the sources of techniques that will be critical for answering tomorrow's questions.

What are tomorrow's questions about RNA processing? One broad class of questions concerns the enzymatic mechanisms of processing. So far only a very few of the processing enzymes have been isolated or even purified away from nonspecific degradative enzymes, and, indeed, at present we would be happy to know just how many different cutting and splicing enzymes exist in a cell. If some of these enzymes operate with small RNA cofactors, the various interrelationships between enzyme, cofactor, and RNA substrate will have to be worked out. From the information accumulated to date (cf. reference [107]), it would seem that the substrate recognition sites for processing enzymes reside only partly in features of primary and secondary structure and that they also depend heavily on features of tertiary structures, i.e., on three-dimensional conformation. This aspect poses some formidable obstacles to progress in this area, because most of the current methods for conformational determination are not well suited for studies of the minute quantities of precursor RNAs that are normally available from cells. In lieu of any significant methodological advances, one can resort to specific genetic manipulations of the substrate and to approaches such as those used to study an enzyme that processes 5S RNA in *B. subtilis* (108, 109). In this case, an efficient in vitro processing system was developed and then used with pre-55 RNA that was specifically modified by partial nucleolytic digestion and ligation of synthetic polynucleotide appendages. With detailed secondary structure models of the precursor and its derivatives, the requirement for particular base-pairing interactions within the substrate molecules can be determined.

Another broad class of questions concerns the role of processing in regulating gene expression. Earlier, I cited examples in which alternative processing modes produce multiple and functionally distinct mRNA molecules from a single set of noncontiguous genetic elements. It will be interesting to know whether such qualitative regulation at the processing level is widespread amongst eukaryotic genes, and, if so, to know how a cell can exhibit preference for one processing pathway over another under different physiological and developmental situations. The role of processing in the quantitative regulation of gene expression is also a phenomenon that deserves further scrutiny. The wastage of pre-rRNA that occurs in certain resting-cell populations and disappears in growing populations has been known for more than a decade ([110], see also [111] for other references), but we still do not understand what actually determines whether a particular precursor molecule will be processed or degraded to its nucleotide constituents. Similarly, there is reasonably good evidence to indicate that mRNA abundance is regulated in part by variation in processing efficiency (112, 113), but the molecular basis of this regulation remains obscure. Answers to these questions will require improved knowledge of the processing mechanisms and also the development of new experimental systems (biochemical or genetic) that will enable us to probe the determinants of processing specificity and to generate protocols capable of revealing cause and effect relationships.

Given the large number of interesting unanswered questions and the vast number of biological systems that have yet to be studied, it seems clear that the field of RNA processing will remain an exciting one for many years to come. One can look forward to the repeated satisfaction that will come when various complex biological phenomena are explained and clarified in straightforward molecular terms. Moreover, on the basis of past developments in this field, we can confidently predict that

future progress will come from unexpected ramifications of conceptual discovery and methodology and that serendipity will play a major role.

Addendum

A delay between the completion of this chapter (in March 1980) and its actual publication has given me an opportunity to consider some of the recent developments in the RNA processing field within the context of my general theme. As anticipated, several important new facts have been revealed by the combined use of recombinant DNA-cloning methodology and various powerful techniques of nucleic acid structural analysis. Some of these facts have clarified uncertainties and helped resolve unsettled issues, while others have given new insights into the ways in which processing can be implicated in the regulation of gene expression and cellular differentiation. In contrast, there has been less progress on the characterization of processing enzymes and the determinants of processing specificity. In these cases the aforementioned technical obstacles still constitute a rate determining step. The following selective survey should give the reader some idea of the pace in this field over the past 18 months.

FORMATION OF 5′-TERMINAL CAPS: It has now been conclusively shown for the genes specifying early and late SV40 mRNAs (114), adenovirus late RNA (115), and mouse β-globin (115) that the 5′-terminal cap site and the site at which transcription is initiated by RNA polymerase II are one and the same. Among the multiple initiation/cap sites ascribed to early SV40 mRNA, a major species is ^{7}mGpppC^{m}U, thus demonstrating that eukaryotic RNA polymerase II, like the prokaryotic RNA polymerase, can sometimes initiate with a pyrimidine nucleotide. Certain cellular mRNAs may also have heterogeneous cap sites, as indicated by the finding of two 5′ termini in ovalbumin (116) and liver α-amalyse (117) mRNAs. Although not yet conclusively demonstrated, this heterogeneity could be the result of imprecise initiation, as was shown for the SV40 mRNA species (114).

A significant addition to our knowledge of processing mechanisms concerns the guanylyltransferase-catalyzed reaction of cap formation. Recent studies of the vaccinia-capping enzyme, one of the few processing enzymes that can be obtained in high purity, have shown that the transfer of GMP from GTP to a triphosphate-terminated polynucleotide involves an intermediate in which GMP is covalently linked to a subunit of the capping enzyme (118). The specificity for GTP as a nucleotide donor seems to reside in the formation of this covalent complex; the basis for specificity of the polynucleotide acceptor is still obscure.

TERMINATION AND POLYADENYLATION: The importance of the AAUAAA sequence in determining the site of 3′-terminal cleavage and polyadenylation of growing transcripts has been firmly established by experiments with deletion mutants of SV40 (119). The cleavage/poly A-addition site is completely abolished in mutants lacking this sequence and is moved proportionately downstream in mutants with deletions to the 3′ side of it. Although the AAUAAA sequence (or a very close relative) is apparently indispensible for proper 3′-terminal cleavage and polyadenylation, it is also clear that other structural features in this region can modulate the efficiency of this process (119).

It is now apparent that the use of endonucleolytic cleavage for transcript termination can apply to cellular genes as well as viral genes. In the transcription of the β-globin gene in both mouse (120) and chicken (121) nuclei the RNA polymerase continues more than 1,000 nucleotides beyond the poly A site.

SPLICING: Despite the fact that well over 100 individual splice junctions have now been identified in a variety of mRNAs, our understanding of the splicing mechanism is still rather primitive. Except for the GU on the intron side of the 5′ splice site and AG on the intron side of the 3′ splice site, there is considerable variability at the other positions of the consensus sequence (Fig. 6). Indeed, in a completely random sequence, the 5′- and 3′-consensus sequences should occur, on average, every 2,000 and 500 nucleotides, respectively, distances which are short compared to the lengths of many known introns (122). Nevertheless, the hypothetical implication of U1-RNA in the splicing reaction has received some support from experiments in which a lupus antiserum that precipitates ribonucleoproteins containing U1-RNA was shown to inhibit the proper splicing of adenovirus mRNA by isolated HeLa cell nuclei (123).

RNA transcripts of recombinant chimeras that contain a 5′ splice site of an SV40 gene and a 3′ splice site of a mouse β-gene can be accurately spliced in vivo (124). Such a result indicates that the two splice sites bracketing an intron need not constitute a unique pair. This has led to the consideration of a processive mechanism in which splicing always occurs between the most proximal pair of splice sites (122). Yet, we now know of several examples of both cellular and viral genes in which a choice between alternative 5′ or 3′ splice junctions must be made in order to produce the correct mRNA product. One way out of this dilemma is to have a stepwise mechanism in which new splice sites are regenerated by having similar consensus sequences at intron and exon boundaries. This mechanism appears to operate in the processing of collagen mRNA (125) and could conceivably work for SV40 early mRNA (122). However, it does not seem applicable to other situations, e.g., the excision of J_{1-3}-C introns in immunoglobulin mRNAs (see Fig. 4).

Additional examples of obligate splicing in the processing of ribosomal RNA in simple eukaryotes have been discovered (126–128). The list now includes the products of nuclear genes as well as those associated with the genomes of cytoplasmic organelles. A comparison of four sets of sequences surrounding the ribosomal RNA splice junctions have revealed two interesting features: common nucleotides (T and G) at the 5′ sides of both 5′ and 3′ splice sites, and a short repeated sequence further upstream of these sites (129). The lack of resemblance of these sequence features and those associated with mRNA and tRNA splice junctions supports the idea that a distinctive splicing mechanism has evolved for each major class of RNA.

ROLE IN GENE EXPRESSION: The importance of RNA processing in regulating the expression of cellular genes is coming to be increasingly appreciated. For example, the prediction (ref. 92) that the mRNAs encoding the membrane and secreted forms of μ-heavy chain are produced by alternative processing of transcripts from the same gene was confirmed by detailed analyses of the 3′-terminal sequences of the appropriate μ-mRNAs and the identification of corresponding sequences in the μ structural gene (130, 131). Moreover, the finding of two AAUAAA sequences, one on the 3′ side of the genetic element encoding the secreted chain, and the other, on the 3′ side of the element encoding the membrane-associated chain, suggested that the two mRNAs might be derived from primary transcripts that are terminated at two different sites (131). An analysis of μ-mRNA precursors in cells producing different proportions of membrane and secreted mRNAs tends to support this idea

(132). An additional role for RNA processing in the developmental regulation of immunoglobulin gene expression is suggested by the simultaneous production in a single cell of mRNAs encoding the same variable region sequence and constant regions of either the μ or the δ class (133–135). The C_μ gene is located between the V_H gene and the C_δ gene. Therefore, in the processing of δ-mRNA, the C_μ sequences are treated as an intron rather than an exon.

An elegant example of the implication of RNA processing in cellular differentiation was provided by an analysis of the α-amalyse genes in different tissues of the mouse (117, 136). Although the α-amalyse mRNAs in salivary gland and liver cells are derived from the same gene, their relative abundance in these two tissues is markedly different. An examination of gene and mRNA structure revealed that the salivary and liver mRNAs possess different 5′ untranslated sequences which are encoded by two widely separated elements located several kb upstream from a common coding segment. One attractive idea is that the wide variation in expression of this gene in the two tissues is related to the differential use of two transcriptional promoters. Obviously without the flexibility afforded by RNA processing, this could otherwise be achieved only by using a duplicate gene, as is the case in pancreatic cells. A hint that such flexibility may be exploited by other cellular genes was provided by the recent observation that the L and L′ subunits of pyruvate kinase are encoded by two distinct mRNAs, presumably derived from a single gene (137).

A remarkable regulatory principle involving RNA processing has been found in the expression of the cytochrome *b* gene in yeast mitochondria (138). In this system the first two exons and part of the second intron of the cytochrome *b* gene code for an "mRNA maturase" which is, itself, responsible for the splicing out of the second intron. This constitutes a negative feedback system in which the activity of the maturase eliminates the mRNA that encodes it, and concomitantly produces cytochrome *b*-mRNA. The 5′ cleavage involved in the removal of the second intron is unorthodox in that it occurs downstream, rather than upstream, of a GU doublet. An inability of the normal mRNA splicing system to cope with this situation might account for the maturase requirement.

One of the most striking recent examples of the necessity for RNA processing in the expression of genetic information has come from a detailed examination of the human mitochondrial genome (139, 140). In this case, the genes for rRNA and various mRNAs and tRNAs are immediately contiguous to each other, leaving essentially no space for conventional transcriptional start and stop signals. The evidence accumulated to date indicates that over 90% of the genome is transcribed as a single unit, and that the various discrete RNA components are generated by a series of precise endonucleolytic cleavages of the growing transcript (140). The tRNA sequences, which punctuate most of the other genes, could conceivably serve as recognition signals for these cleavages.

These exciting new discoveries further illustrate the crucial role that RNA processing plays in mediating gene expression in eukaryotes. It is hoped that they will soon be paralleled by some deeper insights into the mechanistic aspects of the various processing reactions. One cause for some optimism in this regard is the recent progress in the determination of RNA secondary structure, in which accessibility to digestion with selected nucleases is used to discriminate among computer-generated models based on primary structure and minimum energy considerations (141, 142). These methods are suitable for relatively small amounts of material, and are thus capable of providing crucial information about the secondary structure of processing substrates. Such structural information should help considerably to sharpen our concepts of processing mechanisms.

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The Nuclear Envelope and the Architecture of the Nuclear Periphery

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By definition, the eukaryotic cell is characterized by a compartmentation structure that divides the intracellular space into two different regions: (*a*) the nucleus, which contains the genome and the structures involved in transcription and processing of transcription products; and (*b*) the cytoplasm, which contains the translational apparatus, the cell organelles, the endomembranes, and a variety of other particles. This nucleocytoplasmic compartmentation is maintained by a specific eukaryotic membrane structure, the "nuclear envelope" (exceptions are some special situations, such as some forms of nuclear division, certain sperm cells, and some pathological conditions; see below). The existence of a membranelike structure between nucleus and cytoplasm had been indicated in early light microscope studies.

In his description of the cell nucleus in 1833, Brown (1) had already mentioned the possibility that it might be surrounded by a membranelike structure. After decades of lively discussion of the existence, real or artifact of preparation, of a distinct boundary layer between nucleus and cytoplasm, it was Flemming (2), who, in 1882 in a thorough review, summarized the accumulated evidence for "the existence of a special achromatinous lamella, that is a real—though in most types of nuclei very thin—layer of substance, which ... is not merely the expression of the region of contact between nuclear substance and cytoplasmic substance." Extending earlier observations of Hertwig (3) in nuclei of certain protozoa and of Soltwedel (4) in plant cell nuclei, Flemming (2) also clearly distinguished between the nuclear membrane proper as the "outer, achromatinous layer" and an inner "usually interrupted layer of peripheral chromatin" (the "nuclear cortex layer" sensu Hertwig and Soltwedel). Hertwig (3) also described certain fine punctate interruptions in cross sections of the achromationus nuclear membrane structures and discussed the possibility that these might represent pores, which allow exchange between nucleus and cytoplasm, an idea critically discussed by Flemming (2), who correctly pointed to the lack of evidence for the existence of such pores ("Poren in der Kernmembran," 1882; in reference 2). Several authors (2, 5) also noted the plasticity and viscosity of the nuclear membrane, properties that were then demonstrated with special clarity in the nuclear microdissection experiments of Kite (6).

Further support for the existence of a true nuclear membrane structure, which was profoundly different in composition and molecular organization from the nuclear and cytoplasmic zones, was obtained by polarization microscopy. In these studies the nuclear membrane showed negative spherite birefringence, indicative of lamellar arrays parallel to the nuclear surface (7, 8). Disappearance and finally reversion of the character of this birefringence in imbibition series reported by Schmitt (9) then was interpreted to show that the lamellar structures responsible for the negative spherite appearance was a result of form birefringence and that the intrinsic birefringence of the nuclear membrane resulted from layers of molecules, probably lipids, oriented perpendicularly to the plane of the nuclear membrane.

However, the demonstration of the significance and the unique mode of organization of the nuclear membrane has been made possible only by the development of electron microscopic preparation techniques (10–20; for more complete and detailed treatment of literature see reviews in references 21–23). The nuclear envelope as the structure of the nucleocytoplasmic borderland has always been suggestive of being biologically important and, also because of the distinct and intriguingly regular morphology of the nuclear pore complex, has attracted many electron microscopists. Biochemical work on the nuclear envelope has begun relatively late, i.e., after methods were developed that allowed the isolation of nuclear membrane material in sufficient amount and purity from various cells and tissues (18, 20, 21, 23–34).

In the present article we shall discuss some major findings on the organization and composition of the nuclear envelope. In particular we shall focus on those aspects which are specific to the nuclear envelope, and only in passing will we mention properties which this membrane system has in common with other cytoplasmic membranes.

The Nuclear Envelope Is a Membrane Cisterna Interrupted by Pore Complexes

The nuclear envelope is a special perinuclear cisterna of the endomembrane system and is constituted by the inner and outer nuclear membrane enclosing a lumen ("perinuclear space" [14]). The typical structure of the nuclear envelope as

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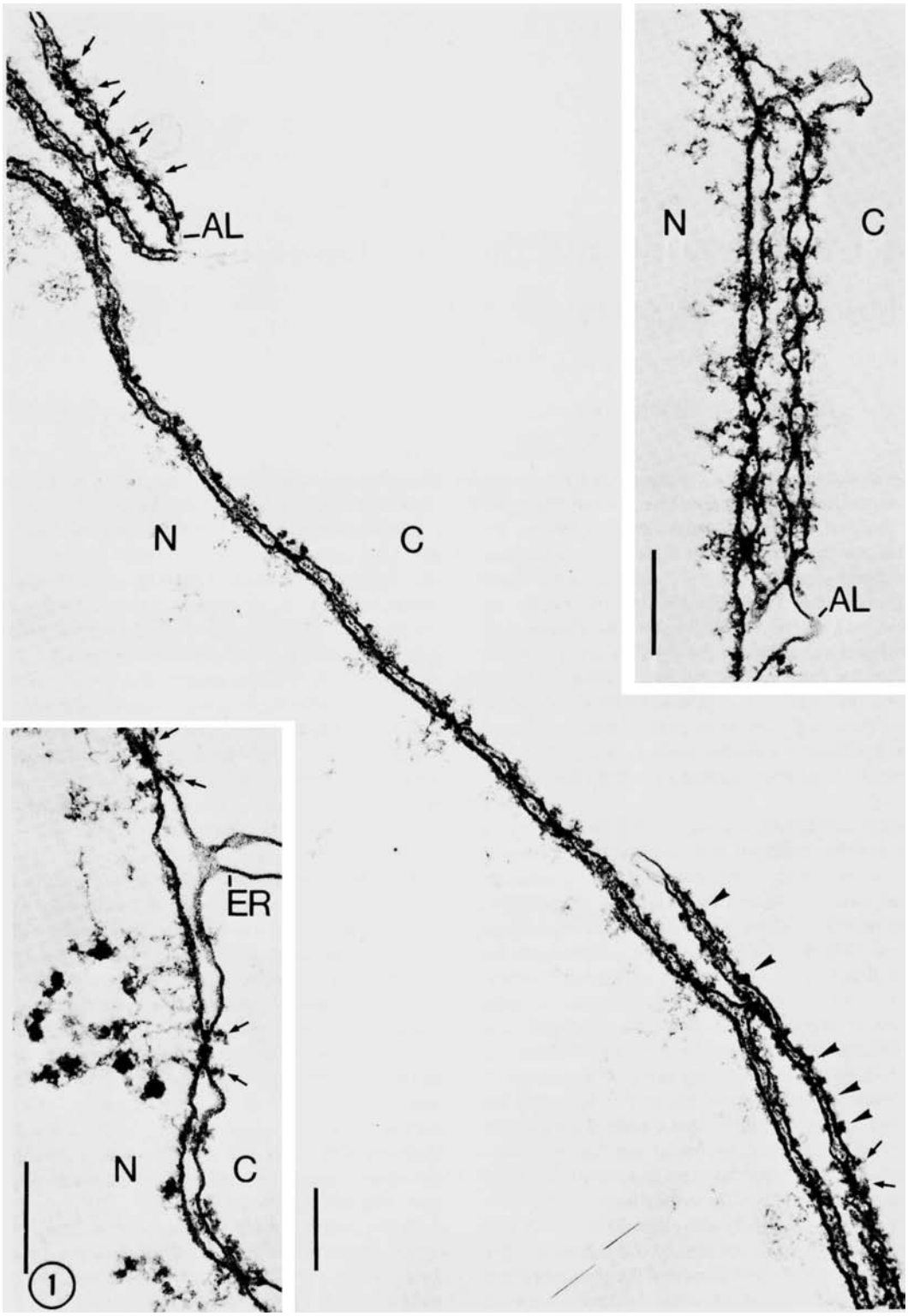

FIGURE 1 Transverse sections through isolated nuclear envelopes and attached cytoplasmic annulate lamellae (AL) from oocytes of various amphibian species (*Xenopus laevis, Pleurodeles waltlii,* lower left insert; *Bufo bufo*, upper right insert). Pore complexes are numerous in both membranes, the nuclear envelope and AL, and have identical ultrastructure: annular granules lie on either pore margin and cones of dense material protrude from the membranous walls of the pore into the pore lumen; often a centrally located granule or rod-like element is recognized (some annulus subunits are denoted by the arrows). Nucleoplasmic filaments terminate at the inner annulus and the central granule of the nuclear pore complex. These annulus-attached fibrils are often arranged in cylindrical arrays and are associated with densely stained granules, probably including ribonucleoprotein material (lower left insert). The juxtanuclear cytoplasmic AL are seen to be in luminal continuity with the perinuclear cisterna (e.g., upper right insert). Direct luminal interconnections with endoplasmic reticulum (ER) and with AL are also frequent (some membrane-associated ribosomes are denoted by arrowheads). C and N represent cytoplasmic and nucleoplasmic side of the nuclear envelope, respectively. Bars, 0.2 μm. × 65,000; upper insert, × 74,000; lower insert, × 85,000.

seen by electron microscopy of sections is presented in Fig. 1. Since the discovery of porelike discontinuities in nuclear envelopes by Callan and colleagues (10, 11), the nuclear pore complex (15) has received special attention as a distinct site for nucleocytoplasmic exchange (16–23). The architecture of the pore complex has been studied with various electron microscopic techniques (ultrathin sectioning, e.g. Fig. 1; metal shadowing, positive and negative staining, e.g. Fig. 2; freeze-cleavage, cf. references 19 and 35; high-resolution surface scanning, cf. references 36 and 37), and pore complex structure models proposed by several authors show remarkable agreement in the essentials (Fig. 3; e.g., 14–24, 38–40). Today it is clear that the pore complex, with its characteristic ultrastructure (see below), is a universal feature of the nuclear envelope in all cells, with the possible exception of late stages of spermiogenesis in some species (although definite proof of the existence of nuclear envelopes completely devoid of pore complexes has not been reported).

The Pore Complex Has a Unique Symmetrical Organization

The pore complex is a highly symmetrical (bilaterally and radially) array of distinct, particulate, nonmembranous substructures associated with the transcisternal orifice of the nucleocytoplasmic pore (Figs. 1–3). It is profoundly different in organization from other similarly sized pore formations in membranes, such as in capillary endothelia, in cisternae of Golgi apparatus, and in the "secondary envelope" surrounding the giant primary nuclei of certain green algae (Fig. 4; for references see 20–22). The pore orifice is constituted by the locally fused transitions of inner and outer nuclear membrane and has an inner-pore width (membrane-to-membrane pore diameter) that appears to be rather sharply defined in a given type of cell or nucleus, but can show considerable variation (range: ~ 60–90 nm) when different types of nuclei and different electron microscopic methods are compared. The most prominent morphologically distinguishable components are recognized in Figs. 1 and 2 and are schematically illustrated in Fig. 3. They include (*a*) two rings (annuli; see references 10, 11, 13–17), located on either pore margin, each composed of eight 10- to 25-nm large granular particles (annular granules; see references 13, 18, 38–41) that are arranged in a precise eightfold symmetry (18); (*b*) eight radially distributed cones or tips projecting from the pore wall into the pore lumen (15, 18, 24, 39, 40) that some authors regard as locally aggregated fibrils

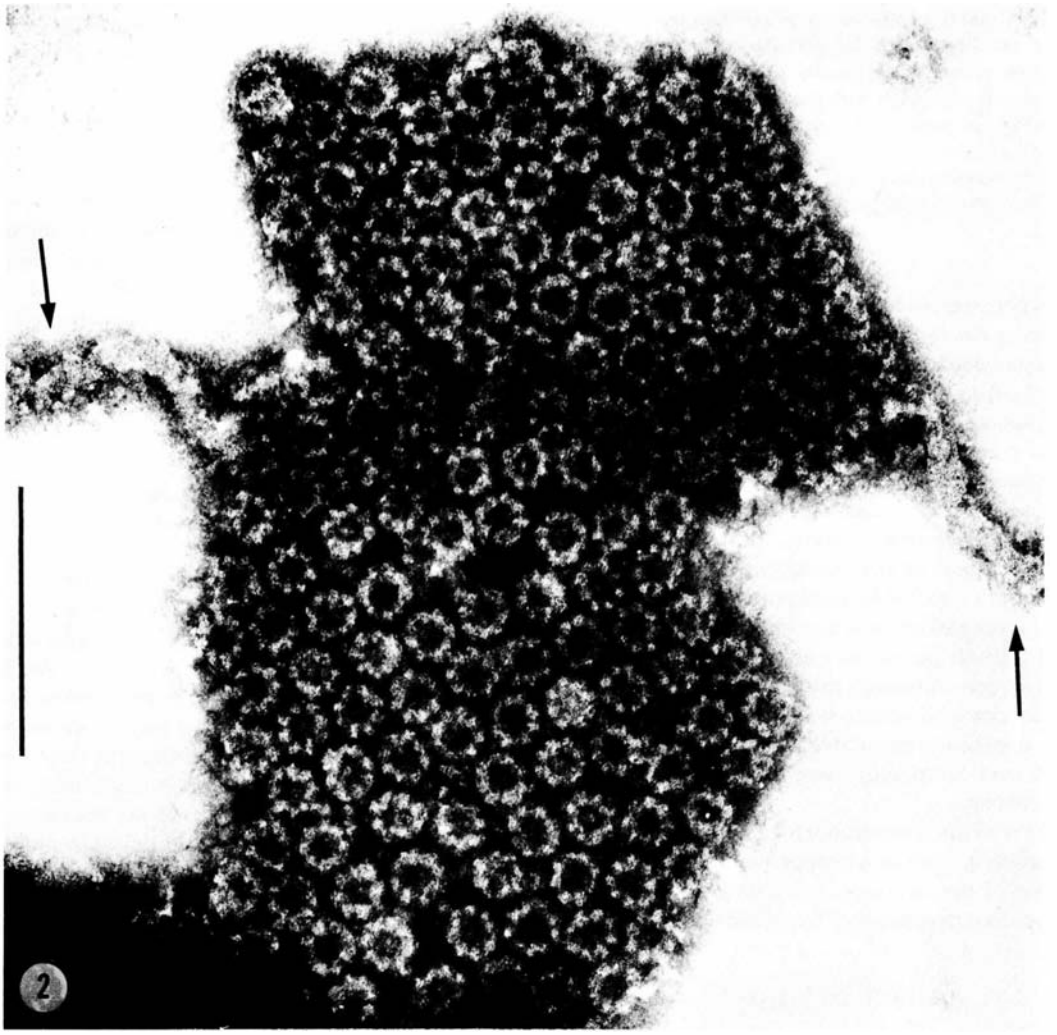

FIGURE 2 Negatively stained (phosphotungstic acid, PTA) cytoplasmic annulate lamella isolated from *Xenopus laevis* oocyte. The ring-like annulus material lying on each pore rim consists of eight symmetrically arranged granules. In the lumen of some pores a central granule is observed. Note the abundance and high packing density of pore complexes in the AL and structural continuities of AL with membranes of endoplasmic reticulum (arrows). Bar, 0.5 μm. × 70,000.

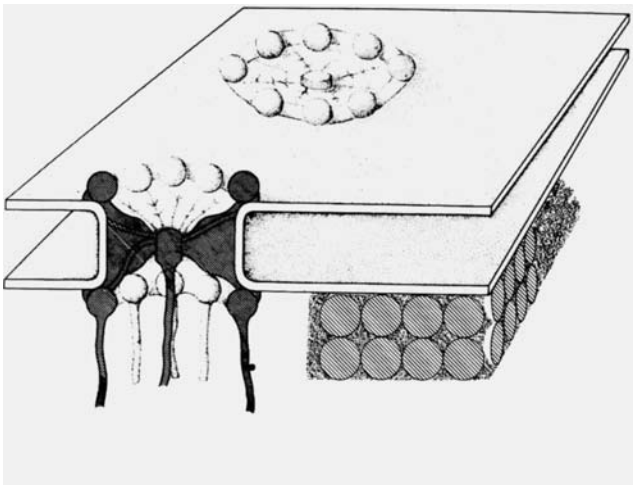

FIGURE 3 Schematic presentation of nuclear pore complex architecture and the association of peripheral condensed chromatin with inner nuclear membrane. The pore complex consists of (*a*) inner and outer annulus, each composed of eight granules of diameter 10–25 nm which are symmetrically distributed on either pore margin, (*b*) eight conical tips which project from the pore membrane wall and also are arranged in an eightfold symmetry, (*c*) a frequently present central granule or central rod, and (*d*) bundles of nuclear filaments which are attached to the granular components. In the interporous region of interphase nuclei (in the right) chromatin strands appear closely associated with the inner nuclear membrane, and this attachment of peripheral chromatin (20- to 30-nm large, higher order globular units are indicated by hatched circles) seems to be mediated by interchromatinous nonhistone protein material (dotted) containing the specific peripheral nuclear "skeletal" proteins (see text).

traversing the pore periphery (for references see 22); (*c*) a centrally located particle, granular or rodlike, of variable diameter and shape, which, however, is not recognized in all pore complexes; and (*d*) tangles of nucleoplasmic 4- to 8-nm filaments that terminate at the inner annulus, that often reveal eightfold radial symmetry, and that seem to be interconnected and to form cylindrical arrays ("channels," "funnels"; 39, for other references see 20–23) extending deep into the nucleus.

Great variations have been reported, in different types of nuclei, of both numbers of pore complexes per total nuclear surface (range from 10^2 to 5×10^7) and pore complex frequency (i.e., pore complexes per/μm^2 nuclear surface; range from 1 to 3 pores/μm^2 to 50–60 pores/μm^2) as well as differences of pattern of distribution. Although correlations of pore numbers and pore frequencies with certain nuclear activities, e.g., transcription, are sometimes suggestive (16–22), the functional associations of pore morphology and number cannot be resolved at the moment.

Pore complexes of the same symmetrical ultrastructure have also been observed in nuclear envelope fragments during mitotic breakdown of the envelope and during reformation of nuclear envelope in anaphase and telophase stages (e.g., Fig. 9*c*; see 17–22).

Pore Complexes Are Not Exclusive to the Nuclear Envelope

Transcisternal pore formations with essentially the same symmetrical architecture as that of the nuclear pore complex are also observed in cytoplasmic cisternae of the endoplasmic reticulum (ER), either as ordered stacks rich in pore complexes (annulate lamellae [AL]; 14, 42; for references see 22 and 43) or as single pore complexes in rough ER, as well as in "intranuclear AL" (for references see 20–22). The only difference from the pore complexes of the nuclear envelope appears to be the absence of polarity because both sides of the pore complex of AL are exposed to the same compartment. Typical cisternae of cytoplasmic AL are seen in Figs. 1 and 2. Such AL have been observed in a diversity of animal (22, 42, 43) and plant (44; cf. references 20–22) cells. Characteristically, the pore complex density can attain higher values in cytoplasmic AL than in the nuclear envelope of the same cell (16, 22, 44, 45). As in the case of nuclear pore complexes, neither the mode of formation of AL and their pore complexes nor their functions are known. Their mere occurrence, however, demonstrates that pore complexes are not exclusive to the nuclear envelope and are not formed only in association with nucleocytoplasmic compartmentation and exchange.

Nuclear Pore Complexes Contain Stable Architectural Components and Are Integrated into a Peripheral Karyoskeletal Framework

When nuclear envelopes are exposed to mechanical stress or to rigorous extraction treatments involving solutions of high ionic strength or containing nondenaturing detergents, the basic structural elements of the pore complex are still identified, even under conditions that result in the disintegration and removal of most of the membrane material proper (for mechanical stability see references 20–23, 29, 35, 41, 46; for resistance to extractions see references 20–23, 36, 46–53). Nuclear envelopes treated with both detergent solutions and high salt concentrations show the persistence of the pore complex studs and interconnecting dense material (Figs. 5 and 6). The latter, located at about the level of the inner nuclear membrane, has been described as a continuous layer ("lamina"; 49, 50, 52) or as a meshwork of filaments (Fig. 5; 46, cf. references 22 and 23). The composition of such skeletal complexes including pore complex structures and interconnecting material (Figs. 5 and 6) has been examined, and a simple polypeptide pattern has been observed (49, 50, 52–55). Characteristic in such preparations ("pore complex-lamina-matrix," PC-L-M) made from mammalian liver (49–54) and from other cells (e.g., references 55 and 56) is the predominance of a triplet of three major polypeptides with apparent molecular weights in the range of 60,000–80,000 and three minor polypeptides of higher molecular weight (cf. Fig. 7, slot 7), which are also recognized as significant protein components in unextracted isolated nuclear membranes (32, 49, 57, 58). An even more simplified protein pattern has been found when such extractions were performed on manually isolated nuclear envelopes from amphibian oocytes (Figs. 6 and 7, slots 1–6; cf. reference 54): in such preparations, which are highly enriched in pore complex material, a protein of apparent moelcular weight of ~ 68,000 is prominent, together with only a few minor polypeptides of higher molecular weight (Fig. 7, slots 2–6). At the moment, it cannot be decided whether these polypeptides are exclusive to the pore complex or to the interconnecting material, or are common to both. Moreover, the possibility that residual nonhistone proteins of peripheral, nuclear membrane-associated structures (Figs. 8 and 9; for references see 20 and 21) contribute to this protein fraction has not been excluded. Complicating this characterization of the components of the nuclear periph-

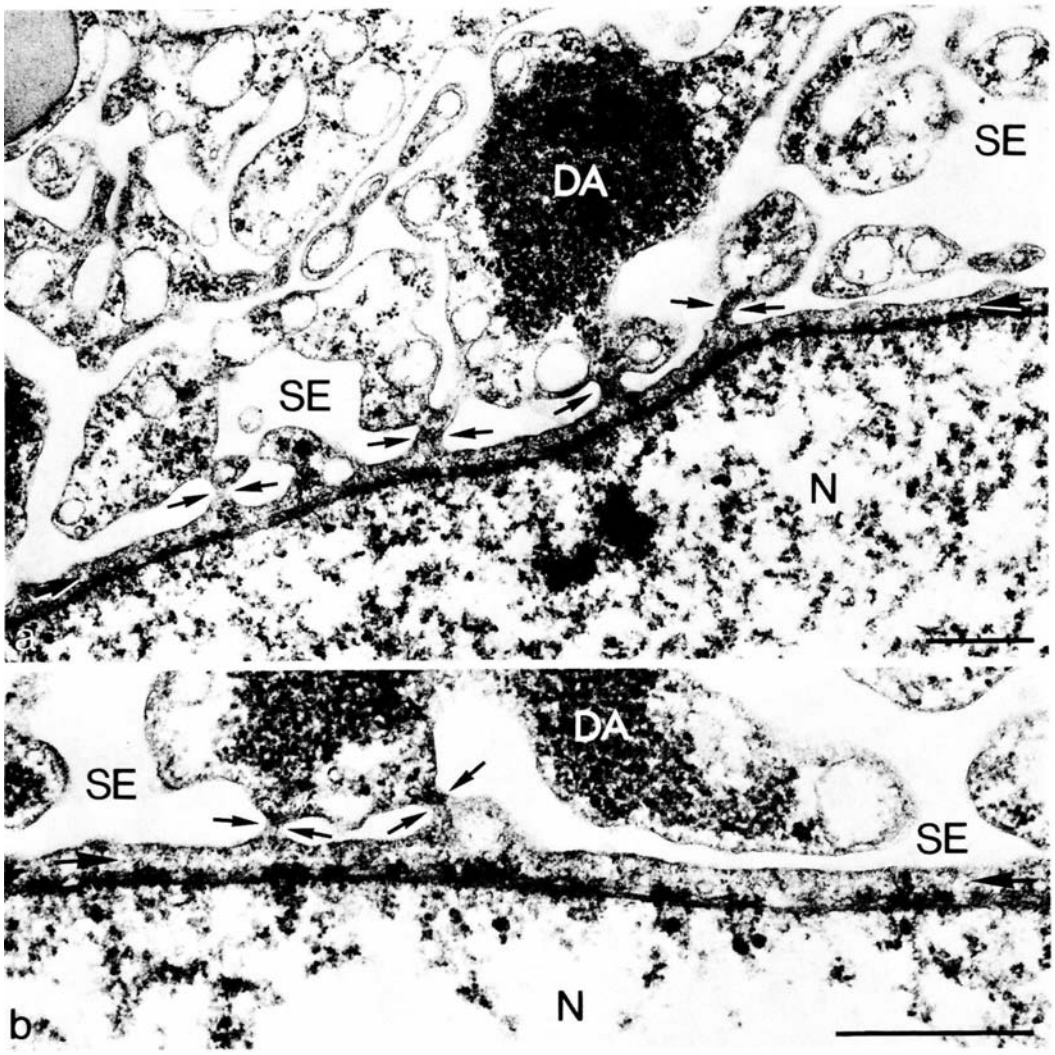

FIGURE 4 The primary nucleus of the green alga *Acetabularia mediterranea*, including the nuclear envelope, is separated from the cytoplasm by a special labyrinthine membrane system which constitutes a "secondary envelope" (SE). The thin intermediate zone sandwiched between the "true" nuclear envelope and this secondary envelope (thick arrows in *a* and *b*) opens into the cytoplasm via narrow channels (some are denoted by the pairs of arrows in *a* and *b*). Membrane continuities of the nuclear envelope with cytoplasmic ER via these channels has not been observed. Large, dense aggregates (DA) are frequently observed in the juxtanuclear cytoplasm. Bars, 0.5 μm. (*a*) × 27,000; (*b*) × 52,000.

ery is the observation that polypeptides of similar sizes, as the triplet proteins mentioned above, have also been reported to be predominant in other nonhistone protein fractions ("nuclear matrix" fractions; cf. reference 51) made from whole nuclei or chromatin (for reviews see references 59 and 60; for differences between PC-L fraction and internal matrix components see references 54 and 61).

On the other hand, in immunolocalization experiments, antibodies directed against the major triplet protein(s) of the PC-L fraction from rat liver have reacted with the periphery of interphase nuclei of different, although not all, mammalian cell types (Fig. 10; cf. references 52, 53, 62), but not with matrix structures of the nuclear interior. Interestingly, this protein of PC-L-M fractions is distributed throughout the cytoplasm during mitosis and has not been localized in metaphase chromosomes (52, 53, 62). Biochemical comparison of the major polypeptides of the nuclear envelope (58) has further indicated that the polypeptides of the triplet group are different, the middle-band polypeptide being a component with a distinct proteolytic cleavage pattern. Clearly, further experimental work is required to elucidate the nature of the skeletal components of the nuclear periphery and the pore complex and their topological relationships. However, the present data already permit the conclusion that the periphery of the interphase nucleus and the nuclear pore complex contain specific proteins that form structures of unusually high stability.

Chromosomes Interact in a Specific Mode with the Nuclear Envelope and the Nuclear Periphery

It has been demonstrated in many cases that in the interphase nucleus the genomic material, i.e., chromosomes and extrachromosomal genes, is not distributed at random but that certain chromosomes or chromosomal regions are arranged in an ordered fashion with respect to the nuclear surface. Certain chromosomes and chromosomal regions are located regularly in the nuclear periphery; prominent examples in many cell types include centromeres and pericentromeric heterochromatin, telomeres and telomeric heterochromatin, perinucleolar heterochromatin, and sex chromosomes (e.g., X and Y chro-

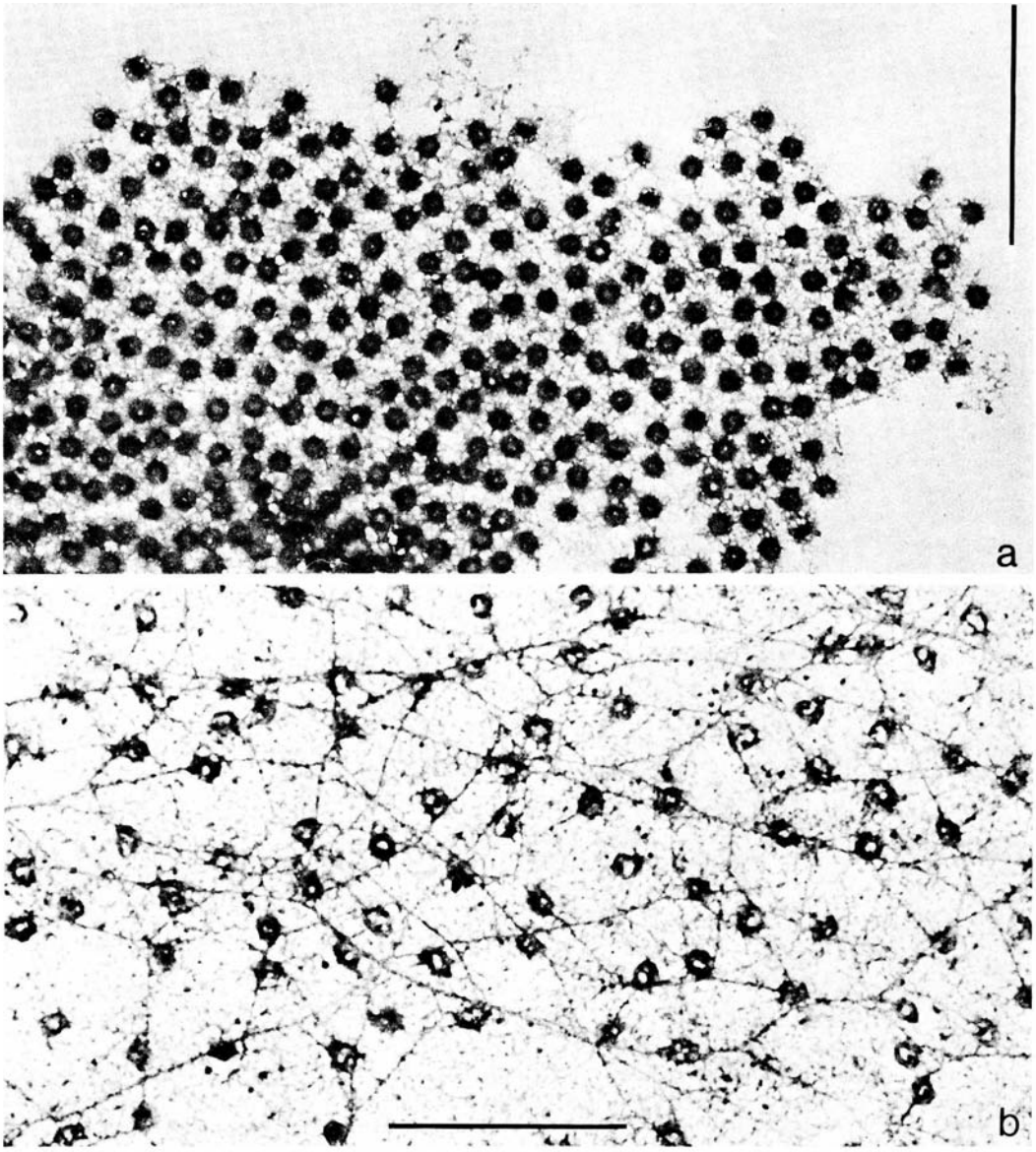

FIGURE 5 Different stages of experimental disintegration of the nuclear envelope isolated from oocytes of *Triturus alpestris* (*a*) and *Xenopus laevis* (*b*) revealing the abundance of pore-connecting fibrils. The preparation shown in (*a*) has been treated with 0.5% NP-40 in water and then centrifuged on an electron microscopic grid, whereas the nuclear envelope presented in (*b*) has been disrupted by spreading on the surface of a water droplet, which results in additional mechanical stretching. Both preparations were positively stained with ethanolic PTA. Bars, 1 μm. × 32,000.

matin; for a more detailed treatment of the literature see references 20 and 21). An especially striking example of this ordered interaction of chromosomes with the nuclear envelope is observed during meiotic prophase of many organisms in which the chromosomal ends are attached to interpore regions of the nuclear envelope; this is particularly well seen in the synaptinemal complexes of such chromosomes (Fig. 9 *d*; 63; for references see 20 and 21). Moreover, nucleic acid hybridization techniques have shown that certain subfractions of DNA (e.g. heterochromatin satellite DNAs) are often preferentially accumulated in the nuclear periphery (for references see 20 and 21). The molecular basis of this localization is not understood. Morphologically, two different situations must be distinguished: (*a*) in diverse types of nuclei, a distinct 15- to 80-nm thick layer of nonmembranous material is seen to be sandwiched between the inner nuclear membrane and the chromatin ("fibrous lamina," 64; for references see 20–23, 65). This laminar material, determined cytochemically to be proteinaceous, has been correlated with the "lamina" structure observed to interconnect pore complexes in isolated, extracted, and detergent-treated nuclear envelopes (49, 50, 52). Thus, generalized nuclear-structure models have been proposed in which the chromatin does not directly border on the inner nuclear membrane but rather is separated from it by a continuous layer containing the triplet polypeptides mentioned above (49, 52, 65). (*b*) On the other hand, chromatin has been shown in various types of plant and animal nuclei to border directly on the inner nuclear membrane, within the limits of resolution of the electron microscope thin-section technique (1–2 nm), with no identifiable fibrous lamina interspersed (Figs. 8 and 9). This absence of a nonchromatinous laminar structure between chromatin and inner nuclear membrane is also seen in cytochemical experiments that result in selective chromatin bleaching (Figs. 8 *c–e* and 9 *b*) and in isolated nuclei, in which the chromatin

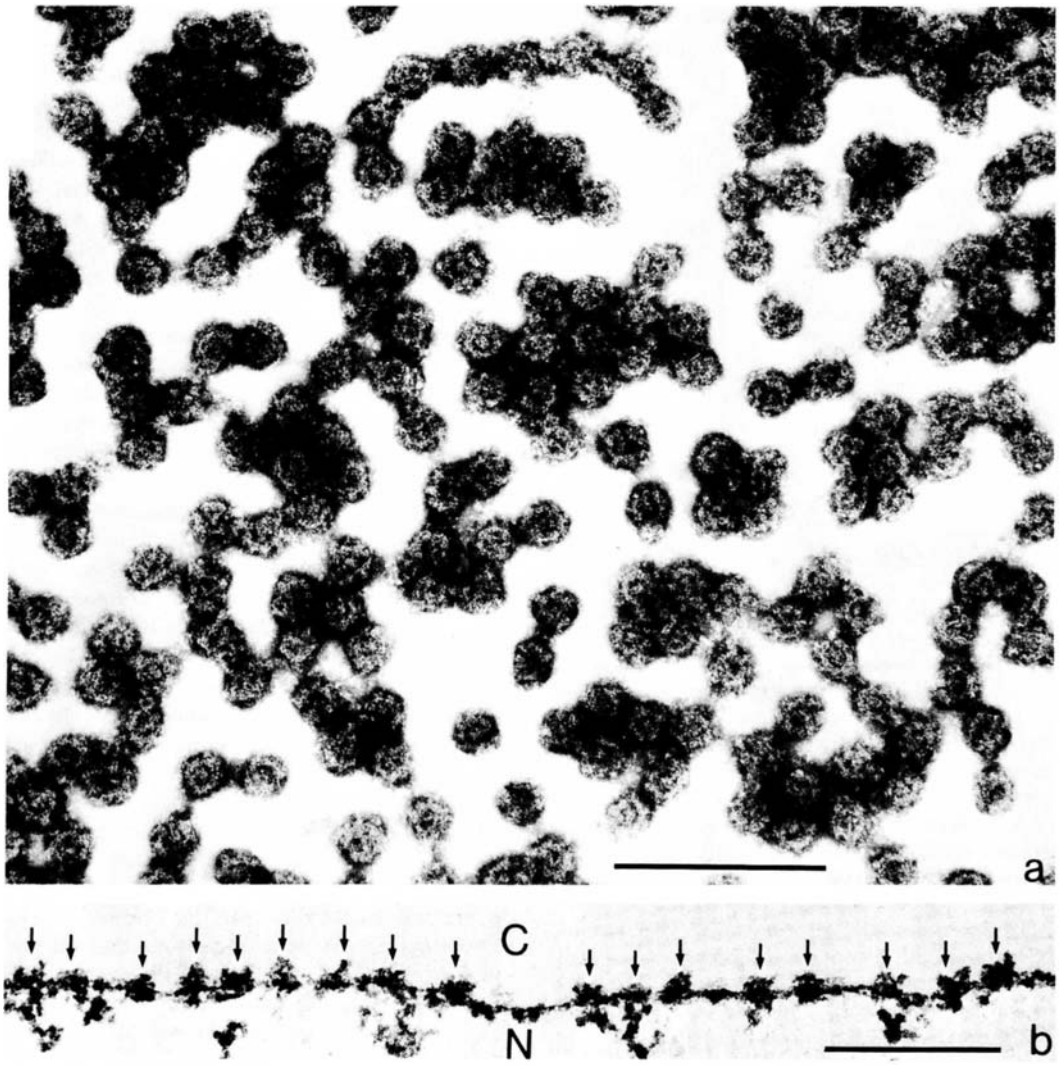

FIGURE 6 Nuclear membrane material from oocyte nuclei of *X. laevis* as obtained after treatment with high salt buffer and detergent and demonstrated by electron microscopy in negatively stained preparation (*a*) and ultrathin section (*b*). The sequential extraction with buffered 1.5 M KCl solution followed by 1% Triton X-100 (for details see text) removes most of the interporous membrane material (*b*) but leaves the basic organization of the pore complex intact (*a, b*); pore complexes in (*b*) are denoted by arrows. C, cytoplasmic side; N, nuclear side. Bars, 0.5 μm. (*a*) × 57,000; (*b*) × 47,000.

has shrunk slightly and separated from the inner nuclear membrane, with only a few thread connections left in an electron-translucent "gap" (Fig. 8 *b*). Moreover, DNA has been shown to be intimately associated with isolated nuclear membranes of various cells (for references, see 20 and 21). Therefore, an alternative explanation is proposed which emphasizes the existence of a proteinaceous, weblike material finely dispersed and associated with both the nuclear envelope and the peripheral chromatin (see right part of Fig. 3), which in certain cells accumulates or, upon chromatin extraction, collapses to form a fuzzy peripheral lamina (see also the left part of Fig. 3 of reference 49). Future experiments will doubtless help to clarify the relationship of chromatin and the nuclear membrane and its possible functional significance. Evidently, strong and specific forces exist in chromosomes to permit association with this type of membrane-attached, nonhistone proteins, and to promote formation of a continuous nuclear envelope. This is best demonstrated in the cycle of dispersion of both nuclear membrane and the PC-L-M proteins during nuclear divisions of the "open" form in which during anaphase-telophase stages elements of the reforming nuclear envelope are assembled on the chromosomal surfaces (Fig. 9 *c*; cf. references 20–23) and PC-L-M proteins are reaccumulated in the forming daughter nuclei.

There is no experimental evidence that pore complexes themselves contain chromatin (for references see 20–23).

The Nuclear Membranes Are Similar in Composition to Membranes of the Endoplasmic Reticulum But Represent an Independent Membrane System

The biochemical composition of nuclear membranes from various plant and animal cells has been compared with that of other cellular membranes (for reviews see references 20–23, 32, 33, 57, 66). These studies have shown that, in cells that allow the direct comparison of membrane fractions, the nuclear membranes are similar to ER membranes in their lipid pattern, in a large number of proteins and enzymes, in the carbohydrate

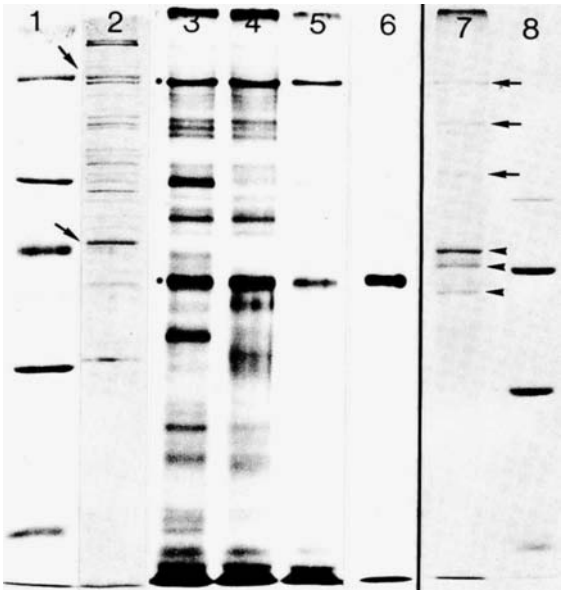

FIGURE 7 Polypeptide composition of unextracted and extracted nuclear membranes of *X. laevis* oocytes (slots 2–6) and rat liver (slot 7) separated by SDS-polyacrylamide slab gel electrophoresis (for references see text). Oocyte nuclear envelopes were manually isolated and cleaned under a stereomicroscope. Proteins of unextracted membranes were separated and stained with Coomassie Blue (slot 2; 170 nuclear membranes; two of the major polypeptides are denoted by arrows; reference proteins separated in the same gel are shown in slot 1, and are, from top to bottom myosin heavy chain (220,000 M_r), phosphorylase a (94,000 M_r), bovine serum albumin (67,000 M_r), actin from rabbit skeletal muscle (42,000 M_r), and chymotrypsinogen (25,000 M_r). Manually isolated nuclear membranes of oocytes were then extracted with various high salt and detergent solutions, resistant membrane components were sedimented, and proteins were radioactively labeled in vitro with [^{3}H]dansyl chloride and visualized by radiofluorography (slots 3–6; slot 3 represents the protein of 22 total nuclear envelopes, slots 4–6 contain the protein equivalent to 40–45 nuclear envelope residues). The polypeptide composition of the unextracted nuclear envelope is shown in slot 3; slot 4 demonstrates the effect of treatment with buffer containing Triton alone. The two major polypeptide bands resistant to sequential extractions with buffers containing 1.5 M KCl and 1% Triton are shown in slot 5. Only one major polypeptide band of the oocyte nuclear envelope has been found to be resistant to simultaneous extraction with 1.0 M KCl and 1% Triton (gel shown in slot 6). The two major resistant polypeptides observed in the pore complex material enriched fractions are denoted by asterisks (slots 3–6) and seem to be identical with the two polypeptides denoted by arrows in slot 2. Slot 7 shows for comparison, the polypeptide pattern of purified nuclear membranes from rat liver which then have been extracted simultaneously with 1.5 M KCl and 1% Triton X-100 (for details and references see text). The three major polypeptide bands reexamined (apparent M_r values relative to those of the reference proteins mentioned below: 74,000; 72,000; 62,000) are marked by arrowheads, the minor components (apparent relative M_r values: 200,000; 160,000; 125,000) of high molecular weights are denoted by short arrows. Reference proteins (slot 8) are, from top to bottom, phosphorylase a, bovine serum albumin, actin, and chymotrypsinogen (slots 7 and 8 have been stained with Coomassie Blue); in other slots of this gel (myosin heavy chain, clathrin (180,000) and β-galactosidase (125,000 M_r), transferrin (76,000 M_r), vimentin (57,000 M_r) and glutamate dehydrogenase (55,000 M_r) were run for comparison.

pattern of their glycoproteins, in their lectin-binding properties, and perhaps also in their pattern of hormone receptors and several components defined as antigens (as to the latter see the examples discussed in references 67 and 68; there is a continuing discrepancy as to the significance of determinations in nuclear membrane fractions of components widely assumed to be characteristic of mitochondria such as cardiolipin, cytochrome oxidase, and oxidative phosphorylation, cf. references 20, 21, 23, 27–29, 32, 33, 66, 69). Certain proteins (32, 57) and enzyme activities, however, seem to be specific for the nuclear envelope. In rat liver, for example, a special nuclear envelope-bound protein kinase system has been described (70, 71), as have differences in the substrate specificity, stability, and drug inducibility of the nuclear membrane monooxygenase system (cf. references 32, 72–74). If proven correct, the nuclear pore complex-bound ATPase (cf. references 20–23, 32) might also represent a speciality of the nuclear envelope. Like rough ER, the nuclear envelope in many, though not in all, cells appears to be a site of membrane-bound protein synthesis, as suggested by the attachment of ribosomes and polyribosomes to the outer nuclear membrane of interphase cells and to both sides of nuclear envelope fragments during mitosis (Fig. 9 *c*; for references see 20 and 21).

The close biochemical similarity of nuclear and ER membranes may well reflect the direct continuity between these two membranes via manifold, mostly tubelike connections (15, 17, 20–23). However, the nuclear envelope can also exist independently from the ER system, and cells have been described that do not show nuclear envelope-ER continuities, such as the vegetative cells of *A. mediterranea* and related green algae (Fig. 4), avian erythrocytes, and late spermiogenic stages of many species (20, 21). In many cell types, the nuclear envelope also shows "transitional elements," i.e., regions with intensive secretory vesicle blebbing from the outer nuclear membrane (20, 21). In cells that do not have an extended endomembrane system, it is obvious that the nuclear envelope makes an important contribution to the total endomembrane functions of the cells. Thus, it seems as if the nuclear envelope not only is a means to nucleocytoplasmic compartmentation, but also can provide the minimum function of the endomembrane system in the eukaryotic cell.

The Nuclear Envelope is Permeable to Small Molecules, But Directs Nucleocytoplasmic Exchange of Particles to the Nuclear Pore Complexes

The nuclear envelope is readily permeable to ions and small molecules (for references see 20 and 21). Observations of dumbbell-shaped structures in the pore complexes (cf. references 20–23; 75) indicate that nucleocytoplasmic transport of particulate material of diameters larger than the pore interior lumen is via the pore complexes by a nonpassive process. The configuration of these particles, presumed to represent ribonucleoproteins, suggests that not the whole membrane-to-membrane diameter is used for such transport events, but only a central channel of a patent diameter of 10–20 nm. Microinjection experiments using various particles and proteins as probes and performed primarily in large cells such as the amoebas and oocytes, have also demonstrated the existence of a size exclusion limit of approximately 18 nm for migration from cytoplasm into the nucleus (76–78). That pore complexes are preferential, if not exclusive, sites for nucleocytoplasmic exchange of particles, and that size limitations exist for such exchange have also been recognized in studies of the infection of cells with certain DNA viruses. After infection, the virus particles are distributed in the cytoplasm, attach to the central

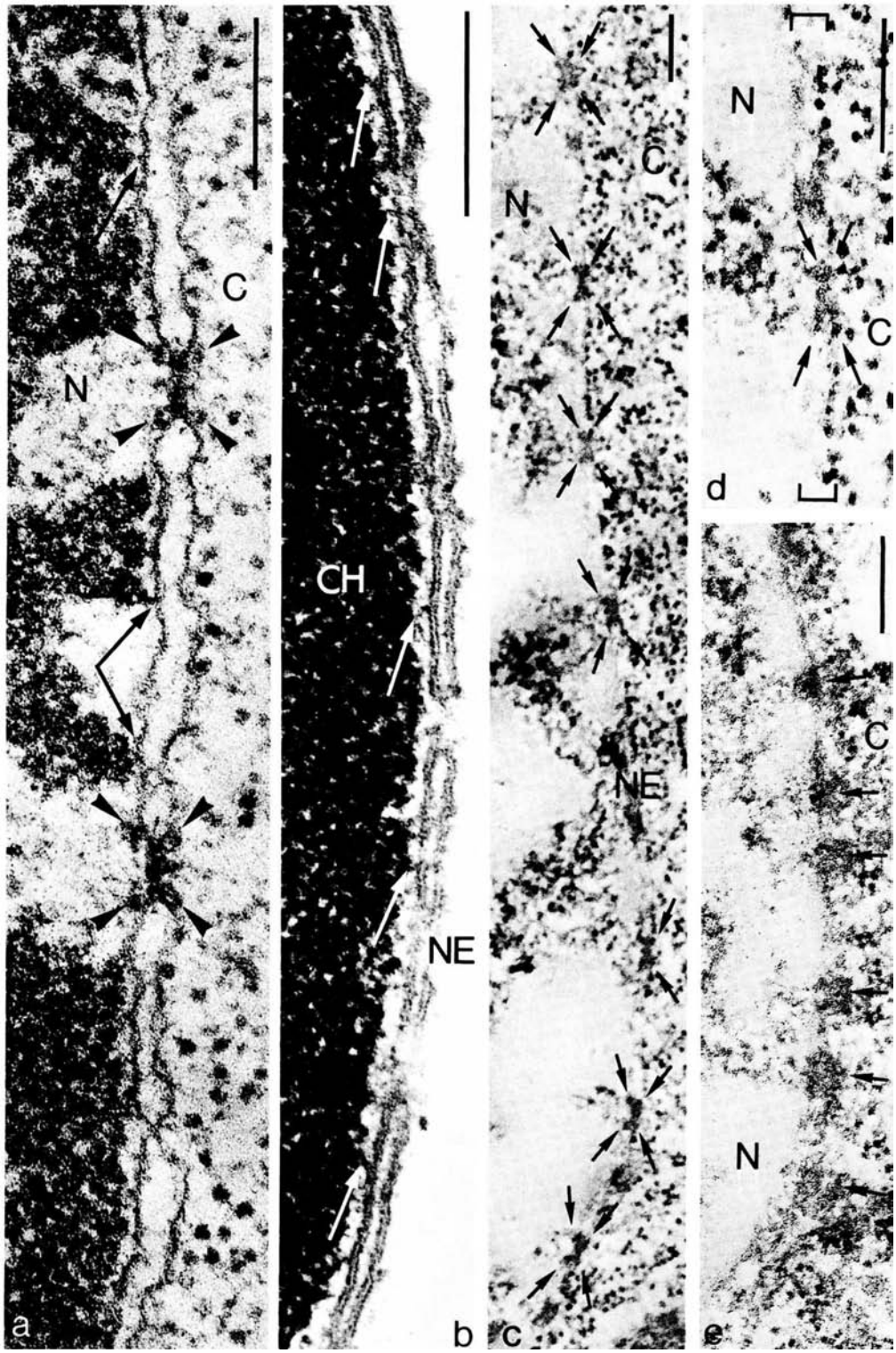

FIGURE 8 Association of the inner nuclear membrane with condensed chromatin as seen in conventionally double-stained (*a*, *b*) or EDTA-treated (*c*, *d*) transverse sections through onion root tip cells fixed *in situ* (*a*, *c*, *d*) or after isolation (*b*). Note the close apposition of peripheral condensed chromatin with the inner nuclear membrane, which often appears to be mediated by short and thin (7–12 nm) thread connections (some are denoted by arrows in *a* and *b*). This peripheral chromatin, which often reveals a composition by tightly packed granular units (see Fig. 3), is regularly interrupted at the pore complexes (arrowheads in *a* and arrows in *c* and *d*) thus forming interchromatinic "channels" which lead to the pores (*a*). Note the absence of a densely stained nonchromatinous layer (*lamina densa*) separating the peripheral chromatin from the inner nuclear membrane; this is especially well seen in isolated nuclei in which some chromatin shrinkage has been occurred (*b*). When the selective staining method of Bernhard is used (*c–e*), chromatin is "bleached" whereas ribonucleoproteinaceous and proteinaceous structures retain the staining. The pore complexes (some are denoted by arrows in *c–e*) as well as the annulus-associated nuclear fibrils and the ribosomes are positively stained. A distinct lamina structure located between the inner face of the nuclear membrane (the contours of the nuclear envelope are indicated by brackets in *d*) and the bleached heterochromatin is not seen, both in onion root tips (*c*, *d*) and in HeLa cells (*e*). NE, nuclear envelope; N, nucleoplasm; C, cytoplasm; CH, chromatin. Bars, 0.2 μm. (*a*) × 115,000; (*b*) × 150,000; (*c*) × 48,000; (*d*) × 100,000; (*e*) × 70,000.

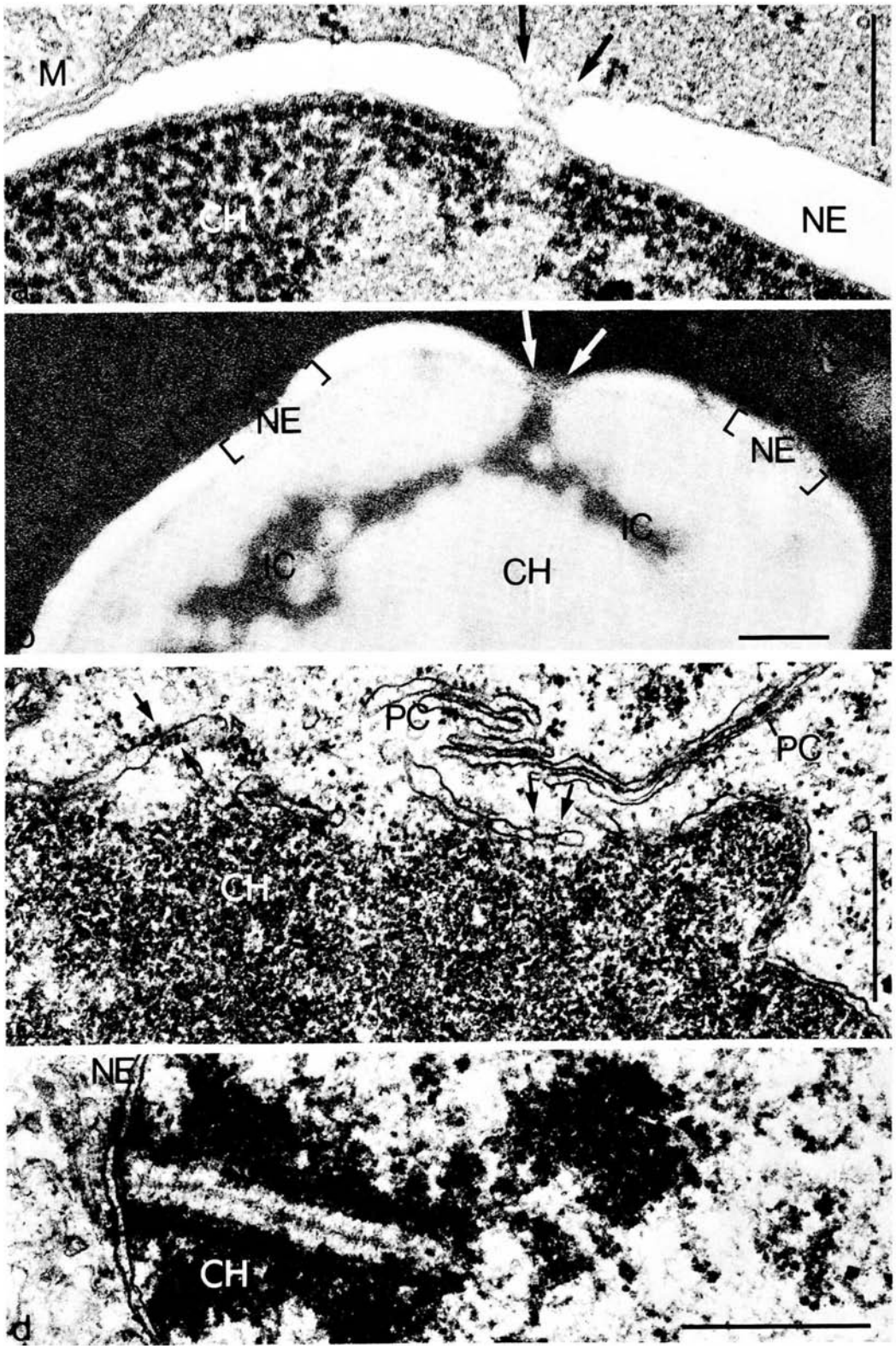

FIGURE 9 Ultrathin sections showing various aspects of chromatin (CH)-to-nuclear envelope (NE) association as seen (*i*) in nuclei of late stages of avian erythropoiesis (*a*, late erythroblasts in chick bone marrow; *b*, erythrocyte in chick leg muscle capillary), after conventional double-staining (*a*) and after treatment with EDTA according to Bernhard's regressive staining method (*b*), (*ii*) during envelope reconstitution on chromosome surfaces of late anaphase stages of mitosis in an experimentally (using dimethylbenzanthracene) induced adenocarcinoma cell in lactating rat mammary gland (*c*), and (*iii*) in form of the specific situation of chromosomal telomeres in termini of synaptinemal complexes in meiotic spermatocytes of rat testis (*d*). The peripheral condensed chromatin is intimately associated with the inner nuclear membrane, and no distinct "lamina" structure is seen in stained and chromatin-bleached nuclei (*a*, *b*). Intranuclear channels (IC in *b*) leading to the pore complexes (arrows) have retained intense uranly staining (*b*). Contours of the two nuclear membranes in (*b*) are demarcated by brackets. During mitosis chromatin and nuclear membrane material are dissociated but begin to reassociate in anaphase-to-telophase on the chromatin of the chromosomal surfaces (*c*). Such fragment units of NE often show ribosomes on both sides (arrows in the left) and typical pore complexes (pair of arrows in the center). In the vicinity of such mitotic configurations often "paired cisternae" (PC) are observed that may include membrane of nuclear envelope fragments. A demonstration of the topological specificity of chromatin-nuclear membrane interaction is presented in the example of the synaptinemal complex (*d*). M, mitochondrion. Bars, 2.0 μm (*a*, *b*) and 0.5 μm (*c*, *d*). (*a*) × 100,000; (*b*) × 70,000; (*c*) × 52,000; (*d*) × 58,000.

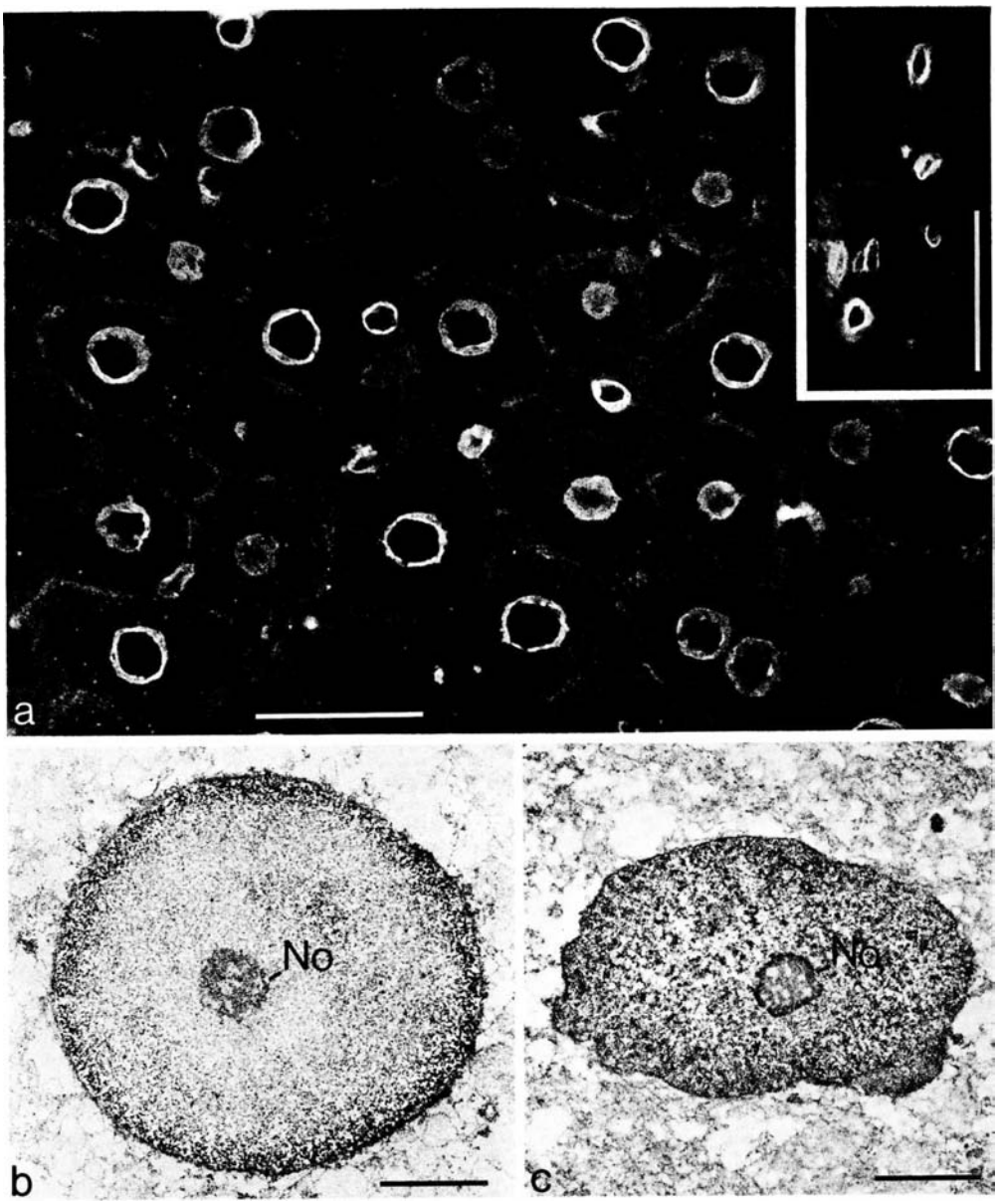

FIGURE 10 Localization of a nuclear envelope-associated karyoskeletal nonhistone protein by indirect immunofluoresce microscopy (*a*) and by immunoelectron microscopy using the peroxidase method (*b*, for details see text) using an antibody against one of the major polypeptides (i.e., the middle band polypeptide of the triplet denoted by arrowheads in Fig. 7, slot 7) from a rat liver fraction enriched in nuclear-envelope-associated material. Frozen sections of rat liver (*a*; for ultrathin section made therefrom see *b*) and rat myocardium (insert in *a*) show a strong peripheral staining of the nuclei. The intense immunostaining is restricted to a relatively thin peripheral nuclear zone corresponding to some layers of granules of the peripheral chromatin (*b*) but does not appear restricted to a thin layer (lamina) interposed between the nuclear envelope and the chromatin. By contrast, sections treated with antibodies against histones (*c*, antihistone H2b) show a uniform staining of the chromatin of the nucleus (*c*). No, nucleolus. Bars, 30 μm (*a*, and insert in *a*) and 2 μm (*b*, *c*). (*a*, and insert in *a*) × 730; (*b*, *c*) × 7,000.

portion of nuclear pore complexes, and release their nucleic acid content into the nucleus, leaving the emptied capsids on the cytoplasmic side of the pore complexes (78–80). This shows that the passage of particles across the nuclear envelope is confined to pore complexes and may involve profound changes in the shape of structures in transit. However, whether the pore complex material itself can exert some control on the nucleocytoplasmic exchange, e.g., influence selectivity and rate, awaits further experimental evidence. In fact, there is not even direct demonstration that the nuclear envelope itself is critical for the maintenance of ordered pathways of biological significance, such as nuclear uptake of certain proteins and cytoplasmic transport of transcription products. Experiments which have shown, in living amphibian oocytes, that neither nuclear accumulation of proteins nor nucleocytoplasmic transfer of ribosomal RNAs are markedly affected when the nuclear envelope is experimentally disrupted (81, 82) point to our fundamental ignorance of the true biological function of the nuclear envelope in intracellular compartmentation.

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II. Protein Turnover and Secretion

Ribosomes and Protein Synthesis

PHILIP SIEKEVITZ and PAUL C. ZAMECNIK

Let us start at the very beginning. Between 1897 and 1899, G. Garnier, in France, published elegant microscope studies describing a basophilic component of the cytoplasm of glandular cells (1). Because of what he thought its role might be in the elaboration and transformation of secretory products, he gave a Greek name to these concepts—ergastoplasm (work plasm). Garnier's research was extended by others—particularly A. Prenant, R. R. Bensley, and A. Matthews—to include other cell types, so that by the early part of this century ergastoplasm came to be a generally accepted term for a specific basophilic area of the cytoplasm. These early studies are extensively reviewed by F. Haguenau (1). The next major advance was to show that basophilia was due to RNA: in 1933, J. Brachet used RNase (2); in 1939, T. Caspersson used ultraviolet spectrophotometry (3); in 1943, J. N. Davidson and C. Waymouth used chemical methods (4). The high correlation which was shown between the amount of RNA in various cells and the postulated protein-synthesizing capacity of those cells led Caspersson in 1941 (5) and Brachet in 1942 (6) to proclaim the importance of RNA in the process of protein synthesis. As can be imagined, this conjecture spurred many scientists in the next decade to try to answer three questions. In what form was this cytoplasmic RNA? Did it really have a role in protein synthesis? If so, what was the role? Various methods were used: extraction and chemical procedures; extraction and physicochemical procedures, such as ultracentrifugation; and, because electron microscopy was becoming more and more refined, visualization. We now know that the RNA is in the form of ribosomes, and that the proteins of the ribosomes are involved in the many individual steps of protein synthesis; however, the function of ribosomal RNA is still elusive.

The intensity of the research in the 1940s is caught very well in Haguenau's chapter on the visualization aspect (1) and in Magasanik's 1955 monograph (7) on the extraction and chemical properties of what were then called "pentose nucleoprotein." Confusion abounded, in good part due to the terminologies developed for the different techniques, such as Garnier's ergastoplasm, K. Porter's "endoplasmic reticulum" (8), A. Claude's "microsome" fraction (9), G. Palade's "small particulate component" (10), and the "nucleoprotein" preparations or particles discovered by various workers. The last are reviewed in M. Petermann's 1964 book (11). However, very soon some sense began to emerge from the confusion. A. Claude, who in 1938 (12) had isolated high-speed pellets, which he later (9) called "microsomes," found in 1943 (9) that this pellet contained most of the RNA of the cytoplasm. Porter (8) showed that his newly discovered membranous network, which he named the "endoplasmic reticulum," could be identified with the high basophilic and high RNA content of the ergastoplasm, and gradually the term ergastoplasm dropped from sight. The electron-microscope images developed by Claude and Porter and, later, by Palade, began to replace the light-microscope images.

At about this time, various investigators (13–16) noticed small, dense, 10–15-μm particles in the electron-microscope images of the cytoplasm of various cells. Palade (10) described these particles as being either attached to the membranes of the endoplasmic reticulum or free in the cytoplasm, and expressed the opinion that the attached particles could account for the basophilic nature and high RNA content of the reticulum. Indeed, Clermont (17) showed that, in spermatids, the RNA-staining region, as seen with the light microscope, could be equated with a free granular region, as visualized with the electron microscope. However, RNA content could not be equated with small granule content until the combined biochemical and cytological work of two laboratories, that of the Zamecnik group (18) and that of Palade and Siekevitz (19, 20), independently showed that these particles could be isolated from the microsome fraction and that the high RNA content of the fraction was due to the RNA content of the granules. Palade and Siekevitz (19, 20) had found previously that Claude's fraction could be equated with fragmented endoplasmic reticulum elements. Thus, by the mid-1950s, a clear progression could be established: the high basophilia of the ergastoplasm visible in the light microscope could be equated with the endoplasmic reticulum visible in the electron microscope; the endoplasmic reticulum could be isolated as fragments in the high-RNA-content microsome fraction; and, finally, it was the small, particulate element of this latter fraction, and not the membranes, that was responsible for the high RNA content. Progress in this field could not have been achieved, though, without the cell fractionation procedures worked out by Claude, G. Hogeboom, and W. C. Schneider in the United States, and by C. de Duve and his colleagues in Belgium; it was their work that laid down the conditions and the criteria for the successful isolation of subcellular components, including the elusive RNA-containing particles.

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Carbon-14 and the Development of In Vitro Protein Synthesis Research

At the end of World War II, work began in earnest on protein synthesis, for carbon-14 had just become available, and a way was opened up for isotopic studies. The ^{14}C had much greater sensitivity than did the heavy isotopes used in the pioneering work of the Schoenheimer group (21), and this label made it possible to examine the incorporation of labeled amino acids into proteins in tissue slices, rather than in whole animals. By 1948 it was known that, in the rat-liver slice, oxygen is necessary for protein synthesis (22) and that dinitrophenol, which prevents the formation of ATP, blocks protein synthesis (23). Lipmann (24) and Kalckar (25) had predicted that amino acids are phosphorylated before polymerizing into a peptide chain, and it appeared that those predictions might be correct. Loftfield et al. (26) showed that proteolytic enzymes lack the requisite specificity for protein synthesis.

It was clear to scientists active in this new endeavor (27–29) that it would be necessary to disrupt cells in order to isolate and characterize the cellular constituents involved in the synthetic reaction. At that time, no macromolecules had been synthesized in cell-free systems. The term "incorporation" was, in fact, chosen to express caution. Finding a labeled amino acid bound by apparent covalent linkage to protein in a cell-free system could not be termed "protein synthesis," when an unequivocal demonstration of the formation of a new protein molecule was still lacking. There was good reason for caution: experiments of the Schoenheimer group (21) had disclosed both the lability of the carbon-nitrogen skeleton of certain amino acids and the transfer of the label into other types of cellular compounds, when introduced into a living system. However, at this time the new starch-column chromatography of Moore and Stein (30) made possible the unequivocal separation of the amino acids in proteins after a ^{14}C-labeling experiment. Thus it could be shown that when alanine, glycine, glutamic or aspartic acids are labeled in the carboxyl position with ^{14}C and used in a tissue-slice or whole-animal incorporation experiment, part of the carbon skeleton of these amino acids appears in other amino acids (29), and, in some cases, in carbohydrates, lipids, and nucleic acids (31, 32). Certain amino acids, such as leucine, isoleucine, and valine are, however, metabolically more stable (33), and, at the termination of an experiment, were found convalently bound in protein, with the label predominantly in the amino acid initially added (33, 34).

The effort to find a cell-free system capable of synthesizing protein (35–38) occupied the attention of several laboratories from 1948 to 1952. Bacterial systems were particularly difficult to free of live cells (cf. reference 39) and eukaryotic tissues, such as rat liver and rabbit reticulocytes, came to be preferred. Fractionation of liver-cell homogenates by the centrifugal methods introduced by Claude (9) and further developed by Hogeboom, Schneider, and their group (40) made fractionations of disrupted eukaryotic cells reliable and reproducible. It became possible to separate cell constituents into four major fractions:

(*1*) a mitochondrial and nuclear-rich fraction that also contains whole cells and large, ruptured cell fragments;
(*2*) a microsomal fraction;
(*3*) a soluble protein and other soluble cell-constituent fractions; and
(*4*) a low molecular-weight fraction obtained from (*3*) as a soluble TCA fraction, or as a fraction resistant to heating to 100°C.

This cell-fractionation technique formed a bridge between the morphologists and the biochemists by providing a means for relating the biochemical events of protein synthesis to recognizable structures. The cell homogenate was at this time regarded as a "biochemical bog, in which much effort was being expended to reach firm ground" (41), and the cell-fractionation technique offered a stepping stone.

A first break in the elucidation of the events involved in protein synthesis came in 1952 (36, 42), when amino acid incorporation into protein was related to oxidative phosphorylation in a cell-free rat-liver system by the separation of the energy-utilizing system of incorporation and the energy-producing system of the mitochondria. Further refinement of this method and the use of gentler homogenization showed that incorporation depends on the presence of ATP and on an ATP-regenerating system (43). Thus, it became possible to dissect the protein-synthesizing system into four constituents: amino acid, an ATP-donating component, a soluble enzyme fraction, and a microsomal fraction (43). This subdivision provided a springboard for further partition. It was found in 1955–56 that the amino acid activation reaction is produced by enzymes—a separate enzyme for each amino acid—in the soluble fraction of the cell (44, 45). As a result, the formation of aminoacyl adenylates was disclosed as the first step in the series of reactions leading to completed protein. It was found at the same time that the microsomal fraction is the site of polypeptide polymerization (18, 46). However, the term microsome is only an operational definition of a high-speed, multicomponent, sedimentable cell fraction, and soon it became clear that the ribonucleoprotein particles of the microsome fraction are the actual marshalling site for polypeptide polymerization (47, 48). The ribonucleoprotein particulate fraction of the microsomes could be separated from other components of the microsomal pellet by the addition of sodium deoxycholate, which solubilized enzymes involved in cholesterol synthesis and in detoxification reactions. This procedure left the ribonucleoprotein particles relatively intact (19, 20, 47), but inactive. Purification of an aminoacyl synthetase made it possible to mix the synthetase with labeled amino acid, ATP, and ribosomes but, surprisingly, no protein synthesis occurred (39). At the same time, however, new evidence suggested that another step exists between aminoacyl adenylate and polypeptide synthesis (49, 50). The existence of transfer RNA—first called soluble or sRNA (51)—was discovered in 1958 (51–53), and it was shown that the activated amino acid transfers its aminoacyl moiety (54) to the common cytidylic-cytidylic-adenylic (CCA) terminus (55) of the tRNA. Purification of an aminoacyl synthetase to near homogeneity (56, 57) showed that the same enzyme catalyzes the formation of the aminoacyl-AMP anhydride and also the transfer of the aminoacyl group to esterification with tRNA. At this time, the presence of an unusual base was found in RNA by Allen (58, 59) and by Cohn (60) and was named pseudouridine (60); later it was found to be in tRNA (61). As for the primary structure of tRNA, a race began in the early 1960s which culminated in the complete determination of the primary structure of alanine tRNA by Holley and his colleagues (61), a key event in the sequencing of any polynucleotide.

Logically, but surprisingly, at least one separate tRNA was found for each amino acid species (62, 63). In the same year that tRNA was discovered, Crick (64) postulated the presence of a trinucleotide intermediate attached to an amino acid that would direct the amino acid to the proper triplet position on nucleic acid in a translation of the genetic code. This "adapter

hypothesis" of 1958 expressed the concept in terms of the interaction of tRNA and the template RNA, considered to exist on the ribosome as a mechanism for ordering the amino acid sequence (62).

Although Crick suggested the presence of a trinucleotide as a translation piece, the tRNA actually found contained approximately 75 nucleotides. It was determined that this polynucleotide acts *in toto* (65). At this point, it became appreciated that the same molecule must both recognize its cognate aminoacyl synthetase and provide an association site with the ribosome (66). Thus, the presence of a coding operation involving recognition of amino acid-specific tRNAs for their cognate aminoacyl synthetases became evident (66) and, in time, evidence for a separation of these functional sites on tRNA began to emerge (67). Initially, there were misgivings (62) that the tRNA might be too large to serve as an amino acid shuttle. Kinetic studies revealed, however, that there were enough tRNA molecules, and that the rate of transfer was adequate for tRNA to act in this capacity (53, 66). It was also found subsequently that a number of peptide chains could grow simultaneously by traveling along in sequence on the same polysome (68). As for the rate of construction of a long peptide chain on the ribosome and its conversion into an antigenically recognizable protein molecule, Loftfield's studies were definitive (33). He found that ferritin synthesis, in the liver of the intact rat, requires six minutes.

In 1956, it was discovered that guanosine triphosphate (GTP) is an essential cofactor in the step between aminoacylation of tRNA and polypeptide polymerization on the ribosomal template (69). Furthermore, a new and separate enzyme (or enzymes) is apparently needed to catalyze the polypeptide chain extension on the ribosome (66, 70). Further questions were answered at this time: Does all protein synthesis occur by a single addition of an aminoacyl unit to a growing chain on the ribosome, or do separate, small, peptide intermediates form and then link together? Loftfield and colleagues (71, 72) showed that there are no small peptide intermediates. But in which direction did the nascent peptide chain grow—from the amino end to the carbosyl end, or vice versa? In an answer to that question, Schweet and colleagues (73), and particularly Dintzis (74), determined in 1960–61 that the chain grows from the amino end to the carboxyl end.

Structure and Function of the Ribosome

Returning now to the physical chemistry of the ribosome: the nature of these nucleoprotein particles, on which the protein-synthetic reaction takes place, was still a mystery. All that was known was their fuzzy image in the electron microscope, that the eukaryotic particles contained about two-thirds protein and one-third RNA, and that, in prokaryotic particles, protein and RNA were equally divided. That the particles could be isolated by means of certain mild, nonionic or ionic detergents, such as deoxycholate, indicated that they were complexes of protein and RNA, so researchers began to study them as high-molecular-weight complexes by the biophysical means of ultracentrifugation. Actually, this tool had been used in the late 1930s and early 1940s, when Wyckoff's group demonstrated 40–90S particles in extracts of silkworm (75) and plants (76) and Sevag's group (77) found 100–125S particles. Perhaps the key papers in this period were those of Taylor et al. (78, 79), who found RNA-containing particles of 69–71S in chick embryos and in extracts of human and rabbit brain, and who noted also the breakup into smaller 60 and 40S particles by increasing salt concentration and increasing pH, and that electron-microscope images showed particles with diameters of ~18 μm, observations that were confirmed by Kahler and Bryan (80). However, it was not until the early 1950s that M. Petermann and her co-workers and Chao and Schachman could show that these particles are ubiquitous, that they are well defined in terms of sedimentation properties, and that they have certain definable and reproducible characteristics. Approximately 10 μm particles of uncorrected 50S and 30S values that contained 50% RNA were found in bacteria, and 80S and 60S particles were found in yeast (81); similar particles with 50% RNA and with an uncorrected 40S value corrected to 75–80S, were found in mammalian cells (82–84). Both these groups made the important discovery that Mg^{++} is necessary for the stability of the particles, and by 1956 and 1957, purified particles had been obtained from yeast (85, 86), liver (87, 88), and peas (89). It was also becoming apparent at this time that these isolated particles are the in vitro counterpart of the ~10–15-μm-diameter particles seen *in situ.*

In 1958, the first symposium of the newly formed Biophysical Society was held, and many papers on the particles—until then called nucleoprotein particles—appeared in the proceedings edited by R. B. Roberts (90). At that symposium, Roberts proposed the shortened name "ribosomes" for particles that contained complexes of one-third to one-half RNA and two-thirds to one-half protein, were ~10–15 μm in diameter, had sedimentation values in the 100–20S range, were found in all cell types, and seemed somehow to be involved in protein synthesis. The name caught on, for, as Roberts put it, "it has a pleasant sound."

Following Brachet's (2) and Caspersson's cytochemical lead (3), Borsook and colleagues (27), Hultin and Beskow (91), and Keller et al. (46) pointed to the possibility of ribonucleoprotein or ribosome involvement in protein synthesis.

Although the ribosome seemed to be a fairly well-substantiated subcellular structure, there was still a good deal of uncertainty in the middle and late 1950s as to the exact nature and structure of the particles *in situ.* The uncertainty came about partly because of the great variety of Svedberg constants that abounded in the literature for particles from many different organisms, and partly because of the confusion created when attempts were made to correlate the term "microsomes," used by the cell-fractionation workers, and the term "ribonucleoprotein particles," later "ribosomes," used by sedimentation researchers. Various authors found sedimentation values of 80, 60, and 40 in liver and spleen, whereas 80, 60, 40, 30, and 20S were reported for microorganisms, even within the same cell. For example, Petermann et al. (92) attempted to correlate what the microscopists called attached and free ribosomes with various ultracentrifugation patterns that showed different sedimentation values. The confusion began to recede when it was learned that the sedimentation characteristics of ribosomes are concentration-dependent and, more important, are dependent on the isolation and centrifugation medium, its pH, and its salt and ion concentrations. When these conditions were recognized, it became apparent that the ribosomal particles can reversibly dissociate into smaller subunits, that these can be partially unfolded, thus accounting for the various sedimentation values, and that that reaction is influenced greatly by the Mg^{++} concentration. This was recorded initially by Chao and Schachman for ribosomes from yeast (85, 86), by Ts'o et al. for pea seedlings (89), by Tissieres et al. for *Escherichia coli* (93, 94), and by Hamilton and Petermann for liver (95). Actually, Petermann's (96) earlier observation of the

many ultracentrifugation boundaries produced by ribosomes under different conditions led to the speculation that many of these entities represent unfolded subunits or associated subunits. When molecular weights were calculated later, based on sedimentation equilibrium, this hypothesis was found to be true. For example, a 60S subunit seen in the preparation of liver ribosomes is indeed a dimer of the small subunits (97–99), and ribosomes with different sedimentation values contain only the two large RNA polymers that are mentioned below, thus implying that these various values represent different conformational forms of the ribosomes (98, 100). Work in many more laboratories accelerated, and by 1964 M. Petermann, in her milestone monograph (11), was able to list an impressive bibliography of papers in which were given the properties of ribosome and the conditions for isolating ribosomes and their subunits from many types of cells.

Thus, by the mid-1960s, a great deal of information had accumulated on the appearance, isolation, chemical and physical properties, and function of ribosomes. Going on from the early work with mammalian tissues, electron-microscope images of ribosomes had been observed in such diverse species as yeast (101), *Neurospora* (102), wheat (103), silkworms and flies (102), hydra (104), frogs (105), and chicks (106). A cell-free, protein-synthesizing system was worked out for higher plants (107), for which the requirements were the same as those for animals, with one notable difference. The chloroplast of the plant was found capable of synthesizing protein autonomously, without the participation of other plant-cell components. M. Simpson's group (108) found this held for liver mitochondria. Also, a correlation had been made (19, 109) between the occurrence of membrane-bound ribosomes and the secretory status of the cells that contain them. Deoxycholate had been shown in the middle 1950s (18, 19) to free membrane-bound ribosomes from the membrane. In 1960, Takanami (110) found that the ribosomes could be precipitated by Mg^{++} and rendered free of extraneous elements. Grinding the bacteria in alumina to obtain ribosomes from bacteria had been introduced in the late 1950s (94, 111) and, by 1965, ribosomes had been isolated from a large variety of sources (cf. reference 11). A great advance in the isolation and characterization of ribosomes was made in 1960 by Britten and Roberts (112), who combined a reverse loading gradient with a sucrose density gradient to obtain sharp ribosomal bands. This technique permitted the physical separation and analysis of macromolecules on the basis of sedimentation rates. Soon numerous papers appeared, describing density gradient profiles of ribosomes and their subunits. The method was useful in showing both the dissociation of ribosomes and the association of protein radioactivity with the ribosomes. It also aided in the bulk isolation of ribosomes and their subunits.

Early Work on mRNA

In 1956, an experiment was performed by Volkin and Astrachan (113) with a puzzling result that had profound implications for an understanding of the mechanism of protein synthesis, although it was not fully appreciated for several years by some investigators already in the field and was unknown to others of recent entry. Volkin and Astrachen provided the first evidence for the presence of "messenger" RNA, a metabolically labile, uncharacterized RNA involved in protein synthesis and distinct from the more stable RNA known to be part of the ribosome. In 1959–60 several groups provided more compelling evidence for the existence of a messenger RNA. Riley et al. (114), Brenner et al. (115), and Watson and colleagues (116), all showed in phage or bacterial systems that the RNA that directs translation could change more rapidly than the life span of the ribosomal particle should have permitted, and must therefore consist of a separate, newly formed strand of RNA that turns over rapidly and associates and dissociates from existing ribosomal particles.

Nirenberg and Matthei (117) accepted this postulation, and reasoned that a simplified, synthetic polyribonucleotide might also serve as an mRNA. In 1961 they used the cell-free bacterial system that had recently been devised by Lamborg and Zamecnik (111), preincubated it to get rid of the postulated existent mRNA, added polyuridylic acid, phenylalanine, and a higher concentration of Mg^{++}, and then observed the formation of polyphenylalanine. The first break in the genetic code had been made. Ochoa and colleagues (118) then found that polyadenylic acid (whose synthesis by a bacterial enzyme had been demonstrated earlier by Grunberg-Manago and Ochoa [119]) coded for polylysine. In retrospect, it is interesting and still puzzling that the complex of initiation, RNA 5′-end-capping, and elongation factors were short-circuited, or fortunately were present in the incubation mixture, and that translation actually occurred in this simplified system. The advance in knowledge produced by this finding was enormous, and by 1966 the entire triplet genetic code was deciphered, the ultimate precision in triplet nucleotide specification having been furnished by the work of Khorana and associates (120).

It was also doubted at this time (121), when it was unpopular to think in such a way, that a triplet code—in which the distinction between the selection of one amino acid and another might rest, in certain instances, on only one or a few hydrogen bonds—would be accurate enough to account for the high fidelity of protein synthesis. Subsequent work (122) provided evidence for a proofreading step at the aminoacylation site. That the overall error level in protein synthesis is somewhere between 1 part in 3,000 and 1 part in 10,000 was determined by Loftfield (123).

Our knowledge about the chemistry of ribosomes was altered in the early 1960s by the unexpected discovery that bacterial ribosomes contain not one, but a large number of different proteins (124, 125); the same result was obtained for ribosomes from yeast (126), pea seedlings (127), reticulocytes (128), and liver (129). Later, Spitnik-Elson (130) found that the 50 and 30S *E. coli* subunits contain 21 and 13 proteins, respectively. Conversely, it was found by Littauer and by Spirin, both of whom used the phenol extraction method, that ribosomes from *E. coli* (131, 132) or from liver (131) contain fundamentally only two high-molecular-weight RNA species, with sedimentation values of ~16 and 23S in the bacterium and of 18 and 26S in liver. Kurland (133) went a bit further. He calculated that the molecular weights of the *E. coli* RNA are 0.56 and 1.1 $\times 10^6$ and found that the 30S subunit contains the 16S RNA, whereas the 50S subunit contains both the 16 and 23S RNA. A similar result was obtained by Aronson and McCarthy (134). Later, molecular weights for reticulocyte RNA were calculated to be 0.5 and 1.5 $\times 10^6$ (135). However, a flurry of papers in the early 1960s produced only confusion, in that, although it appeared that the lower-molecular-weight RNA species are found only in the smaller ribosomal subunit, from whatever source, the larger subunit frequently yields both types of RNA. A greater source of confusion was the observation by many workers of various lower-molecular-weight RNA species, ranging from 12 to 2S. By the mid-1960s, it had become apparent that all these smaller components are breakdown products of

the two larger species, probably as the result of RNase action and possibly from the effects of salt and pH, and that by judicious separation of the larger from the smaller ribosomal subunit, one can obtain only 16 or 18S for the smaller subunit of prokaryotic and eukaryotic ribosomes, respectively, and only 23 and 28S for the larger subunit of prokaryotic and eukaryotic ribosomes, respectively (see references 136–138). The only lower-molecular-weight species then found that has survived such scrutiny to this day is the ribosomal 5S RNA. Even in 1963 it had been recognized (139) as being different from the large ribosomal or transfer RNA of *E. coli* ribosomes. At about this time, a great deal of work from many sources on the base of composition of ribosomes and their subunits always produced the same result, namely the asymmetrical high guanine and lower cytosine content of the RNA (cf. reference 11).

The improvements in the techniques for isolation and stabilization of ribosomes and their subunits led to a better knowledge of their physical properties. For example, in addition to the 30, 50, and 70S *E. coli* particles, a 100S particle was observed during ultracentrifugation procedures. Calculated molecular weights (11) indicated that the 30 and 50S particles form the 70S particle, and that two 70S particles give dimers of 100S. Further proof was the elegant negative-stained electron micrographs by Hall and Slayter (140) and by Huxley and Zubay (141), which showed the cleft between the 30 and 50S subunits of the 70S monomer and also showed that the 100S particles are two 70S monomers bound together by their 30S subunits. The dried-down preparations showed ellipsoids of 20 $\times$ 17 μm.

X-ray diffraction patterns produced by different research groups all suggested a helical conformation of the RNA within the 70 (142–145) and 80S (144–147) ribosomes, confirming the earlier speculation that had been based on postulated hydrogen-bonded structures (148). Because the association-dissociation reaction and the electrophoretic mobilities of ribosomes change with the ionic, particularly the Mg^{++}, environment, Petermann and Hamilton (149) concluded in 1961 that much of the RNA is at the surface of the particle. The nature of the bonds attaching the RNA to the proteins was studied by using protein denaturants like urea (150), and such salts as LiCl (151, 152) and guanidine (153). This led to the conclusion that, in addition to the need for Mg^{++} complexing, as shown by studies with ethylenediaminetetraacetate (EDTA) (86), salt linkages also are involved in holding the ribosomal components together. Indeed, until Martin and Wool (154) published their high-salt, high-Mg^{++} method, eukaryotic ribosomes had not been shown to be reversibly dissociated into active subunits.

While these studies were going on, the physiological role of ribosomes—their involvement in protein synthesis—was also being examined extensively. In 1964, Gilbert (155) found that nascent (radioactive) proteins bind to the larger subunit of *E. coli* ribosomes and, in 1965, Tashiro and Siekevitz (99) found the same binding in liver ribosomes. Gilbert (155) also discovered that tRNA binds to the larger subunit and, in 1963, he and others postulated that the tRNA-nascent polypeptide complex was fitted into a cavity of this subunit (156). This result was a follow-up on earlier studies (18, 157, 158) with eukaryotic ribosomes that established ribosomes as the site of highly labeled, presumably nascent, proteins. However, the situation became complicated in 1962 when it was found that *E. coli* ribosomes to which radioactive nascent polypeptides were still attached resisted dissociation by EDTA. These were given the name "stuck ribosomes" (159–161); in 1965, the same resistance to dissociation was found in liver ribosomes (99). Indeed, even when ribosomes could be dissociated, the presence of the radioactive protein, and presumably also that of tRNA, made the large subunit more compact, so that those subunits which carried the nascent radioactive polypeptide sedimented demonstrably faster than did the bulk of the nonradioactive large subunits (99).

In the early 1960s, as described above, evidence had accumulated for the existence of messenger RNA, a rapidly turning over fraction which attaches to ribosomes (115, 116) and provides information for the amino acid sequence in protein synthesis (117, 162, 163). This mRNA fraction was now found to bind to the smaller ribosome subunit (164). It was also found that the attachment of a natural mRNA fraction (159) or a synthetic polynucleotide (160, 161) to a ribosome preparation leads to the formation of large aggregates. However, the definitive experiments on the nature of these aggregates were published in 1962 and 1963 by Rich's group (165, 166), which coined the term "polysomes" for those structures that previously had been called "heavy ribosomes." These structures sedimented more rapidly than did single ribosome monomers, had the radioactive nascent polypeptide attached to them, and probably were held together by mRNA. These observations were made contemporaneously or confirmed by others (167–170). Chains or clusters of isolated pancreatic ribosomes had been observed some half-dozen years earlier in one laboratory (20) and were seen several years later in another (cf. reference 39). The explanation of their appearance escaped both groups of investigators; not until 1966 (171) were these polysome structures seen *in situ* attached to endoplasmic reticulum (ER) membranes. The visual proof for the polysome structure was provided by Slayter et al. (172), who used the new negative-stain method of Huxley and Zubay (141), a method that came into use for the visualization of many structures other than ribosomes and even for proteins.

By the mid-1960s, then, the role of the ribosome in protein synthesis had been pretty well schematized. It was known that the mRNA molecule has many binding sites for the ribosomal RNA contained in the small subunit, accounting for the existence of polysomes; that the growing polypeptide chain is attached to the large subunit, and also, via its cogate tRNA, to the mRNA; that the tRNA is also bound to the large subunit; and that, upon dissociation of the ribosome, the nascent polypeptide remains stuck to the large subunit. What remained to be done in the late 1960s was to try to fill in the gaps—the specific mechanisms mainly involving ribosomal proteins. Also, an ultrastructural point of the earlier data (19, 109, 157, 158) had to be verified: that the difference between eukaryotic-free ribosomes and membrane-bound ribosomes is that the latter are involved in the synthesis of proteins exported from the cell via the lumen of the ER. The initial steps in export were more thoroughly verified in cell-free systems; they showed that a newly synthesized, purified protein (173) or puromycin-released polypeptides (174) are moved from the surface of the ribosome across the ER membrane into the cisternae of the ER. This morphological correlate to secretion was strengthened when it was found that ETDA removed the small subunit and left the large subunit, with its attached nascent polypeptide chain, still bound to the ER membrane (175).

To digress for a moment on puromycin, these studies had their origin in the novel suggestion by Yarmolinsky and de la Haba (176) that the antibiotic puromycin might act by mimicking a portion of a transfer RNA, thus serving as a bogus acceptor of a growing peptide chain. Because the puromycin molecule resembles the abbreviated end of a tRNA molecule,

it presumably has no way of anchoring the growing peptide chain to the ribosome and acts as a chain terminator. The truth of this hypothesis was demonstrated in a cell-free, hemoglobin-synthesizing system to which a [^{14}C]puromycin was added. A short peptide chain that contained [^{14}C]puromycin at its carboxyl end was isolated (177). A series of inhibitors of protein synthesis were found subsequently, after this demonstration of the molecular mechanism of action of a naturally occurring inhibitor of protein synthesis. Among the most intriguing of these inhibitors was streptomycin, which Gorini and Kataja (178) found to cause a misreading of the genetic code by the ribosome.

Work continued in the late 1960s on ribosomal RNAs. The 23 and 16S RNA of the bacterial ribosomes were shown to be separate entities, and the 23S was eliminated as a possible precursor of the 16S moiety (179, 180). The work of Monier illustrates how one finding leads to another. While on sabbatical, Monier et al. (181) purified the 4S RNA of transfer RNA. He then returned to Marseilles and continued to purify transfer RNA and to study ribosomal RNA. Surprisingly, he found another, distinct RNA that sedimented a little more slowly than the 4S tRNA. This he designated 5S RNA, and Rosset and Monier (139) found it to be tightly associated with ribosomal RNA. This discovery, confirmed by Elson (182) and by Galibert et al. (183), led to a number of investigations into the nature of this component, and it was soon found that 5S RNA is not a breakdown product of the larger species, for it contains no methyl bases or pseudouridine (184, 185), and that only one molecule of the 5S RNA is bound to the large subunit, as compared to the two tRNA molecules which are bound (185).

During this period, work continued on the chemistry of the ribosomal proteins; the chief interest was in their number and characteristics. It was found that they all had about the same molecular weight, from 10,000 to 25,000 (124), that they were virtually all basic (186), and that their number was about 20 in the 50S and 10 in the 30S *E. coli* subunits (186). Traut et al. (187) demonstrated that the proteins separated by acrylamide gel electrophoresis are all different in amino acid composition. The study of the interaction of ribosomal proteins and RNA and the function of the proteins received a big impetus with the finding by Meselson et al. (188) that the use of 5M CsCl made it possible to solubilize some proteins from the *E. coli* 50 and 30S ribosomes, giving "core" particles of 43 and 23S, which are unable to function in protein synthesis. Many scientists began to investigate this phenomenon, and the solubilized, so-called split proteins, different electrophoretically from the core-particle proteins, were found to be involved in both tRNA and mRNA binding to the core particles and in some of the steps of amino acid polymerization (189). Indeed it was found possible (190–192) to add these split proteins back to the core particles to reconstitute the 50 and 30S particles that could combine to form the 70S ribosome active again in protein synthesis. A step forward was made by Spirin and his coworkers (193), who, by using graded concentrations of CsCl, succeeded in degrading the particles stepwise, and who showed that the particles can be reconstituted at each stage by the back addition of the proteins split off at that stage.

A few years earlier Britten, McCarthy, and Roberts (194, 195) had begun to examine the biogenesis of ribosomes by using pulse-labeling with RNA precursors, and they came to the conclusion that ribosomes are formed in a stepwise manner. This is still considered to be the case, even though in their experiments they were probably finding more mRNA than rRNA labeling; these experiments were done shortly after naturally occurring mRNA had been discovered. At the same time, workers began to use protein synthesis inhibitors to try to show the existence of stages in the biogenesis of ribosomes; one of the most popular of these was chloramphenicol, initially used by Nomura and Watson (196). The further use of chloramphenicol, plus the introduction during centrifugation of formalin as a ribosome "fixative" (197), which enabled density and, hence, protein/RNA ratios to be more accurately determined, led to a large number of experiments by many laboratories, particularly those of Osawa et al (198, 199), Kurland et al. (200, 201), and Nomura (200, 202). The 43S chloramphenicol particles lacked some proteins, and they seemed to be the same as those lacking in the CsCl-treated 50S particles (199). Indeed, the chloramphenicol particles seemed to be reconstitutable for protein synthesis (203, 204) in the same manner as that described for the CsCl particles. By the mid-1960s, it was hypothesized, based on the evidence partially cited above, that the ribosomal RNA in each of the subunits complexes with a certain number of proteins to form core particles, which then go through at least two protein-addition stages to finally form the mature subunits.

The 1960s were the high point of investigations on the chemistry and synthesis of ribosomal RNA, and by the end of the decade a great deal of information had been gathered. The high guanine content was further verified, as was the presence of pseudouridine (205), and the presence of methylated bases in many ribosomal RNAs was discovered in 1964 and 1965 (206–208). However, precursor-rRNA splicing to form mature rRNA remained unknown for another decade. Sequencing of the bases began at this time, as did studies on secondary and tertiary structure. As mentioned above, the 1962 work of Robert and co-workers (194, 195) with *E. coli* led to the concept of ribosome precursors; the smallest stable one they could pick up at that time sedimented at 14S. Subsequently, the nascent RNA radioactivity appeared in 30 and 43S particles, and finally ended up in the mature 30 and 50S subunits. Even though some of the radioactivity in the smallest particles, found at the earliest time points, was undoubtedly due to the rapid turnover of the mRNA fraction (116, 209), the idea then postulated of delay points in ribosome biogenesis, at which proteins were added, has held to this day. The early work of Osawa's group (198, 199) confirmed the results, and by 1969 (210) they postulated a sequence of events in *E. coli* which led from the nascent mRNA to a ribosomal 22S, then to 26S, and finally to the 30S small subunit. They also suggested the sequence of 30S to 40S to 50S for the large subunit, and indicated that the methylation of the rRNA took place early in this sequence of events. The use of chloramphenicol accelerated in the later 1960s, and the results from many laboratories agreed with the early formulation, though the exact size, or sedimentation values, of the precursor particles varied among the various experiments.

Ribosomal Biogenesis in Eukaryotes

A step forward in the elucidation of ribosome biogenesis was taken when eukaryotic systems were tested. It had already been supposed, based on many earlier cytochemical (6, 211) and autoradiographic observations (212), that the nucleolus is the site of ribosomal RNA synthesis. Later, the lack of ribosomal RNA in a nucleolar mutant (213) and the electron-microscope localization of rRNA genes in the nucleolus (214) confirmed the early supposition. The results of base composition studies by Edström (215) and of work by R. Perry (216), who used low

concentrations of actinomycin D to selectively block nucleolar RNA synthesis, again supported early data. Later findings (217, 218) showed clearly that a large 45S RNA molecule in the nucleus was the precursor of the mature 18 and 28S RNA of the ribosomal subunits; that this 45S RNS is split to an 18 and a 35 or 32S species (217, 218); that the 35 or 32S RNA is somehow converted to 28S RNA; and that both the 18 and 28S RNA gain proteins in the nucleolus to form 48 and 60S particles, which are then discharged into the cytoplasm, there to form the 80S ribosomes (219–221). Furthermore, various types of experiments toward the end of the decade led to the acceptance of the hypothesis that when the 45S RNA splits to the 18 and 32 or 35S RNA, many nonribosomal stretches are excised, and that this also happens during conversion of the 32 or 35 to the 28S RNA (222–226). Finally, hybridization experiments proved that the 28 and 18S RNAs reside in the same precursor molecule, that the genes for the 28 and 18S RNAs alternate along the DNA chain, and that these genes are interspersed between stretches of DNA not coding for rRNA (227–229). Furthermore, the 45S RNA molecule seems to gather proteins while it is still in the nucleolus, where an 80S particle that contains 45 and some 32S RNA was found (230).

Work continued on the 5S ribosomal RNA in many laboratories (for a review see reference 231). The results agreed, so that by 1970 it was certain that the 5S RNA is present in all kinds of organisms, from bacteria to man, and that it has a high G-C content, is 120 nucleotides long, has no methylated bases, is probably similar to tRNA in possessing significant secondary structure, and is synthesized on chromosomes separate from the 18 and 28S RNA. It should also be mentioned (cf. reference 231) that, by this time, it had become evident that there are distinct genes for the two ribosomal RNAs, that multiple copies of these genes occur, and that they are clustered in the nucleolar-organizing region of eukaryotic cells. This work on rRNA biogenesis continued into the 1970s, and early in the decade it was shown (232, 233) that an RNA precursor, 30S, also appeared in prokaryotes and was cleaved to the 16 and 23S rRNA species. In eukaryotes, the genes coding for 18 and 28S RNA are present in clusters of thousands of copies, arranged linearly along the DNA, with a spacer region, an 18S RNA region, a spacer region, and a 28S RNA (234). In the nucleolus, these RNAs are complexed to some of the structural proteins of the mature ribosome and to some other proteins (235, 236). These ribosomal proteins are synthesized on cytoplasmic polyribosomes, transported to the nucleolus, and assembled there with the rRNA precursors and with the 5S RNA into large ribonucleoprotein particles (230, 237–239).

During the mid-1960s, the recognition of the existence of first one, then a puzzling succession of other, protein "factors" had come about as a result of washing them out of ribosomes, particularly with concentrations of sodium chloride greater than the isotonic level (66, 240, 241), or with cesium chloride washing (188), as mentioned above. The term "factor" was chosen to express the uncertainty as to whether these purified or crude proteins being added back to the washed ribosomes in a cell-free, protein-synthesizing system were acting catalytically or stoichiometrically. From this beginning, a large and bewildering family of factors and cofactors has grown, and continues to increase (241, 242). These are involved in association of mRNA with ribosomes, reassociation of the subunits of the ribosomes, chain initiation, chain propagation, movement of rRNA from the amino acid site to the peptide site on the ribosome, and with chain termination. Certain proteins modulate the rate of protein synthesis. Small nucleotide chains are also being recognized as regulators of protein-chain growth. For example, interferon induction results in the synthesis of a protein kinase and of the unusual trinucleotide pppA2′5′pA2′5′pA and related nucleotides (243), which activate an enzyme that hydrolyzes mRNA and inhibits protein synthesis.

An important advance was made in the early part of this decade, when it became possible to isolate and purify the individual proteins from each of the *E. coli* ribosomal subunits, to begin to characterize them, and to add them back to the stripped ribosomal subunits. The laboratories initially involved were those of Kurland (200, 201, 244), Wittmann (245–247), Traut (186, 187, 248), Nomura and Traub (189, 249), Osawa (198, 199, 250), Spirin (193, 197, 203), and Nomura (202). The existence of these purified proteins gave impetus to studies, first performed by Traub, Nomura, and their collaborators (249, 251), in which individual proteins were omitted from reconstitution experiments, and the resultant "reconstituted" ribosomes were assayed for structure and protein-synthesizing function. For example, proteins split off from the 30S particles were purified and each was added back to the "core" particle; these reconstituted particles were then either analyzed for structure or added to an intact 50S particle to assay for the protein-synthesizing capacity of the resultant 70S particle. It became obvious that much cooperative action exists between various proteins and between the proteins and the ribosomal RNA; some proteins could not be added back until after others had been bound to the core particle. Certain proteins were found to be necessary to complete assembly (249), others were essential for complexing with ribosomal RNA (252); one bound mRNA specifically to the 30S subunit (253), and others tightened the interaction between tRNA and mRNA (254). The two-dimensional gel system developed at that time by Kaltschmidt and Wittmann (246) was a signal contribution in the efforts to separate all the proteins.

Research into the detailed structure of the ribosome, and into which proteins interact with each other and with the RNA—the topology of the ribosome, if you wish—gained impetus through various approaches: immunological (255), protein cross-linking (254), and nuclease digestion. Based on all the studies, assembly maps of the ribosome had been published in the early 1970s (249, 252, 256, 257), and based on the number and molecular weights of isolatable proteins and on the molecular mass of the ribosome, the conclusion drawn from the 50S subunit was that there are at least 28 different proteins (247), a copy of each being present in each ribosome, with the possible exception of one protein. For the 30S subunit, there seem to be some proteins that exist in one copy per ribosome and some with less than one (244, 247, 248, 258), the implication being that some of the 30S ribosomal proteins are bound to the ribosome only when they are functionally necessary for some step in protein synthesis. By the end of the 1960s, much information also had been gathered on the interaction of the tRNA and mRNA molecules with each of the subunits and the dynamics of this interaction during the process of protein synthesis; the result was two models of subunit interaction during protein synthesis: Bretscher's (259) and Spirin's (260).

Work in the early part of the 1970s was concerned with possible specific functions of the subunit proteins, that is, in what binding and functional step each is involved. In the latter part of the decade, emphasis was on another aspect of the research: that not necessarily a specific protein, but rather a whole range of protein-protein and protein-RNA interactions produce the topology of the ribosome and permit protein

synthesis to proceed rapidly. This point of view was amply set forth by Kurland in 1977 (261). By this time, many of the individual proteins of the 30 and 50S *E. coli* ribosomal subunits had been purified and physically characterized, mainly by Wittmann's group (cf. reference 262), and many had been sequenced. The use of new reagents by Traut's group (263) demonstrated that subunit interactions are more extensive than was previously believed. The assembly map indicating moderate interaction between proteins and ribosomal RNA (264) had by now been modified to include the interaction of a larger number of proteins with ribosomal RNA (265). New methods of neutron scattering (266) and fluorescence spectroscopy (267) indicated the degree of proximity of individual ribosomal proteins. But perhaps the most spectacular advance was made possible by immunoelectron microscopy: by making antibodies to the individual proteins, one can, with luck, observe these antibody-protein complexes on the surfaces of ribosomal particles. The two principal groups engaged in these studies have been those of Stöffler (268, 269) and Lake (270, 271). The gross morphology of the ribosomes as given by these two groups is generally similar, but there are differences in the three-dimensional models. When one compares their data on near-neighbors of proteins in the 30S subunit with those obtained by the protein cross-linking method (255, 263, cf. reference 272) and takes into account the possible elongated nature of the proteins, one gets a good deal of correspondence.

Toward the end of the 1970s, the sequence of 5S RNA was worked out (273), that of 16S RNA nearly so (274), and substantial progress had been made in sequencing 23S RNA (275). However, the secondary and tertiary structures of the RNA were still not well understood, particularly when one tried to bring in the contribution of the various ribosomal proteins to these RNA structures as they exist in the ribosome.

Most of the work on ribosome structure and function has been performed with bacterial, particularly *E. coli*, ribosomes, because of their wide availability, but the past decade has produced studies on ribosomes from other sources. For example, the mitochondrial ribosome of eukaryotes, thought for years to be a 70S particle like the *E. coli* ribosome, was found instead to be a 55S particle (276). Another example was the finding that the 80S eukaryotic ribosome contained some 70–80 proteins, as compared with the 50–60 in prokaryotes (277). People in various laboratories have looked for similarities between specific bacterial and eukaryotic ribosomal proteins. However, based on immunological, two-dimensional electropherograms and partial amino acid sequences, there seems to be very little similarity between most of the proteins from the two general sources. The 5S RNA from eukaryotes and prokaryotes does show some sequence similarity (278); nothing can be said about the larger ribosomal RNA species. Recently, finer work has been attempted on mammalian ribosomes, based on the earlier work with *E. coli* particles. Indeed, for some purposes the former are better. Because of their larger size they have been quite amenable to viewing in the electron microscope, and Sabatini's group (279–281) has published striking pictures of the topology of the particles and their subunits and of the fit between the large and small subunits. By the mid-1970s, Wool and collaborators had separated and purified some 33 proteins from the 40S subunit (282) by using the methodology used for *E. coli* ribosomal proteins. They now are trying to decide whether all the proteins are individual entities and true ribosomal proteins, and even are attempting to sequence some of the more interesting ones. A method using 5.8S eukaryotic RNA immobilized on a column (284) has been developed to observe which eukaryotic ribosomal proteins are bound to this RNA (285). A novel reaction in eukaryotic, but not in prokaryotic, ribosomes is the phosphorylation of ribosomal proteins, first found in 1970 (286, 287) and quickly confirmed in many other laboratories. However, the phosphorylation was predominantly of one ribosomal protein (288), and many experiments examined the effects of various physiological conditions, including the conditions for protein synthesis, on the phosphorylation state mostly of this one protein, with inconclusive results (cf. reference 289).

Finally, circling back somewhat to the morphological aspects, a possible solution has been found to the old problem of how proteins synthesized by ribosomes finally reach their destination, in particular how the cytoplasmically synthesized proteins reach a final destination in specific membranes or within specific organelles. Blobel and Sabatini proposed in 1971 (290) that the N-terminal sequence of the nascent protein could be coding for whether a protein becomes attached to, or goes through, a particular membrane. In 1972, Milstein and co-workers (291) found that, in the absence of membranes, a precursor form of immunoglobulin light chain was first synthesized, but in the presence of membranes the normal chain was found; they have postulated that this extra sequence of ~3,000 molecular weight, which was N-terminal, was the coding signal for its membrane attachment, and that the clipping off of this segment allowed the protein to penetrate the membrane, a first step in the process of its secretion. They, and others, quickly confirmed these findings in the case of the synthesis and secretion of immunoglobulins (292–300). Blobel and his colleagues and collaborators elucidated more fully the mechanisms involved (299–310) and, as a result of this work, it has been established that many other proteins, destined either for secretion or for insertion into various cellular membranes, have either an N-terminal segment or a middle segment that is the signal for secretion or insertion, and that is then clipped off by specific membrane-bound proteinases, to finally give the active protein. A recent review (311) gives the more complete history, citing the experiments of many other authors.

Thus to look back over the past 25 years of research on ribosomes and protein synthesis gives one a feeling of almost boundless elation. Researchers in the fields of cell biology, biochemistry, and molecular biology have produced in that time a remarkable picture of the structure of the ribosome, of how the RNA and the proteins probably are interacting, and of the intimate details of protein synthesis. A long road has been traversed from the days of the vague pictures and radioactive uptake experiments. When one considers a particle with so many proteins and such large RNA molecules interlocked in a space some $30 \times 25 \times 20$ μm, a particle whose overall function is to produce, in a very short time, a protein with an exact sequence of amino acids, one might have thrown up one's hands in despair at ever coming to understand the nature of the ribosome.

In retrospect, the history of research into the nature of the ribosome particle began with the recognition by electron microscopists of its structural uniqueness and with their suspicion of its role in protein synthesis. At the same time, biochemists intent on breaking the intact cell to study mechanistic details of protein synthesis became aware that the nascent protein is tightly bound to a sedimentable macromolecule, which appeared to be identical with the one visualized by the electron microscopists. Thus, pinpointing the ribosome as the assembly

site for the growing new peptide chain came about through the confluence of two separate investigative streams in the mid-1950s. There followed a decade of intensive study of the functional role of the ribosome and of its participation with messenger RNA in translating the genetic code. Once again during this period electron microscopy played a key role in identifying the existence of the polysome with its connecting strand of messenger RNA. Recent years have witnessed a cleavage of investigative efforts into two areas. One is the dissection of the protein-RNA structure of the ribosome into its multiple component protein parts and their reassembly into the active complex that comprises the two subunits. The second area, which as yet bears only a slight relationship to the structural findings emerging from the first, is the endeavor to identify and interrelate the many signals and factors essential for initation, propagation, and termination of the peptide chain on the ribosome. It is clear that the mechanism of interaction of the metabolically active proteins and nucleotides with the more fixed structural-protein and nucleic-acid machinery of the ribosome will occupy the attention of future investigators. The depth of our present understanding of the ribosome stands as a monument to the ingenuity and cooperative endeavors of the community of scientists who have been engaged in this quest for the past quarter-century.

TABLE I

Chronology of Significant Events in the History of Ribosomes and Protein Synthesis

Year	Finding	Reference number
1897	Ergastoplasm—basophilia in cells	G. Garnier (1)
1933, 1939, 1943	Basophilia is due to RNA	2, 3, 4
1937–1943	Findings of RNA-containing particles by ultracentrifugal methods	75–80
1941, 1942	Importance of RNA in protein synthesis	5, 6
1943	Isolation and naming of microsome fraction	9
1943	Microsomes contain most of the RNA in cells	9
1948	In vitro incorporation of radioactive amino acids	22, 23, 27, 28
1951–1952	In vitro incorporation of radioactive amino acids using an energy-producing system and microsomes	36, 42
1952–1957	Ultracentrifugal analyses of ribosomal particles	81–89
1953	Discovery and naming of endoplasmic reticulum—identification with high RNA and basophilia	8
1954	Dissection of cell-free incorporation system into several essential components	43
1955	First morphological description of particles the size of ribosomes	10
1955, 1956	Isolation of ribonucleoprotein particles	18–20
1955, 1956	Discovery of aminoacyl adenylates as intermediates in protein synthesis	44, 45
1956	Discovery of need for GTP in protein synthesis	69
1956–1959	Dissociation of ribosomes into subunits	85, 86, 89, 93–96
1956	Identification of microsome fraction as fragmented endoplasmic reticulum	19, 20
1956	The chloroplast as a separate protein-synthesizing system	107
1956	Correlation between membrane-binding of ribosomes and protein secretion	19
1956	First evidence for the occurrence of a messenger RNA	113
1957–1958	Discovery of transfer RNAs	51–53
1957–1959	Identification of ribosome as site of protein synthesis	18, 46–48
1958	"Adapter" hypothesis	62, 64
1958	Coining of name "ribosomes"	90
1958	The mitochondrion as a separate protein-synthesizing system	108
1958–1960	Elucidation of the role of tRNAs	54–57, 62, 63
1959	Use of chloramphenicol as inhibitor of protein synthesis	196
1959	Use of puromycin in elucidating the mechanism of protein synthesis	176–177
1960	Morphological pictures of ribosomal subunits	141
1960	Synthesis of secretory proteins on membrane-bound ribosomes	158
1960–1961	Protein synthesis starts at *N*-terminal end	73, 74
1960–1961	Decisive evidence for existence of messenger RNA	114–116
1960–1963	Physicochemical studies on ribosomes	142–153
1960–1965	Elucidation of size and RNA composition of ribosomes and subunits	134–138.
1961	First indication of a genetic code	117
1961–1966	Complete working out of genetic code	118–120
1961	Existence of two classes of ribosomal RNAs	131, 132
1961–1962	Protein components of ribosomes	124, 125
1962	First experiments on ribosome biogenesis	194, 195
1962	Use of actinomycin D in ribosome biogenesis	216
1962, 1963	The finding of polysome structure	165–170, 172
1962–1966	Initial characterization of prokaryotic ribosomal proteins	186, 189, 193, 197, 198, 200–203
1962–1967	Use of chloramphenicol as a tool in ribosome formation	198–204
1963	Discovery of 5S RNA	139
1963	The binding of mRNA to small ribosomal subunit	164

TABLE I—*Continued*

Chronology of Significant Events in the History of Ribosomes and Protein Synthesis

Year	Finding	Reference number
1963	Error level in protein synthesis	121
1963–1965	Sequence of events in biological formation of RNAs of eukaryotic ribosomes	217–221
1964	Use of CsCl to break up ribosomal subunits	188
1964	First indication of need for various ribosomal protein "factors" in protein biogenesis	189
1964	The nucleolus as site of ribosome formation	213
1964	The binding of nascent protein and of tRNA to large ribosomal subunit	155
1964, 1965	Discovery of methylated bases in ribosomal RNAs	206–208
1965	Use of streptomycin in elucidating mechanisms of protein synthesis	178
1965	Complete primary sequence of a tRNA	61
1966	Movement of nascent secretory protein from ribosomes to ER cavity	173
1966	Attachment of large subunit to ER membrane	175
1966	Role of ribosomal proteins	189
1966, 1967	Elaboration of protein composition of ribosomes	186, 187
1966, 1967	Reconstitution of ribosomes from split products	190–193
1967–1969	Splitting of eukaryotic ribosomal RNA precursors to form final ribosomal RNAs	222–226
1967–1969	Events occurring in formation of ribosomes in nucleolus	230, 237
1967–1971	Further characterization of prokaryotic ribosomal proteins	187, 189, 244–250
1967	Reconstitution of prokaryotic ribosomes using individual proteins	249, 251–254
1969	Sequence of events in biological formation of *E. coli* ribosome	210
1971	Topology of eukaryotic ribosomes	279
1973, 1974	Sequence of events in biological formation of RNAs of prokaryotic ribosomes	232, 233
1974	Proofreading in protein synthesis	122
1974	Arrangement of RNA genes in DNA	234
1974	Assembly maps of prokaryotic ribosomal proteins and RNAs	262–264
1974	Characterization of mitochondrial ribosome	276
1974, 1975	Use of immunology to learn location of prokaryotic ribosomal proteins	268–271
1978	Initial characterization of eukaryotic ribosomal proteins	282, 183
1971, 1979	Movement of nascent proteins to final subcellular destination	311 (review)

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The Discovery of Lysosomes

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Lysosomes ("lytic particles") act as the primary component of the intracellular digestive system in virtually all eukaryotic cells, both plant and animal. First recognized biochemically in rat liver, these organelles are membrane-bounded and contain a variety of digestive enzymes active at acid pH. Their existence and properties became evident during investigations concerning the latency of the enclosed enzymes. Initially defined by the presence of a single enzyme, acid phosphatase, which liberates inorganic phosphate from a number of monophosphoric esters, lysosomes are now known to contain at least 50 acid hydrolases, including various phosphatases, nucleases, glycosidases, proteases, peptidases, sulfatases, and lipases. Collectively, they are capable of hydrolyzing almost all classes of macromolecules according to the following scheme:

$$A - B + H_2O \rightarrow A - H + B - OH.$$

The breakdown products are usually available for metabolic reuse. Functionally, therefore, the lysosome appears to serve as a modern recycling plant (or refuse dump), scavenging and using whatever can be saved, and sometimes accumulating and sequestering indigestible residues as a final resort, sometimes for the life span of the cell.

Customarily, after introducing and characterizing a cellular organelle, one would then present a diagram or electron micrograph and describe its distinctive physical features, so that it would be easily recognized and remembered. In this respect, the lysosome is unique in that its size is variable (from very small to extraordinarily large), and its contents are typically heterogeneous and difficult to predict, because of dependency upon the recent "meal" and the amount of time elapsed since the ingestive event. This is somewhat analogous to the situation of a pathologist at autopsy, attempting to forecast the stomach content of a patient recently dead, in the absence of a reliable history. Indeed, it is this unparalleled aspect of polymorphism, even within the same cell, that makes the discovery of the lysosome different from that of other organelles, as the reader will appreciate in the story to be unfolded.

1949–1952: University of Louvain, Belgium

The trail of the discovery of lysosomes is not a difficult one to follow. "All we wanted was to know something about the localization of glucose-6-phosphatase, which we thought might provide a possible clue to the mechanism of action or lack of action, of insulin on the liver cell"—so explained Christian de Duve upon acceptance of the Nobel Prize for Physiology or Medicine, December 12, 1974, a prize he shared with Albert Claude and George Palade (1). Although the facts of history do not change, the interpretation of history is always changing because the here-and-now reflects the current perspective of the observer. In sketching this brief history of lysosomes, some 25 years after their discovery in 1955, I can visualize the project as a modern-day grant proposal and progress report:

1949: Specific aim: to localize the enzyme glucose-6-phosphatase
Significance: to elucidate the mechanism of action of insulin on the liver.

1952: Progress Report: Unfortunately, no progress has been made on this problem; rather, we would like to report on ... "From Insulin to Latent Acid Phosphatase"....

The lysosome introduced itself in the Laboratory of Physiological Chemistry at the University of Louvain on December 16, 1949 as a crytic form of latent acid phosphatase. The new chairman, Christian de Duve, had just returned from a year of research in St. Louis with the Coris (Nobel laureates, 1947), the discoverers of hepatic hexose phosphatase and with Earl Sutherland, Jr. (Nobel laureate, 1971). He and his students, Jacques Berthet and Lucie Dupret, continued to work on enzymes involved with the metabolism of carbohydrates in rat liver and were able to characterize the hexose phosphatase as a specific glucose-6-phosphatase with a slightly acid pH optimum. In addition, they differentiated it clearly from the nonspecific acid phosphatase acting on glycerol-2-phosphate (β-glycerophosphate) and other phosphate esters upon which glucose-6-phosphatase is entirely inactive. These studies utilized extracts prepared "with typical disregard of cellular organization by vigorous dispersion of the tissue in a high-speed Waring blender in the presence of distilled water." When purification of the enzyme was next attempted, the investigators met an unexpected snag—once precipitated, the enzyme could not be redissolved (2).

At this point, a gentler technique—cell fractionation by differential centrifugation, which had recently been introduced by Albert Claude in 1946 (3)—was employed. Rat liver cells were ruptured with the use of the Potter-Elvehjem homogenizer as a grinding device and 0.25 M sucrose as medium, then further fractionated by several stages of centrifugation. After various procedural modification, the workers succeeded in localizing 95% of the enzyme activity in the microsomal frac-

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tion, thereby establishing the unique distribution of glucose-6-phosphatase in microsomes. (This accomplishment and subsequent experiments by other investigators concerned with the single focus of cytochrome oxidase in the mitochondrial fraction [4] led to the postulates of biochemical homogeneity and unique [sole] location of any enzyme, as discussed by de Duve in *The Harvey Lectures*, 1965 [5]. These two concepts served as working hypotheses in much of de Duve's later research.)

Among the enzymes assayed in the above study, however, was acid phosphatase, largely included for control purposes. To the surprise of the experimenters, acid phosphatase activity in the homogenate was only a 10% of what they had anticipated on the basis of previous assays of preparations subjected to the more drastic homogenizing action of a Waring blender. After 5 days, the same fractions (kept in the refrigerator) were again assayed; this time, the activity of the homogenate was of the right order of magnitude, with a distinct peak in the mitochondrial fraction (see Fig. 1). To quote de Duve: "...we could have rested satisfied with this result, dismissing the first series of assays as being due to one of those troublesome gremlins that so often infest laboratories, especially late at night. This would have been a pity, since chance had just contrived our first meeting with the lysosome." (For a more detailed report, the reader is advised to peruse the charming and adventurous chapter called "The Lysosome in Retrospect" by de Duve [2].) Additional studies demonstrated that results of the first series of experiments were not due to a technical error, but that most of the enzyme content in the "fresh" preparations must have been present in masked form and become activated with storage. Only a few months of work were required to establish that the latency of acid phosphatase was attributable to a membranelike barrier limiting the accessibility of the enzyme to its substrate. "Thus, the lysosome had made itself known to us as a saclike structure surrounded by a membrane and containing acid phosphatase."

At first, the particles containing acid phosphatase were believed to be mitochondria (6). This interpretation seemed reasonable because there were only three fractions—nuclear, mitochondrial, microsomal, and finally the nonsedimented portion, the supernate (see Fig. 1); the acid phosphatase activity clearly sedimented in the mitochondrial fraction. According to de Duve, progress was achieved in this area, again by chance, taking the inconvenient form of a breakdown in the high-speed attachment of the centrifuge. This caused Françoise Appelmans, who was then studying acid phosphatase latency on isolated "mitochondria" to prepare her mitochondrial fractions by a makeshift procedure using a less powerful ordinary table-top centrifuge. She succeeded in sedimenting a sizable amount of particles in this way, but found to her great disappointment that her fractions were almost devoid of acid phosphatase activity. They did, however—as later experiments demonstrated—possess plenty of respiratory activity. Investigations prompted by these findings established that the old "mitochondrial fraction" could be subfractionated into a light and a heavy fraction, containing the particles with acid phosphatase and cytochrome oxidase, respectively (7). Eventually, the particles incorporating acid phosphatase were shown to comprise a distinct group, different from both the mitochondria and the microsomes, and designated "intermediate particles."

Fraction	Phosphatase acide en μg P/20 min.	
	16/12/49	21/12/49
Homogénéisat	10	89
Noyaux	2	10
Mitochondries	7	46
Microsomes	6	10
Decantat	6	9

FIGURE 1 Acid-phosphatase activity in fresh and aged fractions separated from rat-liver homogenates. (Results of Berthet and de Duve [1951], copy of old slide [2].)

1952–1955: Extension to Other Acid Hydrolases: The Lysosome as a Biochemical Concept

In 1952, at the Second International Congress of Biochemistry in Paris, evidence that acid phosphatase belonged to a special type of cytoplasmic particle was presented. At this meeting, a young British biochemist, P. G. Walker, mentioned to de Duve that he had obtained data very similar to the Louvain group's findings on acid phosphatase, but on β-glucuronidase instead (8). With this statement in mind, the Belgian investigators tested a number of enzymes for presence in the key light (L) fraction and for latency. By 1955, five enzymes had been localized in the L fraction (Fig. 2) and proved to be

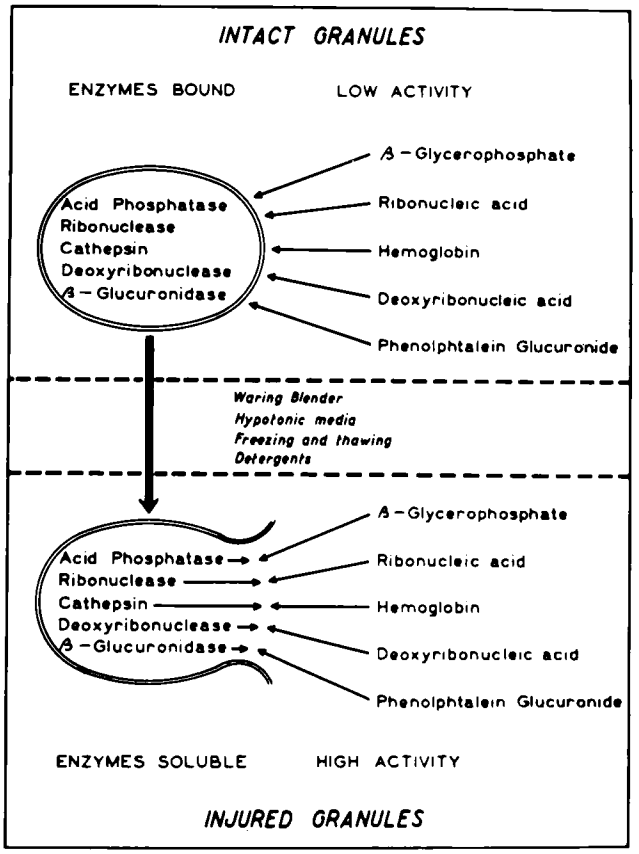


FIGURE 2 Biochemical model representative of rat liver lysosomes as first described by de Duve et al. in 1955. We now know that lysosomes contain at least 50 hydrolases (9), which can act on such diverse macromolecules as nucleic acids, proteins, glycoproteins, polysaccharides, and various lipids.

hydrolytic enzymes with an acid pH optimum (10). Moreover, all acted upon different sets of natural substrates. Such an apparent coincidence was considered biologically meaningful and interpreted to imply that the particles containing these enzymes fulfilled some sort of nonspecific lytic function. Hence the term "lysosomes," denoting lytic particles or bodies, was proposed (10). The lysosomes themselves were perceived as membrane-bounded granules enclosing five acid hydrolases in latent form (Fig. 2).

1955–1956: Morphological Identification of Rat Liver Lysosomes as the "Pericanaljcular Dense Bodies" of Rouiller

Not until 1955 did electron microscopy make its contribution to the identification of lysosomes. Independently of de Duve, a group of cell biologists headed by Alex Novikoff at the University of Vermont had been conducting experiments which involved systematic variations of the cell fractionation scheme in rat liver. They had examined closely a number of enzymes, including (in a remarkably prophetic manner) the use of markers for practically every distinct entity that has since been recognized in rat liver: 5′-nucleotidase (plasma membrane), succinate oxidase (mitochondria), acid phosphatase (lysosomes), urate oxidase (peroxisomes), and esterase (microsomes). Additionally, they had extensively studied the morphology of their fractions by phase-contrast microscopy (11). In 1955, during the Third International Congress of Biochemistry in Brussels, Novikoff visited de Duve's laboratory and was able to obtain the first electron micrographs of cell fractions containing partially purified lysosomes. These specimens were fixed in osmium, and, in addition to known particles (excessively sad-looking mitochondria), the pictures exhibited multitudes of characteristic bodies that had occasionally been observed in intact liver cells and had been termed "pericanalicular dense bodies" by Rouiller in 1954. Their function was unknown; the name signified only their preferential location in cells along the bile canaliculi and their electron density to the beam of the electron microscope (12). Identification of the lysosome activity with these dense bodies, a provisional association at the time, has since been confirmed by a diversity of techniques discussed later. (It happened that microbodies or peroxisomes were also present in such rat liver preparations [see Fig. 3*a*].) The next and extremely helpful step was the development of a reliable staining method for acid phosphatase reaction at light and electron microscope levels (Fig. 3*b*). The basic procedure, evolved by Gomori (14), is performed in two steps—the first yielding lead-phosphate, which can be seen by electron microscopy as dense, needlelike crystals (see Fig. 3*b*). The phosphate released by enzymatic hydrolysis from the substrate (β-glycerophosphate, grade I) at pH 5 is precipitated by the lead ions present in the incubation medium. In the second step, lead phosphate is transformed into lead sulfide by ammonium sulfide, a brown-black precipitate visible by light microscopy. Novikoff (15), Holt (16)and Barka and Anderson (17) effected significant improvements in extending this technique to the fine-structural level. Their work provided independent confirmation of the lysosomal nature of the dense bodies, and subsequently afforded considerable impetus to the study of the existence, origin, morphological features, and functional properties of lysosomes in a broad variety of biological tissues.

1958: Beginning of the Functional Concept

Although the thought that lysosomes might play a role in intracellular digestion was mentioned in the Louvain group's first publication, it is fair to state that few people were ready to accept in 1955 what is now taken for granted, namely, that intracellular digestion is a general function common to virtually all animal and plant cells. The first definite clue to the function of lysosomes came from the work of Werner Straus, who deserves the credit for undertaking studies which would almost certainly have led to an independent discovery of lysosomes. Straus had obtained good evidence that the "droplets" of the proximal tubule of the kidney were a site of storage and breakdown of reabsorbed proteins. By 1954, he had succeeded in subfractionating these droplets and showed them to be rich in acid phosphatase and protease (18), and by 1956, he found other hydrolases similar to those described in liver lysosomes (19). This early work on the kidney provided the first clear link between lysosomal digestion and endocytotic uptake of extracellular materials. Together with a few other data obtained from organs as diverse as brain and spleen, as well as some lower organisms, de Duve presented the first schematic outline of the possible biological functions of lysosomes at a meeting organized by the Society of General Physiologists at Woods Hole, Massachusetts in June 1958 (20). It was postulated that the collection of acid hydrolases present in lysosomes could have but one function, that of acid hydrolysis. Furthermore, an attempt was made to link lysosomes with several natural processes. "These may comprise: digestion of foreign material, engulfed by pinocytosis, athrocytosis (old term for endocytosis) or phagocytosis; physiologic autolysis, as presumably occurs to some extent in all tissues, and particularly as part of the more specialized processes of involution, metamorphosis, holocrine secretion, etc.; pathological autolysis or necrosis." It should be mentioned that digestive and autolytic phenomena had been known for a long time, and their dependence on many of the enzymes found in lysosomes had been at least strongly suspected. However, no satisfactory explanation had been provided heretofore for their inhibition in the heathly cell. In developing the theory of intracellular acid digestion, considerable importance has always been attached to the structure-linked latency of the lysosomal hydrolases, which provided the first satisfactory explanation for the fact that autolysis is largely held in check in most cells, despite their content of highly active hydrolytic enzymes.

1953–1965: The Discovery of Peroxisomes—the Microbodies of Rouiller

We now know that the light fraction of rat liver contains two distinct populations of functional particles—lysosomes and peroxisomes (Fig. 3). The latter are membrane-bounded organelles containing enzymes which catalyze reactions involving hydrogen peroxide, and hence have been termed peroxisomes (21). Three of these catalyzing enzymes produce hydrogen peroxide (urate oxidase, D-amino acid oxidase, and α-hydroxyacid oxidase) and one (catalase) destroys it.

The purification of rat-liver peroxisomes was accomplished with good yield by Wattiaux and co-workers (22), taking advantage of their finding that a preliminary intravenous injection of Triton WR-1339 two days before sacrificing the animal caused a considerable decrease in the equilibrium density of lysosomes in a sucrose density gradient (Fig. 4). When these fractions were examined by electron microscopy, there was no doubt that the microbodies of Rouillier were indeed the particles biochemically characterized as peroxisomes (13, 23). Their morphology in intact rat liver is illustrated in Fig. 5.

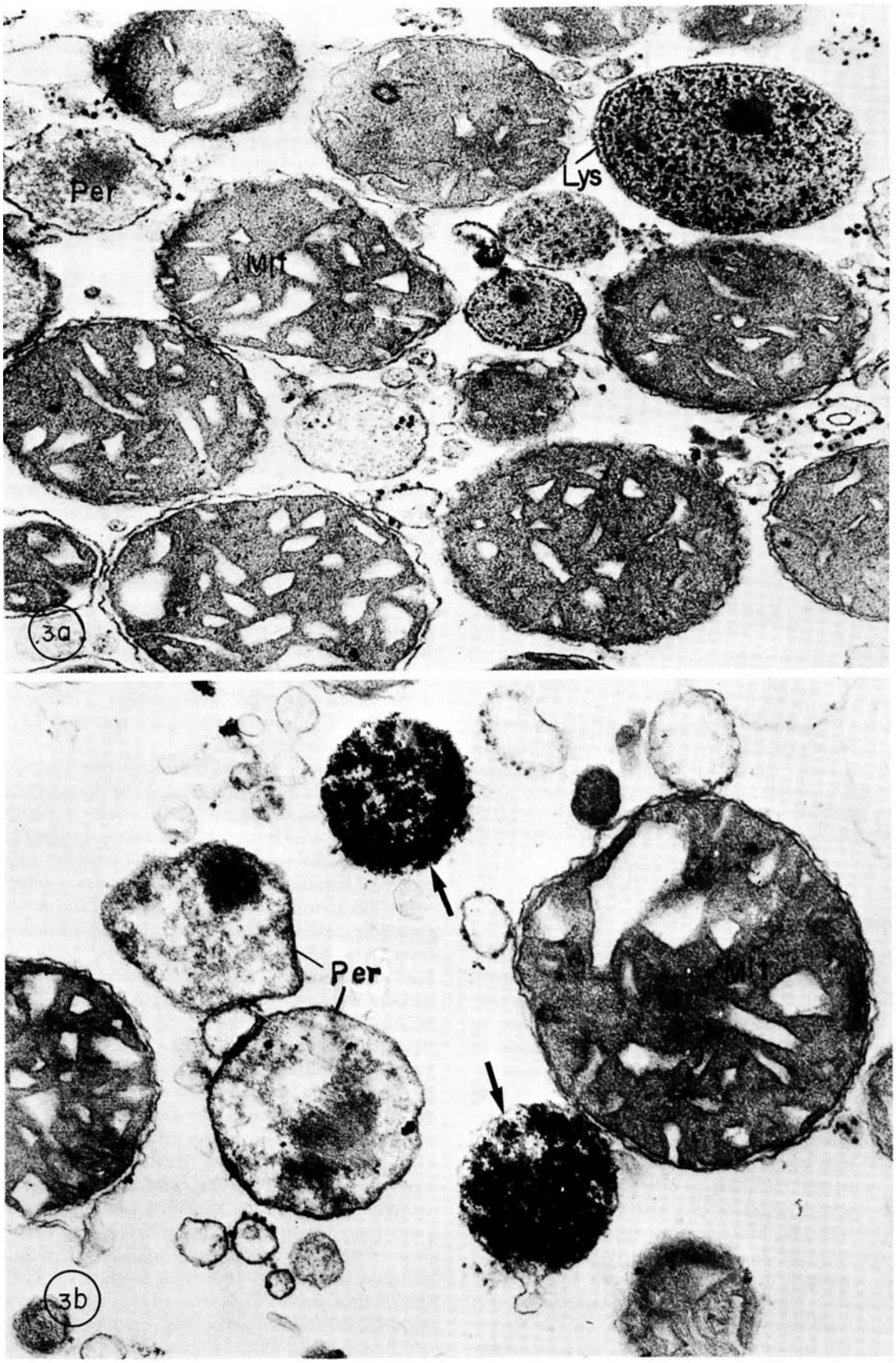


FIGURE 3 (*a*) Electron micrograph of the organelles present in the cell fraction, illustrating the distinctive morphology of dense bodies or lysosomes (Lys), microbodies or peroxisomes (Per), and mitochondria (Mit). This micrograph, however, is not the same as the original (see reference 12), because it was taken in 1967. Organelle morphology has now been much better preserved by glutaraldehyde fixation. × 58,000. (*b*) Same preparation as in *a*, but also incubated for acid phosphatase, which appears as a black precipitate in the dense bodies (arrows), but not in the peroxisomes (Per) or mitochondria (Mit). (From Baudhuin et al., 1967 [13].)

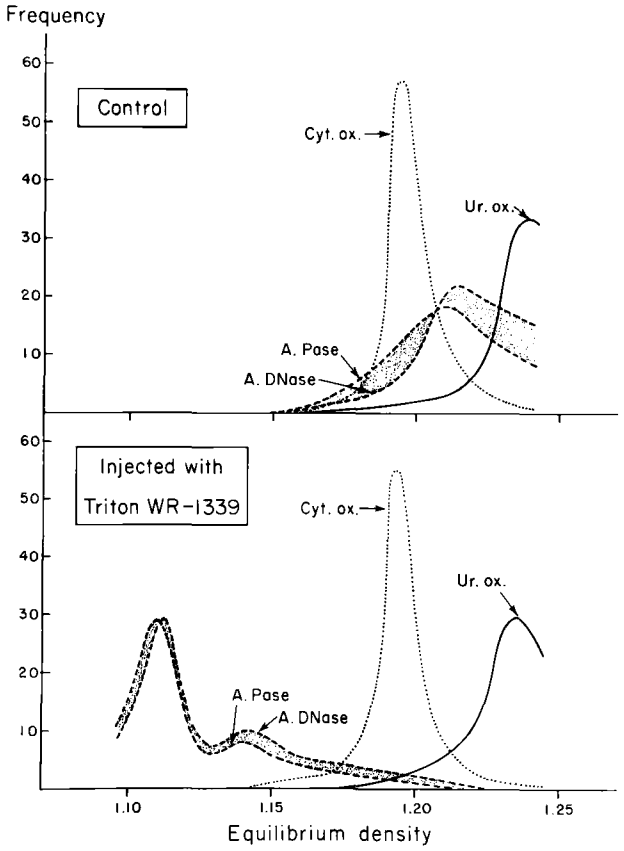


FIGURE 4 Effect of a previous injection of Triton WR-1339 on the equilibrium density of particulate enzymes. Density equilibration of mitochondrial fractions from rat liver in an aqueous sucrose gradient. Upper graph, control; lower graph, animal injected intravenously 4 days previously with 170 mg of Triton WR-1339. Note the selective shift of the lysosomal hydrolases. (Courtesy of Christian de Duve [5].)

1960–1966: The Lysosomal System

As more cells were studied and the ubiquitous distribution of lysosomes in mammalian cells was recognized, it became apparent that the lysosome is not actually a "body," but a part of a remarkably diverse and dynamic system. In addition to their polymorphism, lysosomes were discovered to be unique among other subcellular constituents by the variety of processes, both physiological and pathological, in which they participate. In fact, by 1963, when the Ciba Foundation Symposium on Lysosomes (24) was held, many pieces of the "functional puzzle" were beginning to fit into place. (A number of terms were introduced there that we now use quite frequently: for example, endocytosis, exocytosis, and primary lysosome.) Thereafter, the lysosome became popularized by publications in 1963 in the *Scientific American* (25); in 1964 in *Federation Proceedings*, organized by van Lancker, with contributions from Novikoff et al., Hirsch and Cohn, Swift and Hruban, and Weissmann, as well as de Duve (26); in 1965 in *The Harvey Lectures* series (5); and finally, in 1966 in an extensive review in the *Annual Review of Physiology* (27) entitled "Functions of Lysosomes." The various forms of lysosomes and related particles, together with the different types of interactions that may occur between them and with the plasma membrane, are presented in the diagram below, Fig. 6.

It was now evident that lysosomes, in combination with some closely affiliated vacuolar structures devoid of hydrolases, formed an intracellular digestive system comparable (except for its discontinuity) to the digestive tracts of higher organisms; each separate component of the system was, to some extent, equivalent to a segment of the animal digestive tract. Moreover, it was further established that the material undergoing digestion in this system may be associated with heterophagy or with autophagy. In heterophagy, the material to be degraded is from outside the cell, whereas in autophagy, the material being degraded is of endogenous origin.

The word "lysosome" was chosen on the basis of the classification illustrated in Fig. 6. The choice can be defended, because lysosomes constitute the major functional constituents of the system, and also, usually the most numerous. Their identification, based essentially on the presence of acid hydrolases, is unambiguous. Within the lysosomal group, the primary lysosomes (also variously designated in the literature as pure, true, original, or virgin lysosomes) were distinguished as those containing enzymes which had never been engaged in a digestive event, whereas the secondary lysosomes represented sites of present or past digestive activity. The majority of secondary lysosomes are believed to have an acid pH, which activates their enzymes and allows them to function at optimal pH.

The most important components of the system that lack the acid hydrolase were the prelysosomes, with their contents of unattacked debris, generally destined for future digestion within lysosomes. At that time, the only well-known prelysosome belonged to the heterophagic line or phagocytic pathway: it was commonly called a phagosome (27). Postlysosomes, defined as degenerate telolysosomes that have lost their enzymes, were also included.

By the time of the comprehensive 1966 review (27), 330 references could be cited, indicating the vigorous investigative interest in lysosomes. Indeed, it is not easy to summarize the multiple and diverse contributions that have aided our understanding of the lysosomal system. Certainly, however, the following scientists have afforded significant new information.

(*a*) The contributions of Alex Novikoff and his co-workers should be mentioned first. In the late 1950s he had progressed from "once being a fledgling biochemist," using "grind-and-find" techniques, to becoming deeply submerged in microscopy and cytochemistry, where "seeing is believing" (28). It was Novikoff who assisted the lysosome, a biochemical entity, to its official entry into morphology and cell biology. Shortly after the Woods Hole meeting in 1958, Novikoff, who had accepted the invitation to write a chapter on mitochondria for *The Cell* edited by Jean Brachet and Alfred Mirsky, persuaded the editors to allow him to add a separate chapter on lysosomes (15). Largely stimulated by the work of Novikoff and his associates (29–33), over the years many investigators have sought to determine the formation and identification of primary (pure) lysosomes in many tissues and have pondered their relationship to the Golgi apparatus and endoplasmic reticulum (ER). He introduced the acronym GERL (34). "The specialized region of ER is referred to as GERL to suggest that it is ultimately related to the Golgi saccule (G), that it is a part of ER, and that it forms lysosomes (L)" (29, 32). It is valid to state that, along with the charismatic and articulate de Duve, the energetic and intuitive Novikoff continually brought the lysosomal system to the attention of a broad range of scientists.

(*b*) Hirsch, Cohn, and their colleagues at The Rockefeller Institute (now The Rockefeller University) clarified the manner in which lysosomes participate in digesting material engulfed

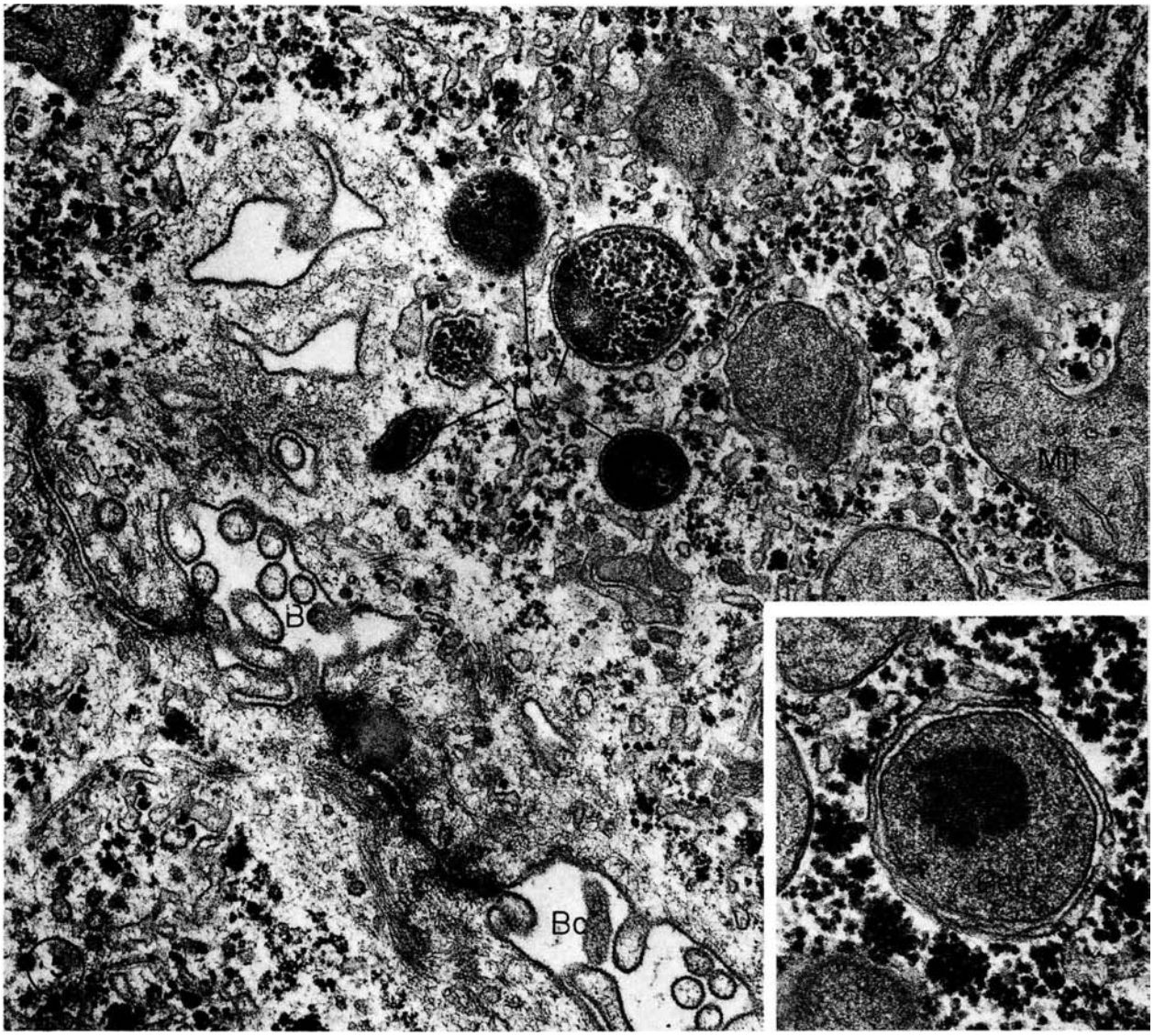


FIGURE 5 Electron micrograph of rat liver illustrating the morphology of the dense bodies (Lys) near the bile canaliculi (Bc). Inset illustrates the morphology of a peroxisome (Per) with a crystalloid in matrix. × 50,000. (Courtesy of Daniel S. Friend.)

by phagocytic leukocytes. After establishing the lysosomal nature of the neutrophil granules, they demonstrated that these granules discharge their enzymes into the phagocytic vacuoles when the cells ingest bacterial and other particles (35, 36). Furthermore, in both neutrophils and macrophages, degradation of isotopically labeled bacteria occurred, as evidenced by the appearance of breakdown products of lipids, nucleic acids, proteins, and carbohydrates (37).

This work on amoeboid phagocytic leukocytes naturally reverted to a reanalysis (2, 26) of the discovery of phagocytosis by Elias Metchnikoff in 1883. During his exploration of intracellular digestion in lower animals and unicellular organisms, Metchnikoff recognized that the interiors of food vacuoles were acid, and assumed that they contained soluble enzymes called cytases. Although this vacuolar acidity is now a cornerstone of the lysosomal concept (26), the exact mechanism by which secondary lysosomes are acidified has still not been completely explained, but the participation of a proton pump appears likely (38, 39).

(*c*) In 1963, H. G. Hers (40) and his co-workers in Belgium were the first to identify a true, inborn, lysosomal storage disease. This was glycogen-storage disease, type II, wherein α-glycosidase, capable of degrading glycogen, is absent (Fig. 7*a*), and the liver contains large glycogen-filled vacuoles (Fig. 7*b*)—as would be expected if accumulation of the polysaccharide were due to lack of digestion within lysosomes. This condition and many others of similar etiology (a primary defect of one lysosomal hydrolase) have now been described. As a matter of fact, by 1973, Hers and van Hoof, editors of *Lysosomes and Storage Diseases* (41), could record at least 21 individual pathological entities—such as Gaucher's disease, with a defect in β-glucosidase, or Niemann-Pick disease, with missing sphingomyelinase. The list continues to grow (42). The clinical appearance of the primary defect in lysosomal protein results in intralysosomal accumulation of all complex molecules that require the missing enzyme for their degradation. Further research on these pathological conditions has now yielded valuable new data on the synthesis and transport of normal lysosomal enzymes and the presence of receptors (reviewed by Neufeld in reference 43), and will be discussed later.

(*d*) Marilyn G. Farquhar and her associates at the University of California, San Francisco described a unique type of

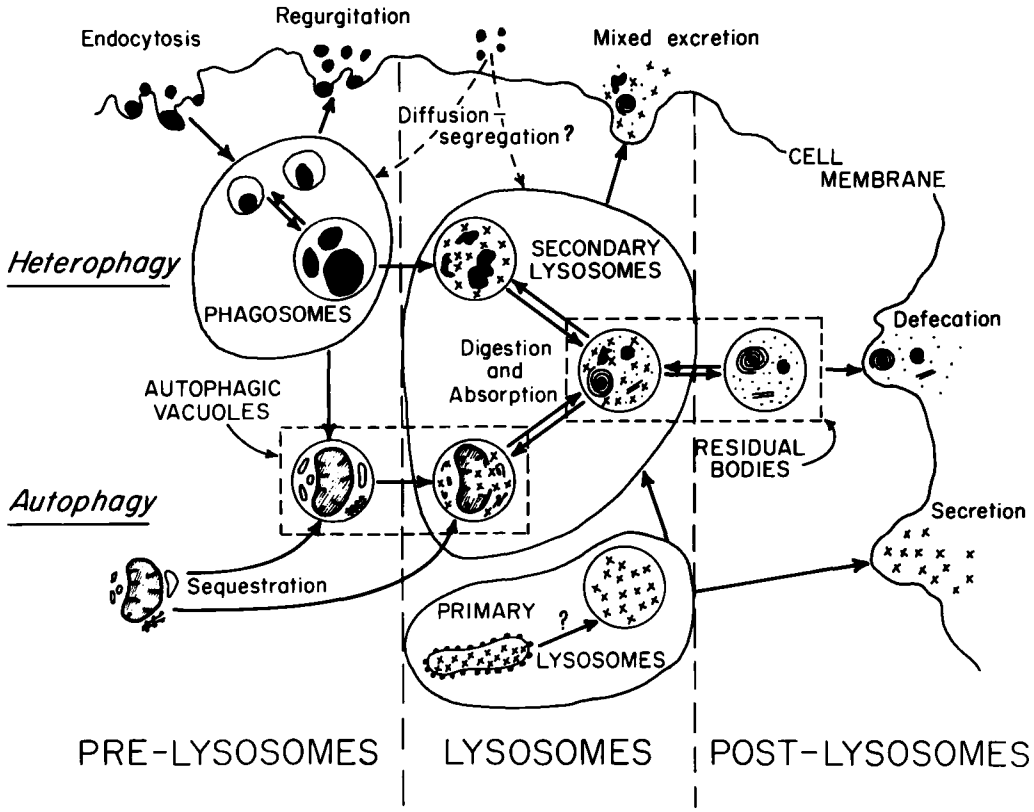


FIGURE 6 Synthetic diagram illustrating the various forms of lysosomes and related particles and the different types of interactions which may occur between them and with the cell membrane. Each cell-type is believed to be the site of one or more of the circuits shown, but not necessarily at all sites. Crosses symbolize acid hydrolases. (Reproduced from de Duve and Wattiaux, 1966 [27].)

autophagy, and established the origin and identification of different forms of primary lysosomes. The significant findings of Smith and Farquhar (44) indicated that certain pituitary secretion granules may fuse with lysosomes under particular circumstances, and that this mechanism probably serves to dispose of excess secretory products when the stimulus for their discharge is lacking (Fig. 8). It should be emphasized that this is not a nondiscriminate process involving segregation of entire areas of cytoplasm, but rather a selective fusion process between the secretory granules and lysosomes. The process was designated as *crinophagy* by de Duve (2) to distinguish it from autophagy (45). Research on lysosomes in blood leukocytes by Bainton and Nichols (see review, reference 46) established that some leukocytes are unusual because they store lysosomal enzymes in morphologically distinct structures demonstrable as large storage granules (Fig. 9*a*). In most other cell types in which primary lysosomes have been identified, they take the form of small Golgi complex-derived vesicles, often coated (Fig. 9*b*), which transport hydrolytic enzymes from the Golgi complex to multivesicular bodies, some of which then become secondary lysosomes, as reported by Friend and Farquhar (47). It should be emphasized, however, that not all Golgi complex-derived vesicles are lysosomal in nature, nor are all small coated vesicles lysosomes.

After 1966, the development of lysosomal functions in physiological and pathological processes can be followed to the fullest extent in a series of books, *Lysosomes in Biology and Pathology*, edited by John T. Dingle and Honor B. Fell from the Strangeways Research Laboratory, Cambridge, England, and beginning with the first number from 1969 and continuing through the sixth, published in 1979 (48). A recent, more concise survey by Eric Holtzman in 1976 (9) is also to be highly recommended. In addition, and perhaps most important, was the initiation of the Gordon Research Conferences on Lysosomes in 1967. The titles of the presentations alone indicate much of the chronological development of new data, as follows:

1967: "Biochemical and Structural Aspects of Self-Degradative Processes in Cells" (chaired by Christian de Duve).

1968: "Lysosomes and Host Defense" (chaired by Zanvil Cohn and Samuel Dales).

1969: "Lysosomes and Storage Diseases" (chaired by Alex Novikoff and H. G. Hers).

1970: "Autophagy" (chaired by James Hirsch and Michael Locke).

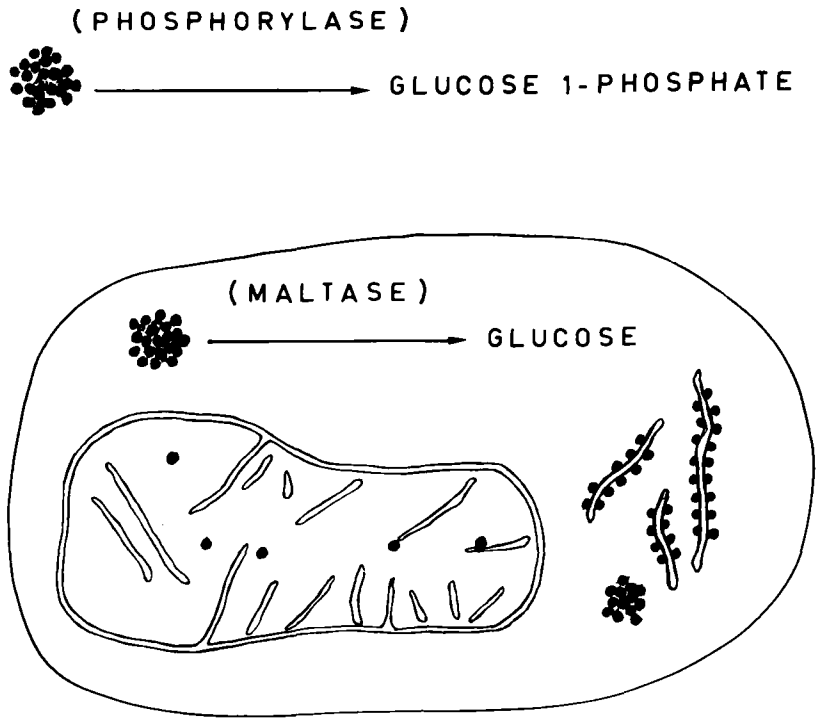
1972: "Immunity and Tissue Injury" (chaired by Gerald Weissmann and Stephen Malawista).

1974: "Lysosomotropic Agents" (chaired by de Duve).

1976: "Intracellular Turnover of Proteins and Eukaryotes and Prokaryotes" (chaired by Eric Holtzman and John Dingle).

1978: "The Origin of Lysosomal Enzymes" (chaired by Oscar Touster and Dorothy Bainton).

1980: "The Role of Lysosomes in the Uptake, Storage, and Recycling of Membranes and Membrane-Bound Molecules" (chaired by Dorothy Bainton and Samuel Silverstein).



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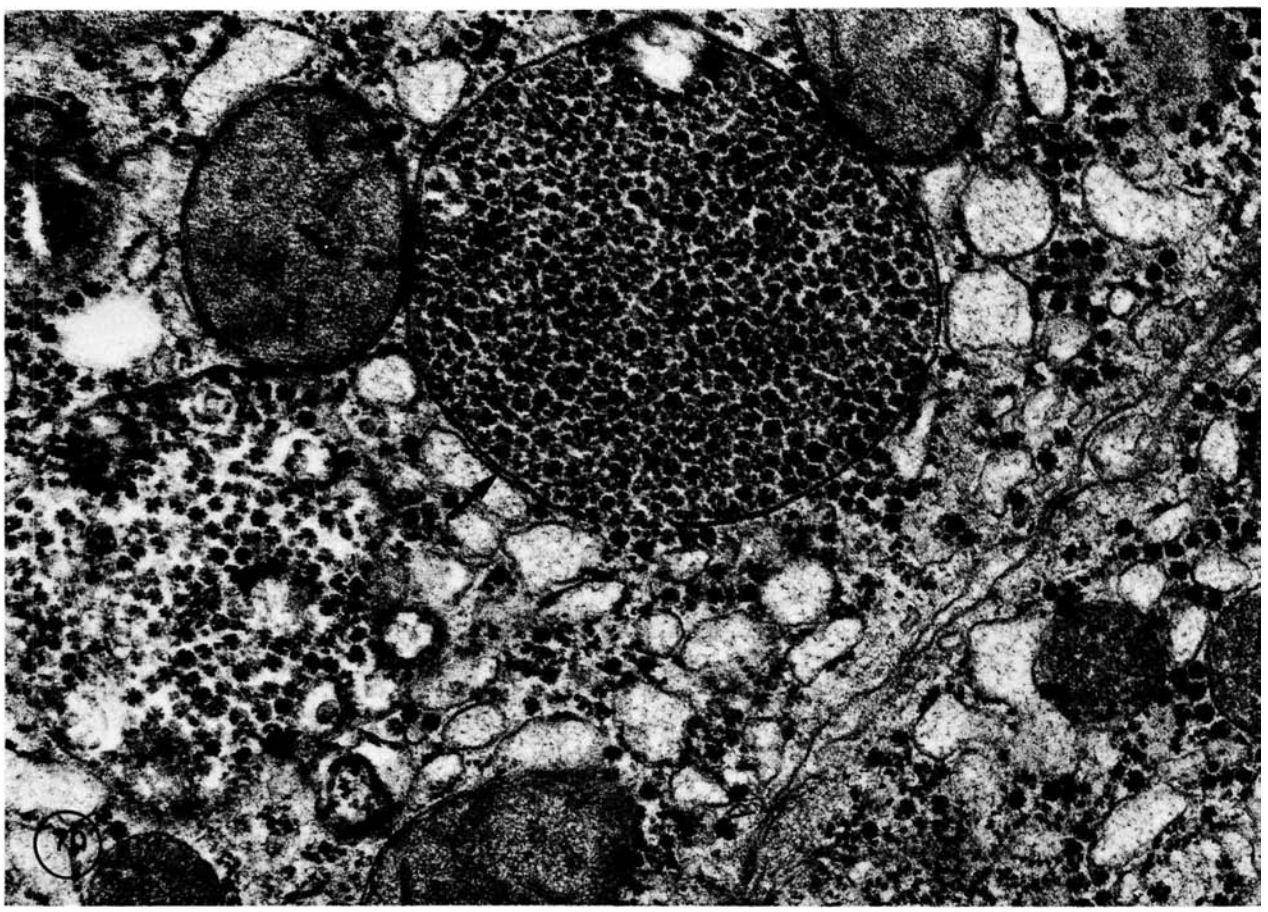

FIGURE 7 (*a*) Schematic representation of tne two pathways of glycogen degradation within cells. The upper one is cytoplasmic; the lower one is within the lysosome. (*b*) Part of a liver parenchymal cell from a patient with glycogen storage disease, type II. One vacuole, a lysosome, is filled with α-particles of glycogen (arrow). (Courtesy of Hers and van Hoof [41].)

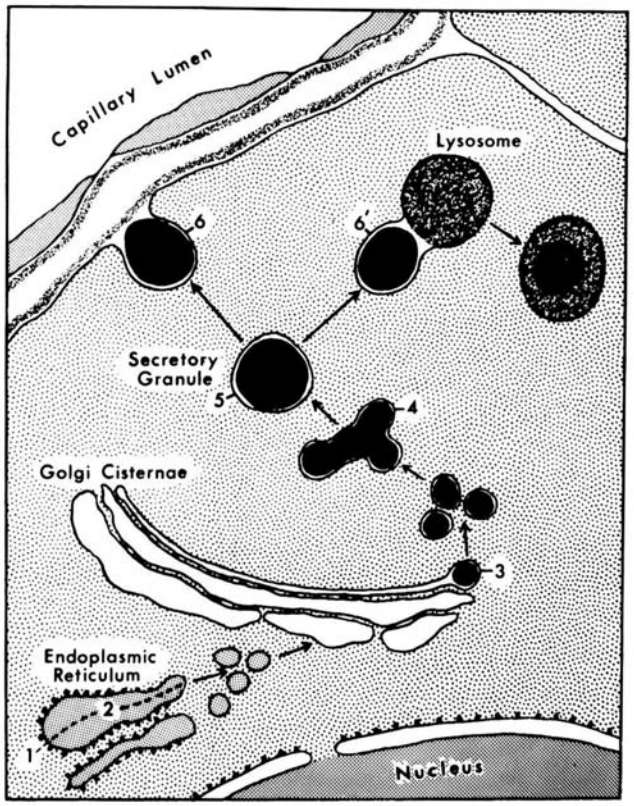

FIGURE 8 Diagram of the events of crinophagy as studied in mammotrophic cells of the rat anterior pituitary gland. Mammotrophic hormone is believed to be synthesized and transported through the cells as outlined in steps 1–6. If the secretory activities of the cells are suddenly discontinued, as takes place when the pups are separated from the lactating rats, the cells dispose of the excess stored hormone by fusion of the granules with lysosomes (6'). (Courtesy of Smith and Farquhar [44].)

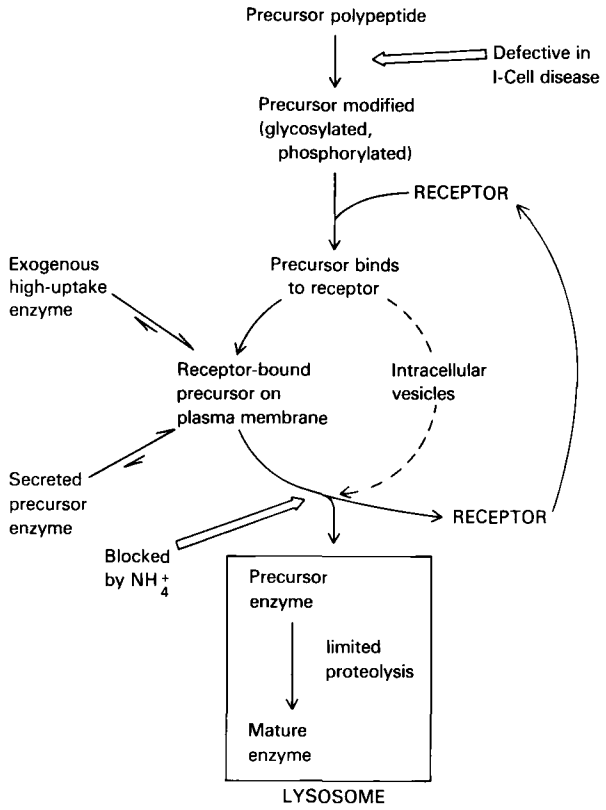

FIGURE 10 Schematic representation of the history of hydrolases in cultured fibroblasts. The present data indicate that precursor polypeptides are introduced into the endoplasmic reticulum, where they are glycosylated and phosphorylated. The precursor chains, presumably assembled at some point into enzyme molecules, bind to receptors, which convey them to lysosomes. Once inside organelles, the enzymes undergo restricted proteolysis. Small amounts of precursor can also be found in the extracellular spaces. (Courtesy of Elizabeth F. Neufeld [43]).

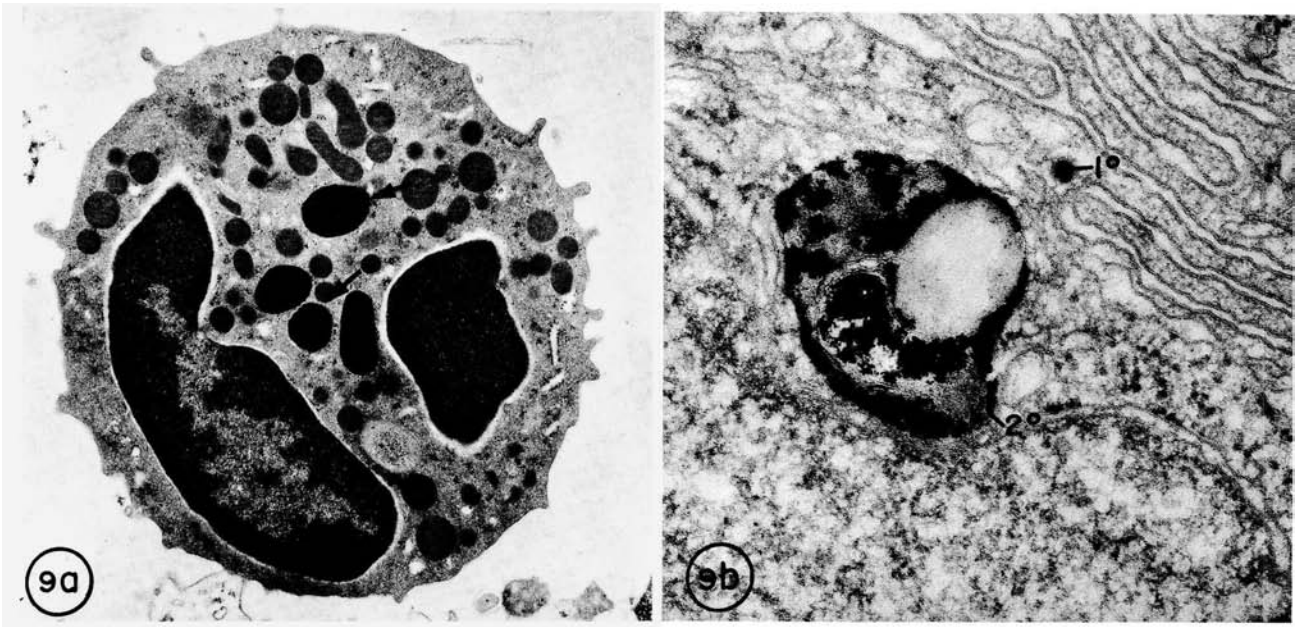

FIGURE 9 Two different forms of primary lysosomes. (*a*) Polymorphonuclear leukocyte. The cytoplasmic storage granules are morphologically and chemically distinct. Only the large, dense storage granules (arrows) contain acid hydrolases and correspond to the primary lysosomes of this cell type (see review, reference 46). It is now clear that relatively few cells store lysosomal enzymes in morphologically distinct structures recognizable as granules. In most cell types other than leukocytes, cytochemical staining has allowed the identification of the primary lysosome as small vesicles, so-called Golgi vesicles, which are sometimes coated. × 14,000. (*b*) Note the small acid-phosphatase-positive coated vesicle (1°) and a much larger secondary lysosome (2°). 9b × 60,000. (Courtesy of Dr. Daniel S. Friend.)

In recent years, interest has focused on the chemistry and biosynthesis of lysosomal enzymes. All lysosomal enzymes are glycoproteins, with the exception of cathepsin B1 and lysozyme (if the latter is indeed a true lysosomal enzyme). Although more than 50 different hydrolytic enzymes have been detected in lysosomes, only a few have been purified to homogenity. There are no known amino-acid sequences of lysosomal enzymes. So far, the one most fully characterized is β-glucuronidase. All of the limited number of lysosomal enzymes studied thus far contain mannose, galactose, and perhaps surprisingly, glucose. Almost all additionally contain fucose (49). What is known about biosynthetic routes of lysosomal enzymes—e.g., (*a*) How is the polypeptide formed? (*b*) Are "pre" and "pro" forms involved? and (*c*) What are the kinetics of this process? A few inroads have currently been forged in this area in the laboratories of Neufeld, Figura, Blobel, Sabatini, and Kornfeld.

In brief, hydrolase transport to lysosomes can now be regarded in the general context of the transport of secretory proteins. As glycoproteins, acid hydrolases would be expected to enter cisternae of the rough endoplasmic reticulum (RER); this has been verified by in vitro translation of cathepsin D (50). Thus, the nascent enzymes should be equipped with signal peptides to facilitate their entry into the RER. Such a signal has been found in the study of cathepsin D by Erickson and Blobel.[1] Where are the precursor polypeptides shortened? Neufeld and her co-workers have shown that the process is relatively slow; in fact, the slowness of the pace suggests that it may occur only after the hydrolases have become lodged in lysosomes (43, 51). Thus, the details of glycosylation, phosphorylation, and proteolytic cleavage and their kinetics are just beginning to emerge (see Fig. 10 and refs. 43, 51–53).

One major question involves the mechanism of delivering the recently synthesized enzymes to the lysosomes and sorting them out of the normal secretory pathway. It is possible that the manner of sorting is carried out by receptors. This is an area in which ideas are in flux. Although receptors for lysosomal enzymes were first encountered on the plasma membrane surface (43, 54), Sly and his co-workers (55) at Washington University in St. Louis have recently discovered that the majority of high-affinity receptors for β-glucuronidase are intracellular. This led them to propose that most newly synthesized lysosomal enzymes rely on the phosphomannosyl recognition marker for intracellular segregation from other products of the RER. From this viewpoint, receptor-bound enzymes would gather in specialized vesicles derived from the ER or Golgi complex and be delivered to lysosomes presumably by fusion. It is also possible that the vesicles could fuse with plasma membrane, exposing receptor-bound enzyme to the exterior of the cell, and that portions of the membrane carrying receptor-bound enzyme might subsequently be internalized through endocytosis (43). Binding of the hydrolases to receptors on the membrane seems to be mediated by an ionic signal, mannose-6-phosphate (54, 55). George Jourdian and his associates at the University of Michigan in Ann Arbor are well underway in their isolation and characterization of the liver-cell membrane receptor that binds β-galactosidase (56). All of these synthetic pathways are still little explored, but can be anticipated to result in significant new information in the near future.[2]

[1] Erickson, A., and G. Blobel. Personal communication.

[2] Varki and Kornfeld have recently found the precise location of phosphorylated mannose residues on oligosaccharides (1980. *J. Biol. Chem.* 255:10847–10858).

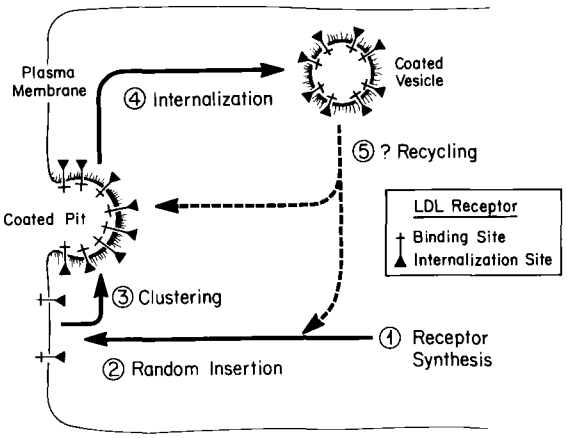

FIGURE 11 Working model for the mechanism by which LDL receptors cluster in coated pits on the plasma membrane of human fibroblasts. The postulated steps are as follows: (1) synthesis of LDL receptors on polyribosomes; (2) insertion of LDL receptors at random sites along noncoated segments of plasma membrane; (3) clustering of LDL receptors in clathrin-containing coated pits; (4) internalization of LDL receptors occurs as coated pits, which invaginate to form coated endocytic vesicles; and (5) recycling of internalized LDL receptors back to the plasma membrane. Step 5 may occur in lysosomes. (Courtesy of J. S. Goldstein et al., [58]).

Finally, one new aspect of lysosome function is now being charted—their role in the intracellular degradation of physiologically important molecules that regulate growth, nutrition, and differentiation in cells. Receptor-mediated endocytosis is now known to occur in many cell types for selective and efficient uptake of macromolecules (57, 58). These include certain transport proteins, such as low-density lipoprotein (LDL), transferrin, and transcobalamin II, as well as peptide hormones such as insulin and epidermal growth factor, asialoglycoproteins, and lysosomal enzymes. It is now clear that receptor-mediated endocytosis occurs in a great variety of cell types, and that many internalized proteins are delivered to lysosomes and degraded there, whereas others are not degraded, but instead are delivered to cellular structures other than lysosomes (see Fig. 11). The compartments responsible for this selective sorting of internalized proteins are presently being investigated (58, 59). In conclusion, it has become evident that the lysosomal system is not just a garbage dump. Rather, through the process of selective endocytosis, multiple biologically active substances, such as hormones, enzymes, LDL, antibodies, and toxins are herded into the cell and may or may not be degraded by lysosomes (58).

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The Golgi Apparatus (Complex)—(1954-1981)—from Artifact to Center Stage

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To the cell biology student of the 1980s, it may come as a surprise to learn that until the late 1950s, the existence of the Golgi apparatus as a bona fide cell organelle was seriously questioned. Surprise would be in order on two accounts: first, the discovery of the Golgi apparatus by Camillo Golgi (1), for whom it is named, took place nearly a century ago; and, second, now no one questions that the Golgi apparatus is a distinct cell organelle, or is unaware of its participation in a wide variety of cellular activities. Indeed, the Golgi apparatus, or Golgi complex as it is often called, not only occupies the cell center, but it also has moved toward center stage, because it has been shown to be involved in so many cell activities. In this review we will describe the recent history of the Golgi apparatus—the developments that led from its position as a suspected artifact to the situation at present when it is rapidly becoming a main center of attention.

Brief Historical Perspective

The Light Microscope Era (Before 1954)

The period before the mid 1950s was characterized by controversy concerning the reality of the Golgi apparatus, with the scientific community divided into nonbelievers and believers. The acceptance of the status of the Golgi as a bona fide cell structure depended on whether one believed that the metallic impregnation methods (involving use of silver or OsO_4), which Golgi and others used to demonstrate the apparatus, were staining a common structure with variable form and distribution in different cell types, or alternatively, that these methods resulted in artifactual deposition of heavy metals on different cell structures in different cell types. The Golgi controversy lasted until the introduction of the electron microscope into biological research, in the early 1950s. Shortly thereafter, the believers began to outnumber the nonbelievers, and by 1963, even the most skeptical had become converts (see Whalley [2]) and Beams and Kessel (3) for details of the history of this period).

The Renaissance (1954-1963)

Electron microscope studies published before 1954 had verified the existence of a distinctive Golgi region in cells; but due to the technical limitations of the preparatory techniques at the time, the images obtained did not extend knowledge of its organization beyond what was known from studies with the light microscope. In 1954, however, the 'lamellar' nature of the Golgi was recognized and described in papers by Dalton and Felix (4), Sjöstrand and Hanzon (5), Rhodin (6), and Farquhar and Rinehart (7). It is Dalton and Felix who deserve the major credit for convincing the scientific public of the reality of the Golgi apparatus, and whose work (4, 8) had the greatest impact at the time. They established that the Golgi apparatus consists of several distinct fine structural components (lamellae, vesicles, and vacuoles), and, accordingly, introduced the term Golgi 'complex' for this organelle; they showed that variations in the form, amount, and disposition of these components occur in different cell types; and they demonstrated deposition of metallic osmium in its lamellar components, thereby relating the newly discovered fine structure to the light microscope studies of the classical Golgi literature which relied heavily on metallic impregnation methods.

The period that followed was characterized largely by detailed morphological descriptions of the fine structure of the Golgi apparatus (or complex) in everyone's favorite tissue. The electron micrographs and the details recorded improved with the introduction of better techniques for specimen preparation. Information on the function of the Golgi complex was limited, however, to noting the topographical association between this organelle and forming secretion granules. Attempts to use cytochemical techniques (other than heavy metal impregnation) or to isolate usable Golgi fractions were still to come. It is during this period that the ubiquity of the Golgi complex, its general structural characteristics, and detailed organization in a variety of cell types were established.

The Modern Period (1964-1973)

During the late 1960s and early 1970s, additional techniques were applied to the study of the Golgi apparatus which added new dimensions to our overall understanding of Golgi structure and function. These procedures included techniques for phosphatase cytochemistry, which yielded new information on the heterogeneity of Golgi elements; autoradiography, which provided the first information on the movement of secretory proteins through the Golgi complex and on the involvement of the organelle in glycoprotein synthesis and in sulfation; and

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techniques for isolating Golgi fractions and (later) subfractions, which made possible biochemical analysis of Golgi components. The last development was greatly facilitated by the discovery of a reliable marker enzyme activity—galactosyltransferase—which is limited in its intracellular distribution to the Golgi apparatus and therefore could be used to monitor the effectiveness of fractionation procedures. Most of the known Golgi functions, which are summarized below, were established during these years.

The Current Period (1973 to the Present)

Currently all these procedures and approaches are being applied—usually in combination—to many different kinds of cells. The focus of current work is to determine the interactions between Golgi components and other cell compartments (ER, lysosomes, plasmalemma) in order to delineate the role of the Golgi complex in such basic and apparently diverse cell processes as secretion, membrane biogenesis, lysosome formation, membrane recycling, and hormone uptake.

Organization of the Golgi Complex

GENERAL DESCRIPTION: The collective electron microscope studies carried out over the past 25 years have established that the Golgi complex consists of a morphologically heterogeneous set of membrane-limited compartments that have common recognizable features and are interposed between the ER and the plasmalemma. Its constant and most characteristic structural component is a stack of smooth-surfaced cisternae (or saccules), which usually have flattened, platelike centers and more dilated rims (Figs. 1–6). Often the cisternae are slightly curved, with one side of the stack oriented toward the rough ER and the other facing the plasmalemma (Fig. 3) or the nucleus (Figs. 1 and 2). Typically, the former side is associated with small vesicles, and the latter with secretory granules or vacuoles in secretory cells (Figs. 1–4 and 7 and 8). Thus, the whole structure has a clearly recognizable polarity, and a number of terms have been introduced and are widely used in the literature to describe its polarity: (*a*) convex vs. concave side; (*b*) proximal vs. distal; (*c*) forming vs. mature; (*d*) entry vs. exit; and (*e*) cis vs. trans (9). We prefer and use the terms cis-trans because (*a*) and (*b*) are not always applicable (due to variations in shape and intracellular organization), and (*c*) and (*d*) assume more than we know at present about the function of both cis and trans Golgi elements.

It is now recognized that in addition to specific Golgi elements, the Golgi region is crowded with other cell structures, such as coated vesicles (Figs. 5, 8, and 9), lysosomes (Figs. 7 and 15), and, in many cases, centrioles with their associated satellites and microtubules (Fig. 2).

In their early studies on the organization of the Golgi complex, Dalton and Felix (8) recognized many of the main features that characterize this organelle: (*a*) its multiple components—flattened cisternal sacs (then referred to as lamellae)

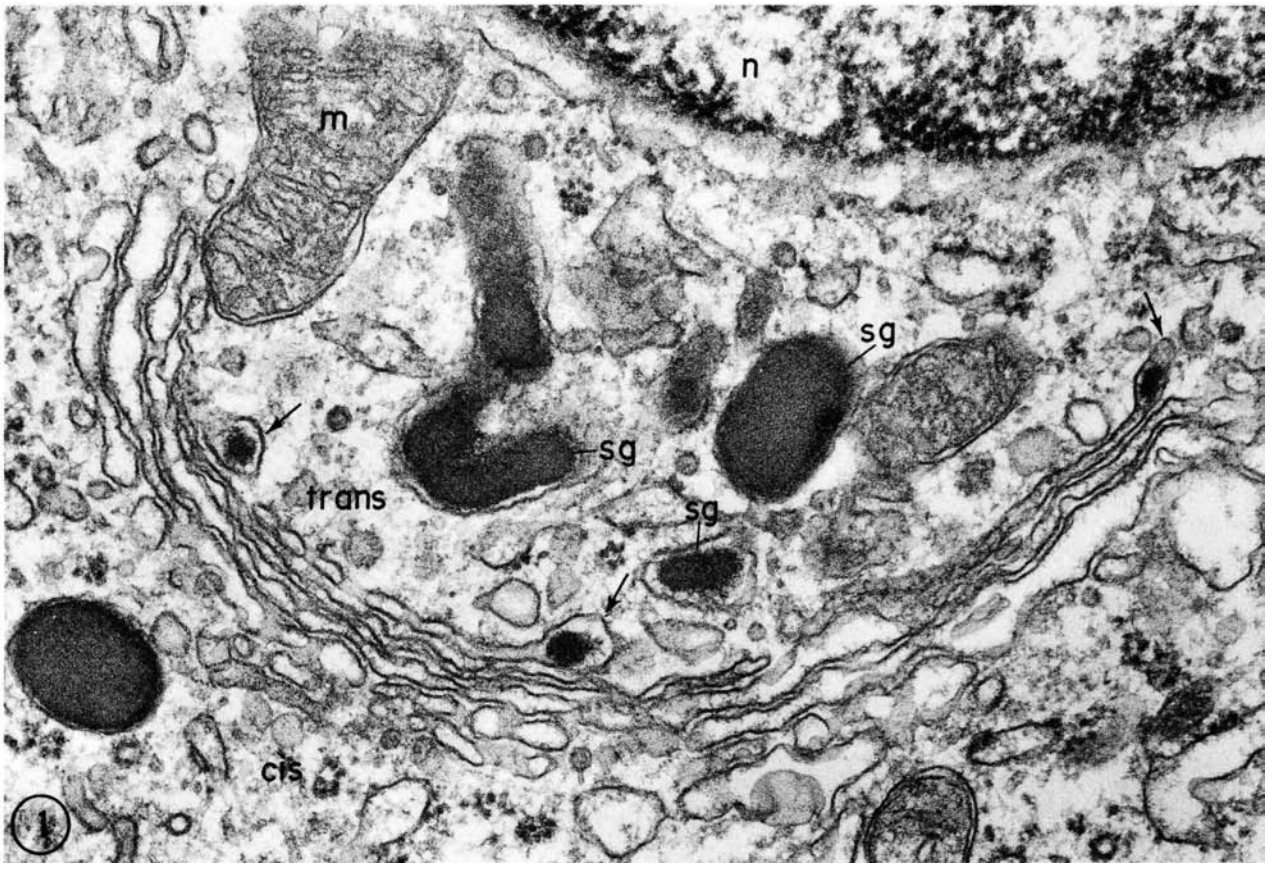


FIGURE 1 Golgi region of a mammotroph or prolactin-secreting cell from the anterior pituitary gland of a lactating rat. A stack of three to five slightly curved Golgi cisternae occupies the center of the field. The secretory granules (85% prolactin) arise within the trans cisternae along the concave face of the Golgi stack. In this field, three small (100–200 nm) prolactin granules are seen condensing within three of the transmost cisternae (arrows). The polymorphous secretion granules (sg) seen above result from the fusion and aggregation of several of the small Golgi-derived granules (as diagrammed in Fig. 26). n = nucleus; m = mitochondrion. × 67,000. From Farquhar (51).

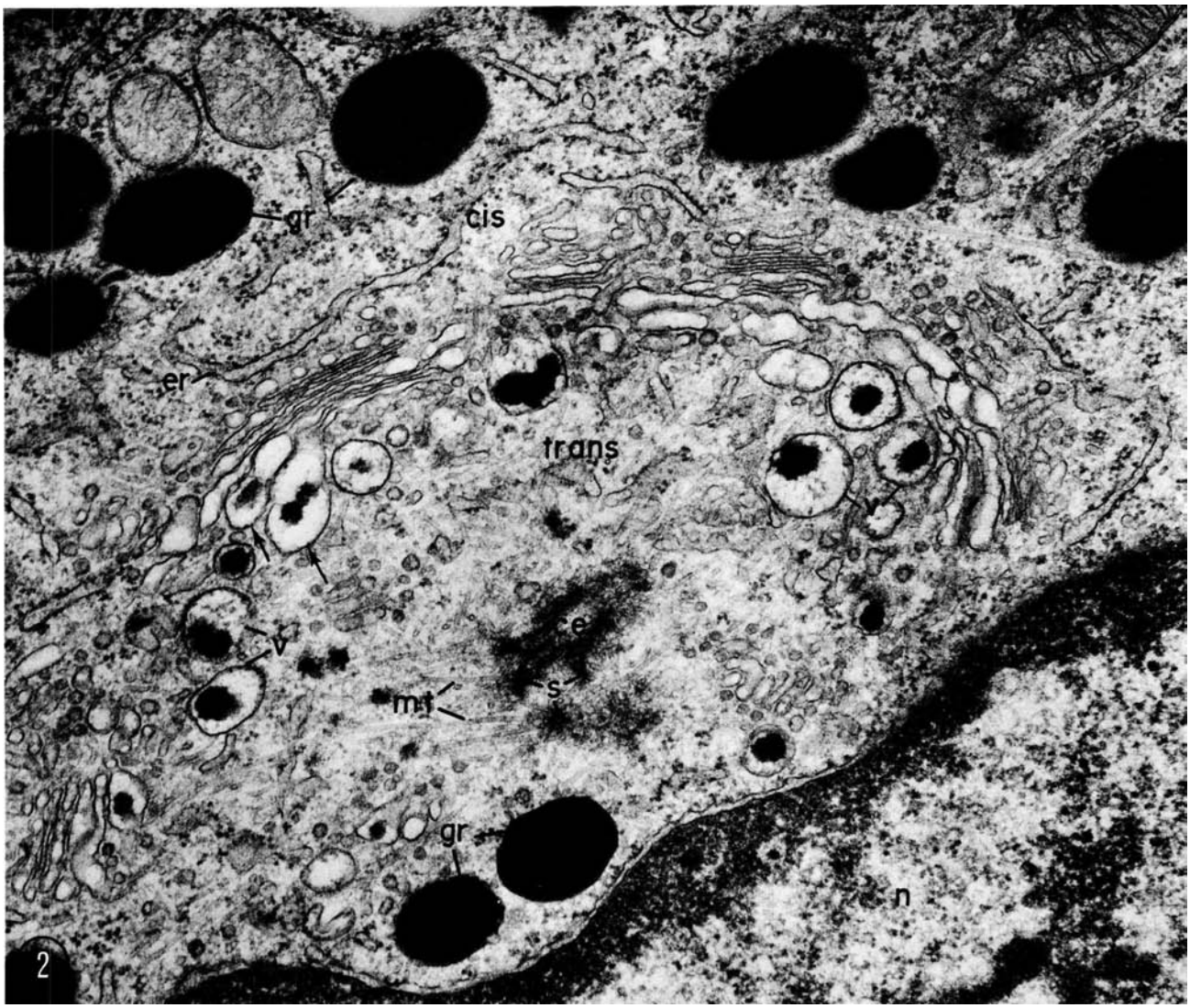


FIGURE 2 Golgi region of a developing PMN leukocyte (promyelocyte stage), illustrating the formation of azurophil or primary granules along the trans side of the Golgi complex. This complex consists of a stack of five to eight slightly curved Golgi cisternae which partially encircle a centriole (ce). The dilated ends of several of the transmost cisternae (arrows) are seen to contain condensing secretory products (i.e., lysosomal enzymes and peroxidase in these cells). Numerous dense-cored vacuoles (v), are also seen along the trans Golgi face; they are presumed to arise by budding from the dilated rims of the adjacent Golgi cisternae. Several of these vacuoles fuse, their contents aggregate and undergo further concentration, resulting in the formation of the compact and uniformly dense azurophil granules (gr). The assembly process is very similar to that involved in the formation of prolactin granules (see Figs. 1 and 26). s = centriolar satellites; mt = microtubules; n = nucleus. × 50,000. From Bainton and Farquhar (122).

as well as vacuoles and vesicles; (*b*) the high variability of the relative amounts of these elements in different cell types; (*c*) the frequent identity of vacuolar elements with dilated cisternae; (*d*) the absence of ribosomes (then referred to as small granules of Palade) from Golgi membranes; and (*e*) the fact that some of these membranes were thicker (8–10 nm) than the membranes of the rough ER (then called ergastoplasm).

Still other organelles, such as ribosomes, glycogen, mitochondria, peroxisomes, and rough and smooth ER, are found in the Golgi area but are usually excluded from the region where the stacks are located (Figs. 5 and 7); Mollenhauer and Morré (10) have referred to this region as the Golgi 'zone of exclusion'. They and others have noted that the matrix in which the Golgi complex is embedded is denser than the rest of the cytoplasmic matrix and has a fibrillar-granular appearance (Fig. 7). At present no information is available about the composition of this matrix material.

GOLGI STACKS OF ANIMAL CELLS: The early light microscope studies established that the intracellular distribution of the Golgi apparatus varies from one cell to another. In neurons, where it was originally discovered, the apparatus appears as a perinuclear network, whereas in exocrine glands it forms a ring-like structure between the nucleus and the apical cell surface. Electron microscopists have utilized cytochemical staining on thick (~ 1/2 μm) sections with tilting to study the three-dimensional interrelationships of Golgi cisternae in the cell. Using this approach, Novikoff et al. (11) and Rambourg et al. (12) have presented evidence that the cisternae in animal cells are extensively interconnected, and have suggested that the Golgi complex consists of a single set of stacked cisternae.

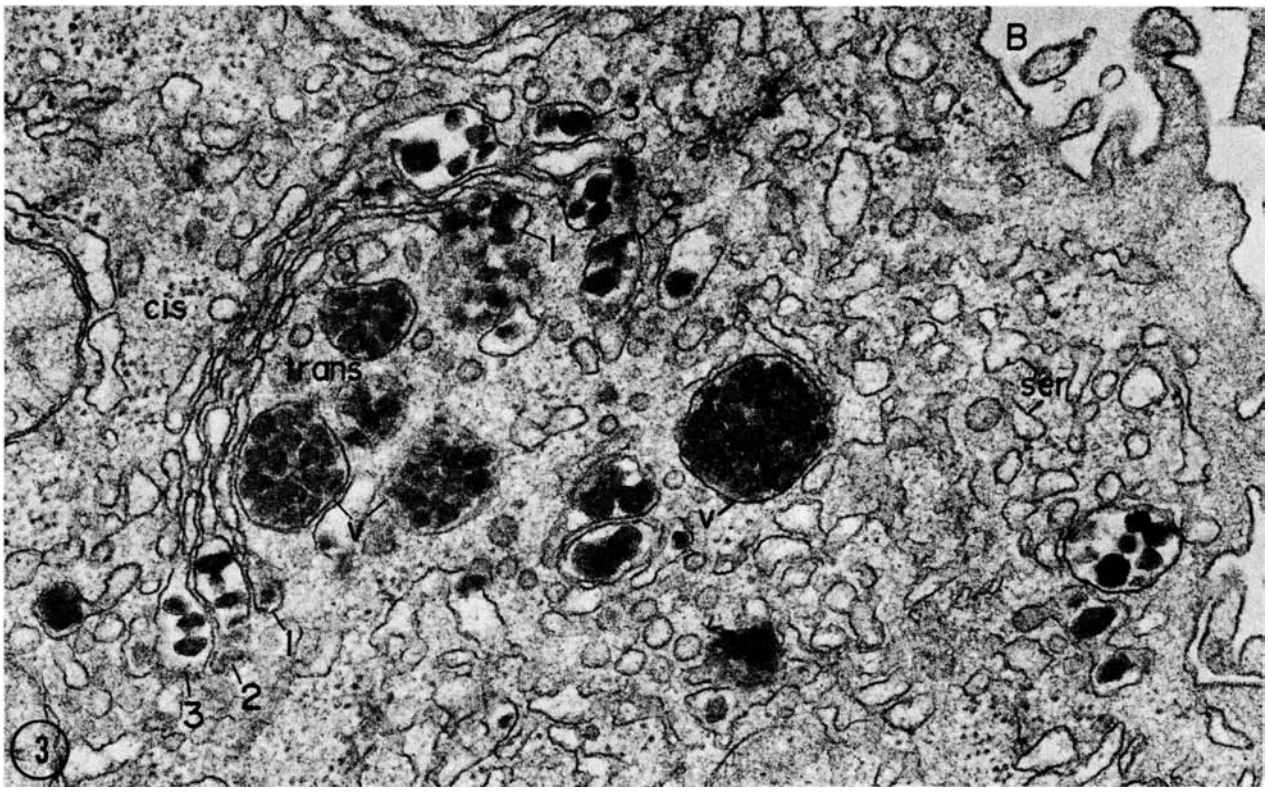


FIGURE 3 Golgi complex of a hepatocyte from an ethanol-treated rat. This complex consists of a stack of three to four slightly curved Golgi cisternae which face the bile canaliculus (B). Clusters of lipoprotein particles are seen in the dilated rims of three cisternae (1, 2, 3) in the trans part of the Golgi stack and in numerous secretory vacuoles (v) located on the trans side of the stack. The accumulation of lipoprotein particles in the rims of Golgi cisternae is a normal occurrence in the hepatocyte, but it is greatly increased following ethanol treatment. ser = smooth er. × 50,000.

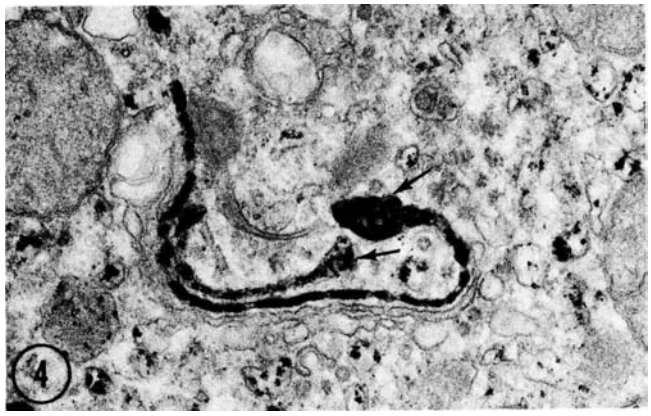
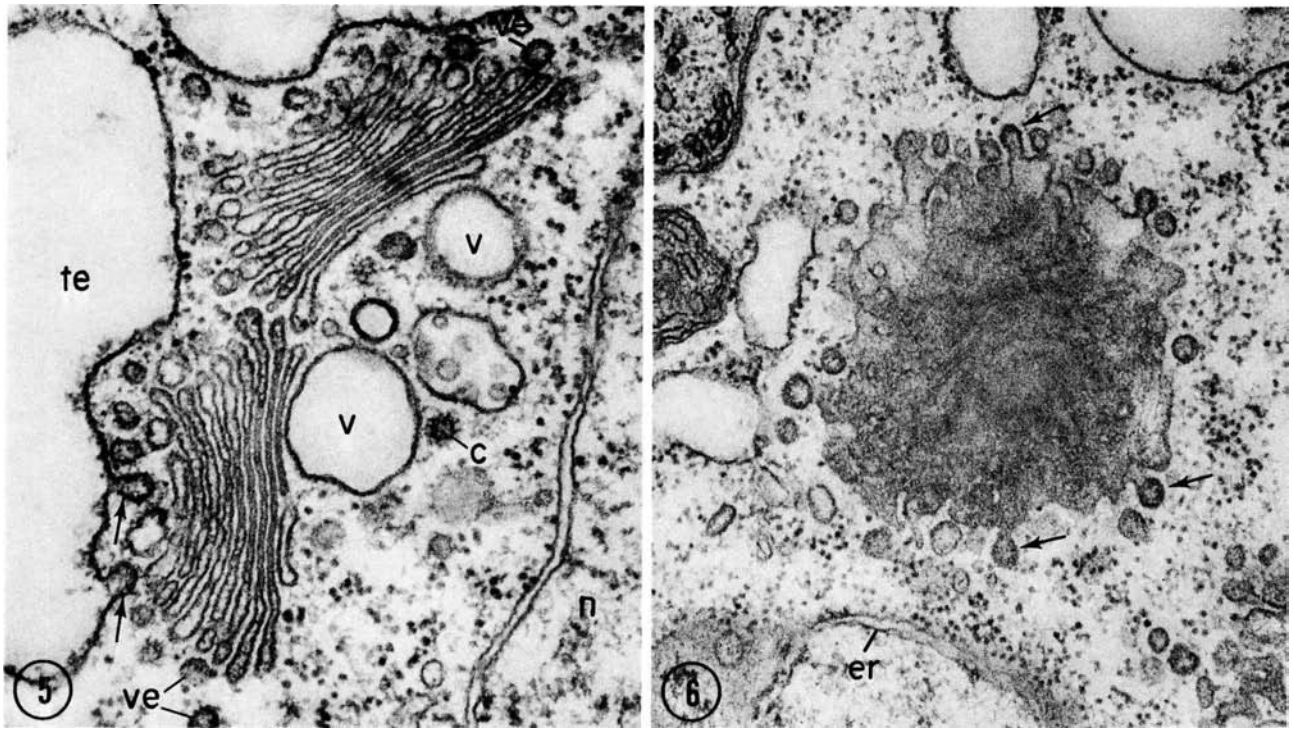


FIGURE 4 Golgi complex from a normal rat hepatocyte in a preparation reacted for TPPase. Reaction product (lead phosphate) is seen within two of the transmost Golgi cisternae, the dilated rims of which (arrows) also contain lipoprotein particles. × 30,000. From Farquhar et al. (20).

GOLGI STACKS OF PLANT CELLS (DICTYOSOMES): Structures which proved to be Golgi in nature had been studied for years in plant cells under the name of dictyosomes. Electron microscope studies during the 1950s established that each dictyosome is present in multiple copies and corresponds to an individual Golgi stack. The detailed organization of the Golgi in plant cells has been extensively studied, especially by Whalley, Northcote, Mollenhauer, Morré, and their associates (see Whalley [2] for a review). Some time ago, the latter two authors called attention to the similarities between Golgi complexes in animal and plant cells (13). In many (but not all) plant cells, there are distinct differences in the thickness of the membranes of the cisternae across the stack, with those on the cis side being thin (ER-like) and those on the trans side being noticeably thicker (plasmalemma-like), a feature first noted by Grove et al. (14). This led to the idea, proposed by Morré and co-workers (15), that a gradual increase in membrane thickness takes place as the cisternae move across the stack (see below).

OTHER MORPHOLOGIC FEATURES: Besides the general features that are applicable to most if not all Golgi complexes, other features have been described which occur less regularly. Examples are the rings of beads between the ER and Golgi cisternae in certain insects (16), and dense nodes of intercisternal material that occur in the cytoplasmic matrix between Golgi cisternae in some protozoans (17). After the discovery of coated vesicles, it was recognized that clathrin-coated vesicles are commonly seen in the Golgi region (18, 18a) and, in addition, that coated regions commonly occur on the rims of the Golgi cisternae and on condensing granules or vacuoles (Figs. 5, 8, and 9).

Finally, on the trans side of many (but not all) Golgi stacks, cisternae of characteristic morphology have been described; they are often separated from the stack (Figs. 10, 13, and 14), and their appearance varies from straight (rigid lamellae) to tubular and tortuous (Figs. 13 and 14). Novikoff and his coworkers, first described these cisternae in 1964, and, based on the observation that acid phosphatase activity is often associated with them, postulated that they constitute a link between



FIGURES 5 and 6 Features of dictyosomes or Golgi stacks found in plant cells (the green algae *Chlamydamonas rheinhardi*). Fig. 5 shows two dictyosomes each with 9 parallel cisternae cut in cross section. Characteristic features of these Golgi complexes are the presence of large vacuoles (v) and coated vesicles (c) associated with their trans side, transport vesicles with fuzzy-coats which bud (arrows) from transitional elements (te) of the rough ER on the cis side, and numerous vesicles (ve) associated with the dilated rims of the cisternae. Fig. 6 shows an obliquely sectioned Golgi stack seen *en face*. It illustrates the presence of numerous vesicles which appear to be in the process of fusing with, or budding from the cisternal rims (arrows). Fig. 5—× 80,000; Fig. 6—× 70,000.

the *G*olgi, and *ER*, and *L*ysosomes. Accordingly, Novikoff introduced the acronym GERL as their designation (see Novikoff et al. [11, 19]). The present status of the GERL concept is discussed further below.

Composition of the Golgi Complex

CYTOCHEMICAL STAINING: The first evidence of compositional heterogeneity among cisternae in the Golgi stacks came from the results of cytochemical staining procedures which demonstrated qualitative differences in staining for various enzymes and other components among Golgi cisternae (Figs. 9–12). These differences are best documented in the case of the hepatocyte (Table I), in which staining has been carried out both *in situ* and on Golgi fractions. The earliest studies of this type were those of Novikoff and Goldfischer (25), who demonstrated that thiamine pyrophosphatase (TPPase) and nucleoside diphosphatase activity (NDPase) represent cytochemical markers that could be used to study the form and distribution of the Golgi apparatus in many, but not all cells (in hepatocytes the ER also contains these enzymes [24]). In subsequent work, Novikoff and co-workers showed that these activities were restricted in their distribution to 1–2 cisternae on the trans side of the Golgi stack (Figs. 4 and 11), and that acid phosphatase (AcPase) was also restricted to one or two of the transmost cisternae (Figs. 10 and 15). Later, based on the study of thick (1/2 μm) sections as well as thin sections, they also demonstrated that AcPase and TPPase are present in different cisternae in many cell types (11, 19). Friend and Murray (23) showed that the classical osmium impregnation procedures preferentially stain one or two of the cismost cisternae in many cells (Fig. 12), and recently, Smith (26) found that intermediate cisternae selectively stain for nicotinamide adenine dinucleotide phosphatase (NADPase) in the ameloblast and several other cell types. In work from our laboratories, it was demonstrated that several other enzymes—5′-nucleotidase (20) and adenylate cyclase (21)—are present in virtually all cisternae, both cis and trans, within the stack. Rambourg and LeBlond (22) found that all Golgi cisternae stain with periodic acid-silver methenamine (PA-silver) (which stains complex carbohydrates), but staining is graded (increasing from cis to trans) across the stack.

Results of cytochemical staining also provided the first indication that, in addition to the differences in composition across the stack, there may be differences in the composition of the membrane of a given cisterna (20, 21, 27). Specifically, our finding that lead phosphate reaction product for both 5′-nucleotidase (20) and adenylate cyclase (21) was concentrated along the dilated rims of isolated Golgi elements (Figs. 16 and 17) and was missing or present in much lower concentration in the flattened centers of Golgi cisternae in liver fractions suggests that the dilated rims may have a composition different from that of the flattened centers. These findings also provide the first clear demonstration that these two plasmalemmal marker enzymes are indigenous to Golgi elements.

Based on the location of the reaction product (on either the inside or outside of Golgi membranes), cytochemical staining has provided suggestive evidence on the orientation or sidedness of the active site of several enzymes. For most enzymes studied (TPPase, AcPase), the lead phosphate reaction product was localized inside the cisternae where it was associated either

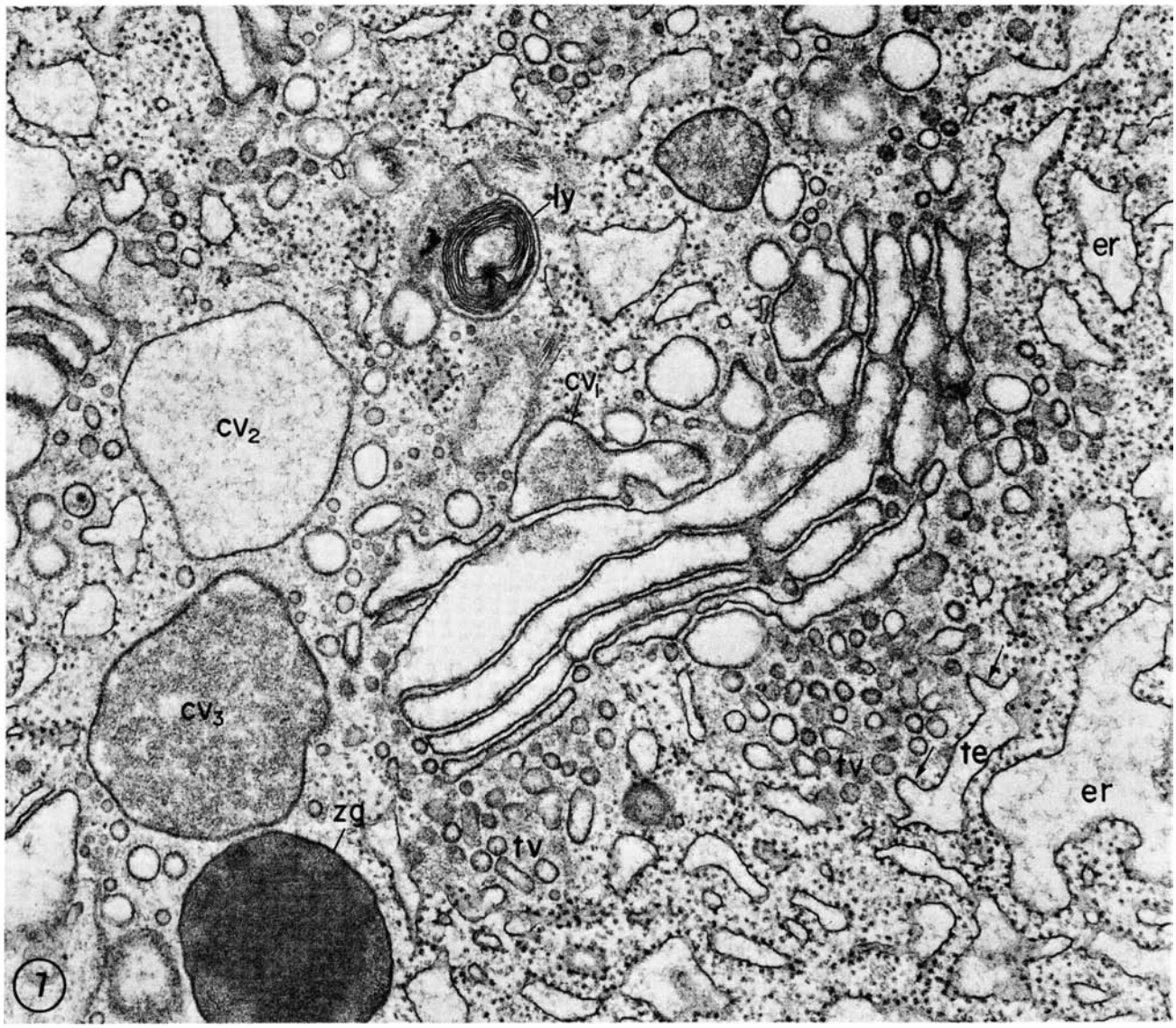


FIGURE 7 Golgi region from an exocrine pancreatic cell (guinea pig). Characteristic features of this Golgi complex are the presence of a stack of four to five slightly dilated Golgi cisternae associated with condensing vacuoles (cv_1) on its trans side, and a profusion of small peripheral Golgi vesicles, or transport vesicles (tv), along its cis side. These vesicles are assumed to bud (arrows) from the transitional elements (te) of the rough ER and to transport secretory products to the condensing vacuoles by a route still unknown. The condensing vacuoles gradually fill with secretory proteins (mostly pancreatic zymogens), undergo progressive concentration, thereby becoming increasingly dense (cv_{1-3}), and eventually become mature zymogen granules (zg). Note that there is a zone around the Golgi cisternae and transport vesicles in which the cytoplasmic matrix is denser than elsewhere in the cell, and from which ER elements (er) and ribosomes are excluded. ly = lysosome. × 38,000.

with the inside of the membranes or the cisternal content. In a few cases, however, 5′-nucleotidase (20) and adenylate cyclase (21), reaction product was found on the cytoplasmic side of the dilated rims of certain Golgi cisternae. It is of interest that the reaction product for 5′-nucleotidase was localized on the *outside* of cisternae and the *inside* of secretory vacuoles (20, 28). Thus far, biochemical assays on cell fractions have largely substantiated the cytochemical observations pertaining to sidedness; when reaction product was localized to the inside of Golgi membranes, the enzyme activity was latent and detergent treatment (to permeabilize the membranes) increased the activity, whereas when the reaction product was localized on the outside, addition of detergent had no effect (28) on the activity measured.

Among the components demonstrated cytochemically, most are enzymes that can be assumed to be associated with Golgi membranes; however, a few (such as AcPase and substances which stain with PA-silver) may also be associated with the cisternal contents. In addition to the localization of these presumptive Golgi components, there are also several examples of cells in which secretory products, primarily peroxidases, have been localized by cytochemical or immunocytochemical procedures.

To summarize, cytochemical findings have provided information on the existence of specialization among Golgi components and have indicated that differentiation exists across the stack, at least between the extreme cis and trans cisternae. In addition, the evidence has suggested that differentiation also exists within individual cisternae. The functional significance of these specific localizations remains largely unknown.

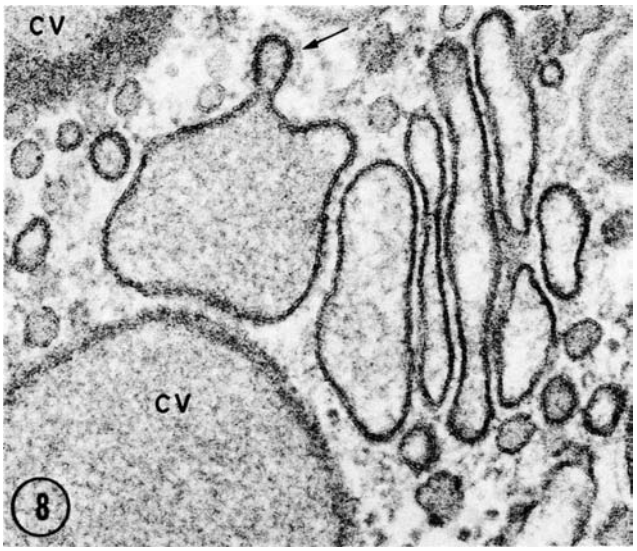
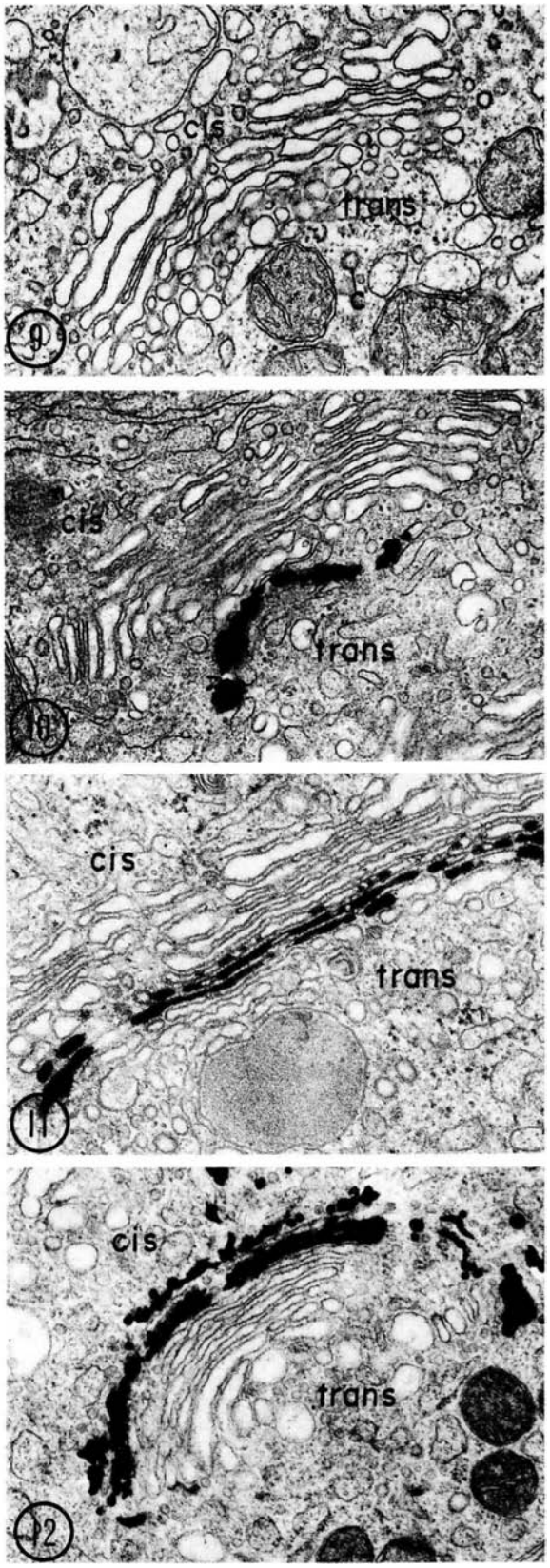
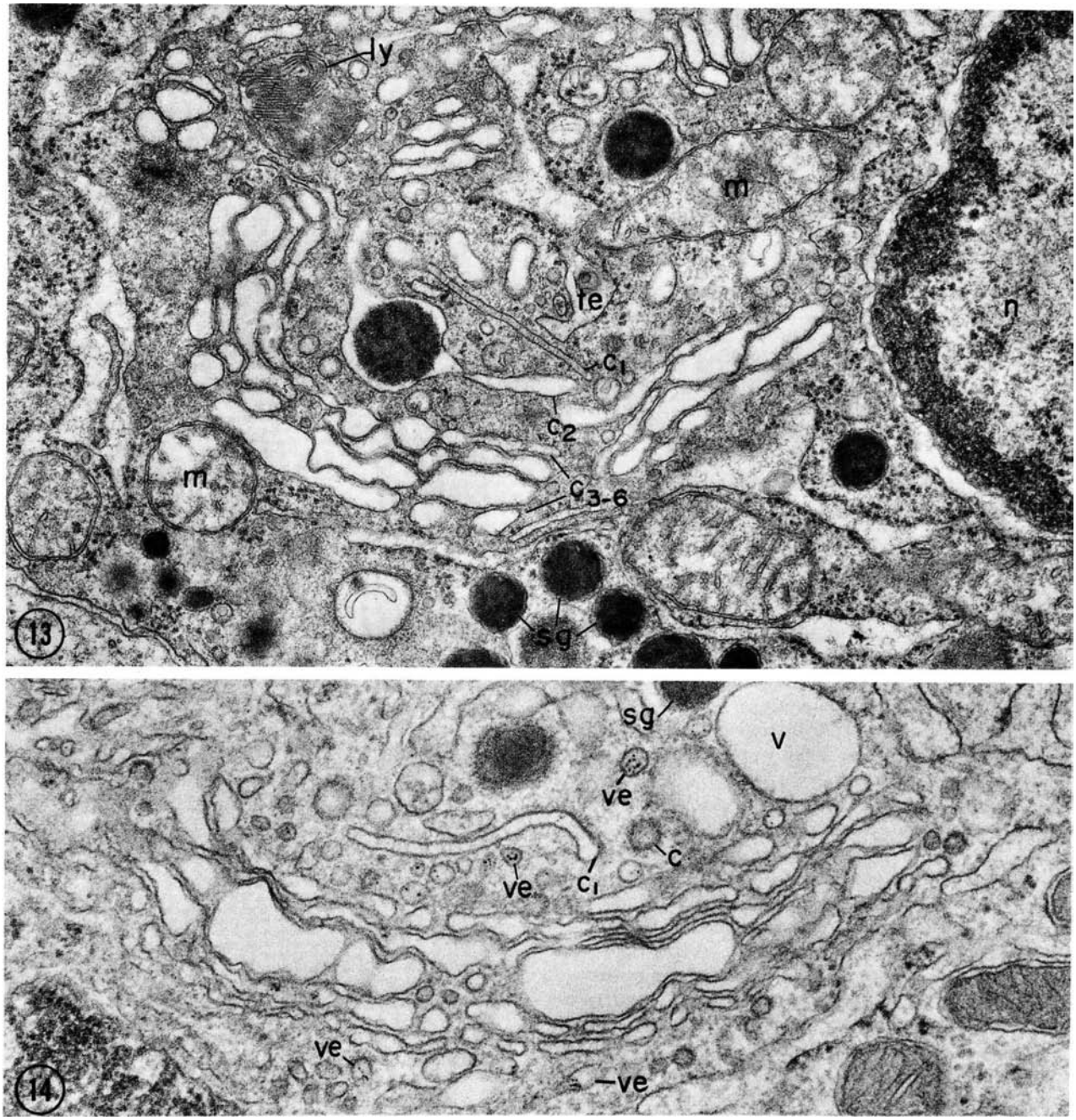


FIGURE 8 Another Golgi complex from an exocrine pancreatic cell showing 4–5 cisternae (to the right) and a condensing vacuole with a budding (or fusing) coated vesicle (arrow). Two other condensing vacuoles or granules (cv) are also present nearby. × 95,000.

METHODS FOR PREPARATION OF GOLGI FRACTIONS AND SUBFRACTIONS: The earliest attempts to isolate Golgi fractions can be attributed to Schneider and Kuff in 1954, who used the rat epididymis as starting material (29). The fractionation was monitored by light microscopy, and the results were puzzling because they seemed to indicate that there was DNA in the fractions. This unusual finding proved to be an artifact of the assay procedure created by the presence of carbohydrates in Golgi elements. Some time elapsed until Morré and his collaborators conducted a series of more fruitful fractionation attempts, first on plant cells (30) and later on rat liver (31, 32). They succeeded in isolating Golgi fractions from liver homogenates by a combination of differential and rate sedimentation procedures. The fractionation was monitored by electron microscopy (32), which revealed that many cisternae remained stacked, and the fractions were examined for a variety of enzymatic activities, mostly phosphatases (24, 33). Shortly after Morré's initial (30) work, B. Fleischer, S. Fleischer, and H. Ozawa (34) and Fleischer and Fleischer (35) simplified and improved the fractionation procedure and demonstrated the presence of a high concentration of galactosyltransferase activity in Golgi fractions from bovine and rat liver by using an exogenous acceptor (*N*-acetylglucosamine). The discovery of galactosyltransferase in Golgi fractions, and its apparent absence from other cell membranes, was an important development in Golgi history because it provided a much-needed marker enzyme for monitoring cell fractionation. Earlier attempts to prepare Golgi fractions had relied exclusively on morphological criteria for the identification of Golgi elements and had been hampered by the lack of a quantitative criterion



FIGURES 9–12 Golgi complexes from the epidydymis (rodent) in which the Golgi complex consists of 8–10, parallel stacked cisternae with numerous associated vacuoles and vesicles some of which are coated (c). Here they are seen either unstained (Fig. 9) or reacted for cytochemical procedures which stain the cisternae in the stack differentially. Fig. 10 shows reaction product for acid phosphatase (β-glycerophosphatase) in a single cisterna on the trans side of the stack which is set off from the rest, and which has the properties ascribed to GERL by Novikoff and co-workers (19). Fig. 11 shows reaction product for TPPase in two of three of the parallel transmost cisternae in the stack. No cisternae comparable to the AcPase-positive cisterna in Fig. 10 is seen. Fig. 12, from a preparation impregnated with OsO_4, shows osmium deposits in two of the cismost cisternae in the stack. From Friend (153). Fig. 9—× 40,000; Figs. 10 and 11—× 30,000; Fig. 12—× 24,000.



FIGURES 13 and 14 Golgi regions in two somatotrophs from the rat anterior pituitary, illustrating some of the variations encountered in the morphology of the cisternae present in the Golgi region. In Fig. 13, the first cisterna (c_1) on the trans side of the stack is straight rather than curved, and is set off from the rest. It has the morphology ("rigid lamella") ascribed to GERL by Novikoff and his associates; there is strict parallelism of the adjoining membranes which appear somewhat thicker than those of the rest of the cisternae. c_2, which is also set off slightly from the Golgi stack, contains a forming secretion granule. c_{3-6} are slightly curved and more dilated. Fig. 14 shows another Golgi stack with another cisterna (c_1) on the trans side set off from the rest with features similar to those of c_1 in Fig. 13. Numerous vesicles (ve) are present both on the cis and trans sides of the stack; some of these are coated vesicles (c). The cells were incubated with cationized ferritin prior to fixation, and many of the vesicles contain the tracer. sg = secretory granule. Fig. 13—× 36,000; Fig. 14—× 50,000. Fig. 13 is from Farquhar (51).

for yield and purity because no enzymes were known to be exclusively restricted to the Golgi complex. The work on glycosyltransferases was extended by Morré et al. (36) to demonstrate *N*-acetylglucosamine transfer to unspecified endogenous receptors, and by Schachter and co-workers (37, 38), who demonstrated the presence of other (sialyl and fucosyl) terminal glycosyltransferases by using appropriately prepared, natural glycoprotein acceptors for these glycosyltransferases.

Subsequently, a number of variants of either Morré's or the Fleischers' procedures have been published (39). Most of the fractions obtained retain stacked Golgi cisternae (Fig. 18).

The recovery of galactosyltransferase activity in Golgi fractions prepared by these procedures was no better than 30–40% (in reference to the homogenate). Hence, attempts were made to improve yield by overloading the Golgi elements with lipoprotein particles, thereby modifying their density (9, 40).

Overloading was induced by acute ethanol intoxication of the animals (rats). At the beginning, the galactosyltransferase recovery appeared to be nearly complete (40), but later, better-controlled assays showed that the yield was no better than 50 to 60% (41). This procedure was capable of resolving (by flotation in a density gradient) two or three fractions of increasing density. The light Golgi fractions were enriched in trans vacuoles or secretory droplets filled with lipoprotein particles (Fig. 19), whereas the heavy Golgi fractions had a higher concentration of cis, predominantly cisternal elements (Fig. 20). These fractions have been used for a variety of enzymological (40, 41) and cytochemical (20, 21, 28) studies and for investigating the transport of secretory proteins within the Golgi complex (42).

TABLE I

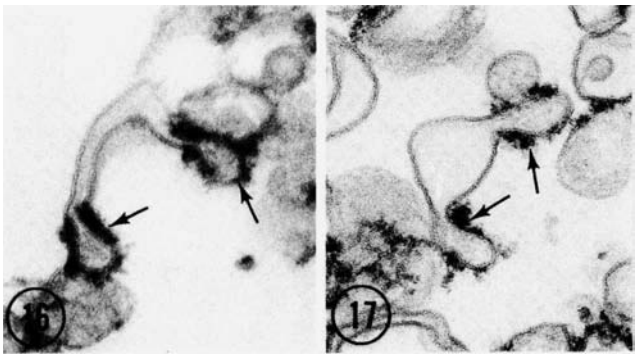
Cytochemical Reactions of Golgi Cisternae in the Hepatocyte

	Cis	Trans	Reference
5′-Nucleotidase	+	+*	20
Adenylate cyclase	+	+	21
Periodic acid-silver methenamine	+	+‡	22
OsO_4 impregnation	+	–	23
Acid phosphatase	–	+	19, 20
Thiamine pyrophosphatase	–	+	19, 20, 24
Glucose-6-phosphatase	–	–	20

* Both cis and trans elements were reactive, but a difference in sidedness of reaction product was detected: it was present on the outside of the membrane of cis elements (concentrated on the dilated rims) and on the inside of the membrane of trans elements.

‡ A gradient of increasing reactivity from the cis to the trans side was detected. From Farquhar (115).

In defining Golgi fractions, investigators have relied on galactosyltransferase as an accepted Golgi marker as well as on the absence (or low concentration) of microsomal (ER) markers, primarily glucose-6-phosphatase and NADPH-cytochrome P450 reductase. A complication arose when it was found (41) that assays carried out immediately upon fractionation showed the presence of microsomal enzyme activities in unexpectedly high concentrations. Further work indicated that the corresponding Golgi activities were lost rapidly during storage, presumably as a result of lipid peroxidation (41). This



FIGURES 16 and 17 Golgi cisternae from Golgi subfractions (GF_3) prepared by the method of Ehrenreich et al. (9) and reacted for 5′-nucleotidase (Fig. 16) or adenylate cyclase (Fig. 17) prior to fixation. Reaction product (lead phosphate) is concentrated on the dilated rims of the cisternae (arrows) and is absent from their central regions. × 85,000. Fig. 16 is from Farquhar et al. (20), and Fig. 17 is from Cheng and Farquhar (21).

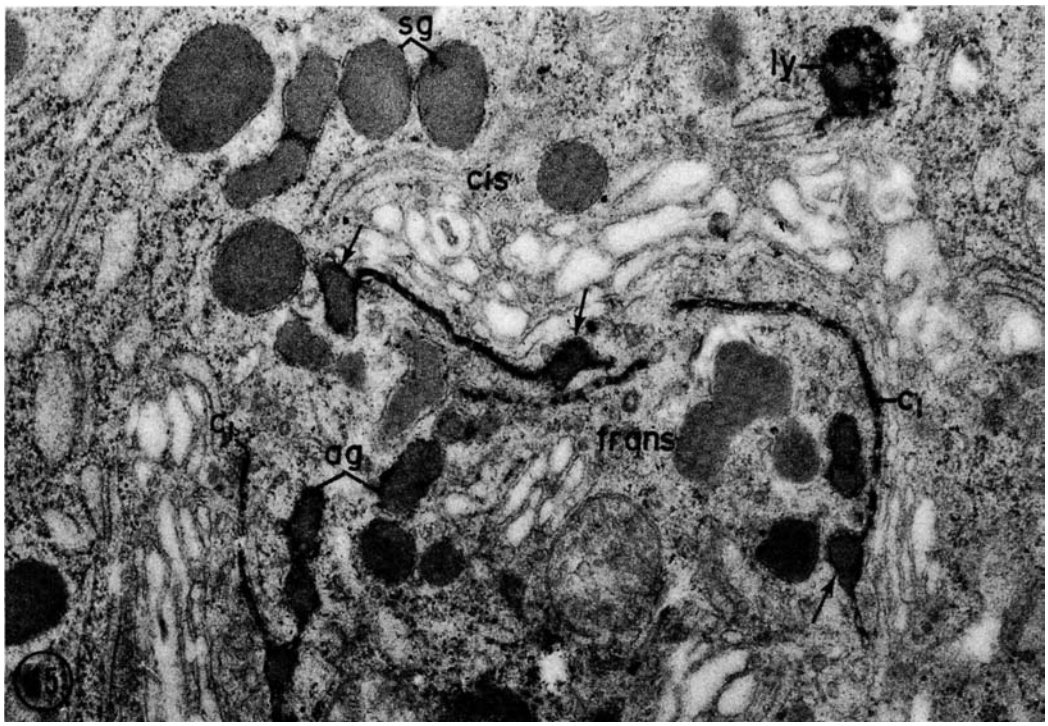
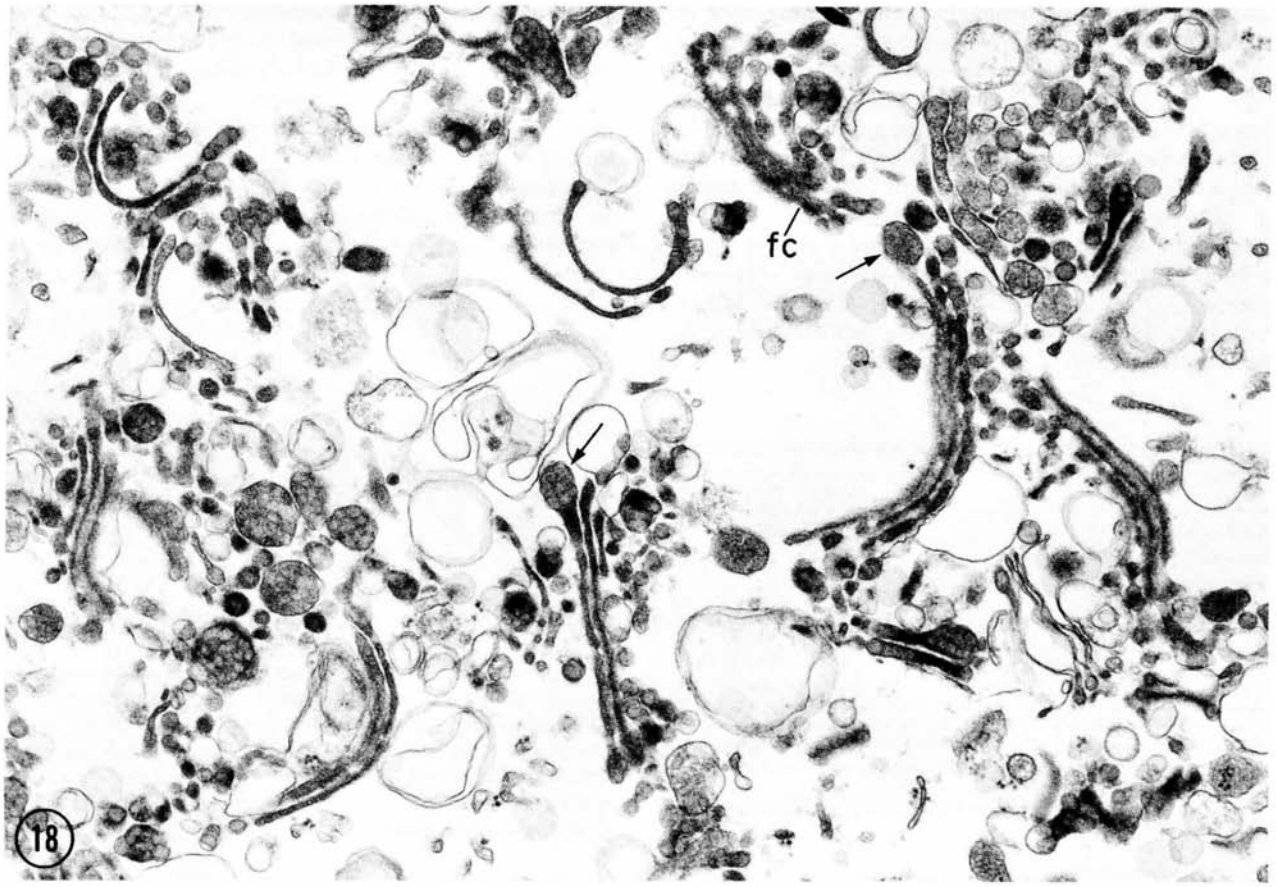


FIGURE 15 Golgi region of a prolactin-secreting cell from a lactating rat (similar to that in Fig. 1); preparation incubated for acid phosphatase. Condensing secretory granules (arrows) and reaction product for AcPase are present in the same Golgi cisterna—i.e., the innermost cisternae (c_1) along the trans side of the stack which is less dilated than the rest. In some places (to the right), the reactive cisterna seems to be included in the regular stack, and in other places (to the left) it appears to be set off from the stack. AcPase reaction product is also seen around some of the immature or aggregating granules (ag) found on the trans Golgi face, at the periphery of a few of the mature granules (sg) present on the cis Golgi face, and in a lysosomes (ly). × 30,000. From Smith and Farquhar (154).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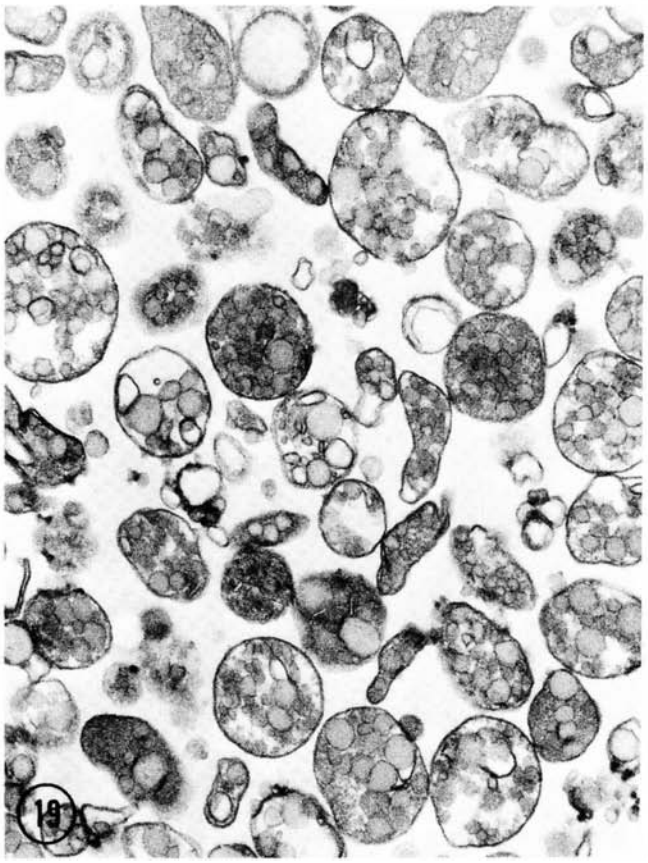
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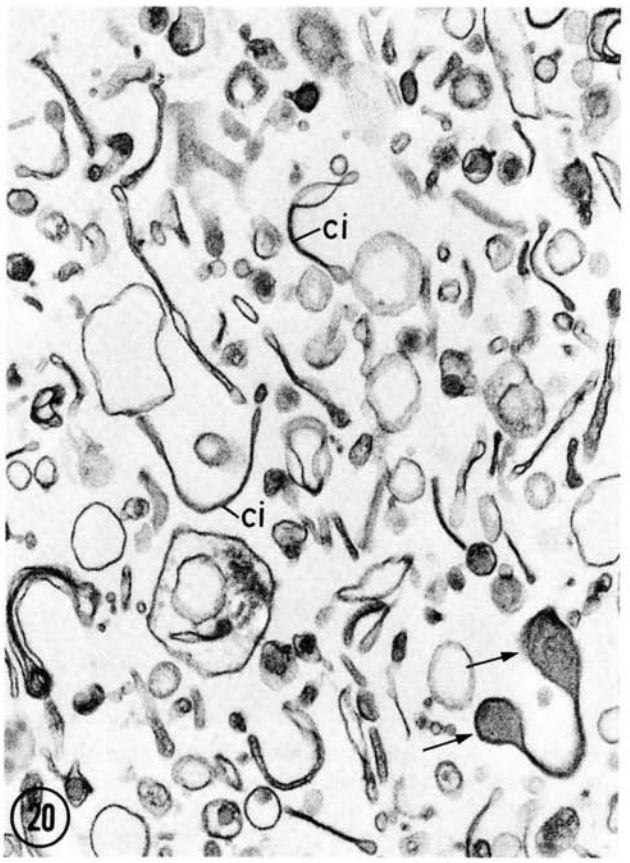
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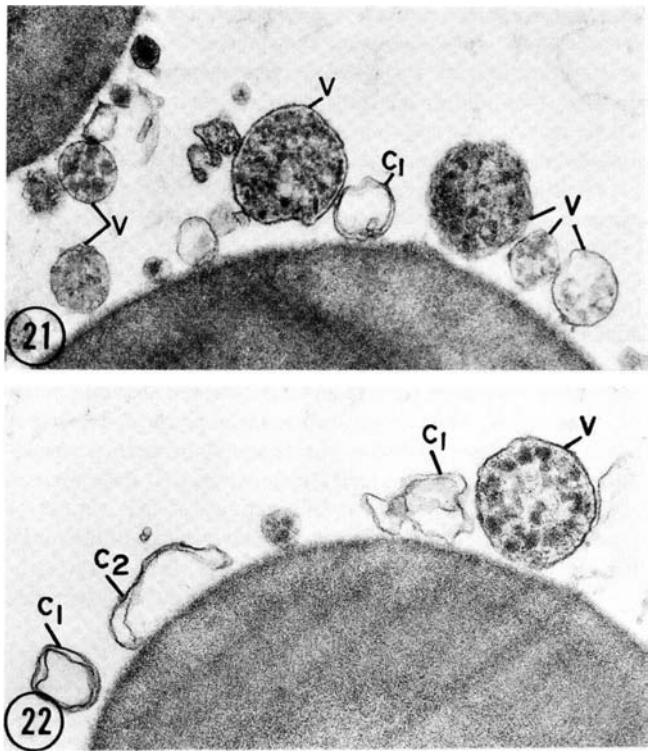
raised the question of whether these microsomal, marker-enzyme activities (like the plasmalemmal markers studied earlier [20, 21]), were indigenous to Golgi fractions, or instead represented contamination of the fractions with ER components.

To solve this problem, Ito and Palade (43) developed an affinity separation procedure. It uses an antibody to NADPH-cytochrome P450 reductase insolubilized to polyacrylamide beads, and allows biochemical assays as well as an electron-microscope survey of immunoadsorbed and nonadsorbed particles. When applied to a light Golgi fraction, the procedure revealed that bona fide Golgi elements—both lipoprotein-loaded secretory vacuoles (~ 58%) and cisternae (14%)—had the reductase in their membranes (Figs. 21 and 22). The affinity adsorption technique was extended to other enzymatic activities, and the results showed that a wide spectrum of microsomal enzymes was present in recognizable, immunoadsorbed Golgi vacuoles, whereas glycosyltransferase activities preferentially remained with the nonadsorbed vesicles. The tentative interpretation of these findings is that Golgi elements have distinct domains; the distended rims of at least some of the Golgi cisternae have 'ER-like' membranes, whereas the central part of the cisternae has an apparently 'Golgi-like' membrane rich in glycosyltransferase activities. It seems probable that the ER-like membrane represents the shuttle containers that transport secretory products from the ER to the Golgi complex. Current thinking (see below) assumes the existence of another membrane container (the equivalent of a secretion-granule membrane) recycling between the Golgi complex and the plasmalemma, but at present there is no information concerning its nature in hepatocytes.

Affinity separation techniques, based on insolubilized specific ligands, are expected to provide further information about the biochemical heterogeneity of Golgi elements and its functional implications. It should be pointed out that although galactosyltransferase activity is considered a marker for Golgi membranes, not only do some morphologically recognizable Golgi elements lack this activity (43), but also a substantial amount (40–50%) of it remains in a residual microsomal fraction in elements of still unknown morphology.

BIOCHEMISTRY OF GOLGI MEMBRANES: Data concerning the biochemistry of Golgi membranes are still limited, partly because of the difficulties encountered in the separation of bona fide Golgi elements from their membrane containers which shuttle between the complex and the ER or plasmalemma. The lipid composition of Golgi membranes appears to be quantitatively different from that of both the ER membrane (more sphingomyelin, less phosphatidylcholine) and the plasmalemma (less cholesterol, less sphingomyelin) (44–46). The electrophoretograms of Golgi membranes reveal a protein composition different qualitatively and quantitatively from that of ER and plasmalemma (35, 45), but the results are in need of extension and improvement.

Enzyme assays established the existence of compositonal overlap between ER and Golgi membranes (15, 45), at least in the case of the fatty acid desaturase system (NADH-cytochrome b_5 reductase); however, from the results of the affinity separation already mentioned (43), the overlap appears more extensive. It includes both the cytochrome P450 system and glucose-6-phosphatase.



FIGURES 21 and 22 Affinity technique for the separation of constituents of Golgi fractions on beads. Goat anti-rabbit IgG was covalently attached to polyacrylamide beads, rabbit anti-NADPH-cytochrome *c* reductase antibody was immunoabsorbed to the beads coated with the first antibody, and Golgi fractions (GF_{1+2}) were reacted with the beads. Recognizable Golgi elements immunoadsorbed to the antireductase-coated beads are secretory vacuoles (v) containing lipoproteins, and cisternae cut either in transverse section (c_1) or in perpendicular section (c_2). × 31,000. From Ito and Palade (43).

Enzymes involved in proximal glycosylation and translocation of nascent polypeptide chains remain unchallenged markers for ER membranes. The same may apply for enzymes involved in triacylglycerol and phospholipid synthesis as indicated by the work of van Golde et al. (46). As already mentioned, terminal glycosyltransferases as well as sulfotransferases (see below) are restricted to Golgi membranes.

Established Functions of the Golgi Complex

PACKAGING OF SECRETION GRANULES: The central role of the Golgi apparatus in secretion was recognized long ago by light microscopists (reviewed by Bowen [47]). Early electron microscopic studies carried out in the 1950s by Sjöstrand and Hanzon (5), Haguenau and Bernhard (48), and Farquhar and

FIGURES 18–20 Golgi fractions from rat liver. Fig. 18 illustrates a fraction prepared from the liver of a normal rat by the procedure of Leelavathi et al. (39), which yields Golgi elements that remain stacked. Lipoprotein particles can be recognized in the dilated rims of many of the cisternae (arrows). Figs. 19 and 20 are Golgi subfractions prepared by the method of Ehrenreich et al. (9) from livers of ethanol-treated rats. Fig. 19 shows a light Golgi fraction (GF_2), and consists mainly of secretory vacuoles filled with lipoprotein particles. Fig. 20, from the heaviest Golgi fraction (GF_3), consists either of whole cisternae or the central parts of collapsed cisternae (ci). A few cisternae contain lipoprotein particles in their dilated rims (arrows). Fig. 18—× 20,000; Fig. 19—× 27,000; Fig. 20—× 36,000. Figs. 19 and 20 are from Ehrenreich et al. (9).

Rinehart (7) noted the close association between secretory granules and Golgi elements, and shortly thereafter several investigators (49, 50) published electron micrographs in which material resembling the contents of secretory granules was clearly recognized within Golgi elements. Subsequent morphological and autoradiographic studies (reviewed in 2, 3, and 51–53) established that in most cell types concentration and packaging of secretory products usually occurs in the dilated rims of the transmost cisternae (Figs. 1–4); however, in a few cell types (exocrine pancreas and parotid of some species), concentration takes place in specialized condensing vacuoles, which are separate from the stacked cisternae (Figs. 7 and 8). In either case, concentration results in the production of a storage granule with a condensed content and a membrane acquired in the Golgi complex. That concentration takes place in many (but not all) cell types has been corroborated by both autoradiographic (52, 54, 55) and cell fractionation (52) data demonstrating greatly increased specific activity of the content of forming and mature granules, as compared to that of the rough ER and Golgi cisternae (Figs. 23 and 24). Recent autoradiographic data obtained by high resolution autoradiographic analysis indicate that concentration up to 200 times that of the ER is achieved in granules of pituitary prolactins (55).

The basis of our current understanding of the overall route of intracellular transport taken by secretory products and the position of the Golgi complex along that route was provided by the combined morphological, autoradiographic, and cell fractionation studies that were initiated by Caro and Palade and further developed by Jamieson and Palade (reviewed in 52 and 57) on the exocrine cells of the guinea pig pancreas, which is diagramed in Fig. 25. With the in vitro systems used by Jamieson and Palade (52, 57, 58), temporal and spatial resolution were increased by using well-controlled, pulse-chase experiments. Moreover, the results of the experiments could be quantitated by autoradiography or by cell fractionation. Their work supports the following model: secretory proteins synthesized in the rough ER are transported to the Golgi region in small vesicular containers which are assumed to function as shuttles between the transitional elements of the ER (Fig. 7) and Golgi elements. Their studies did not establish the route taken by secretory products through the Golgi (see below), but their autoradiographic findings (54) demonstrated clearly that

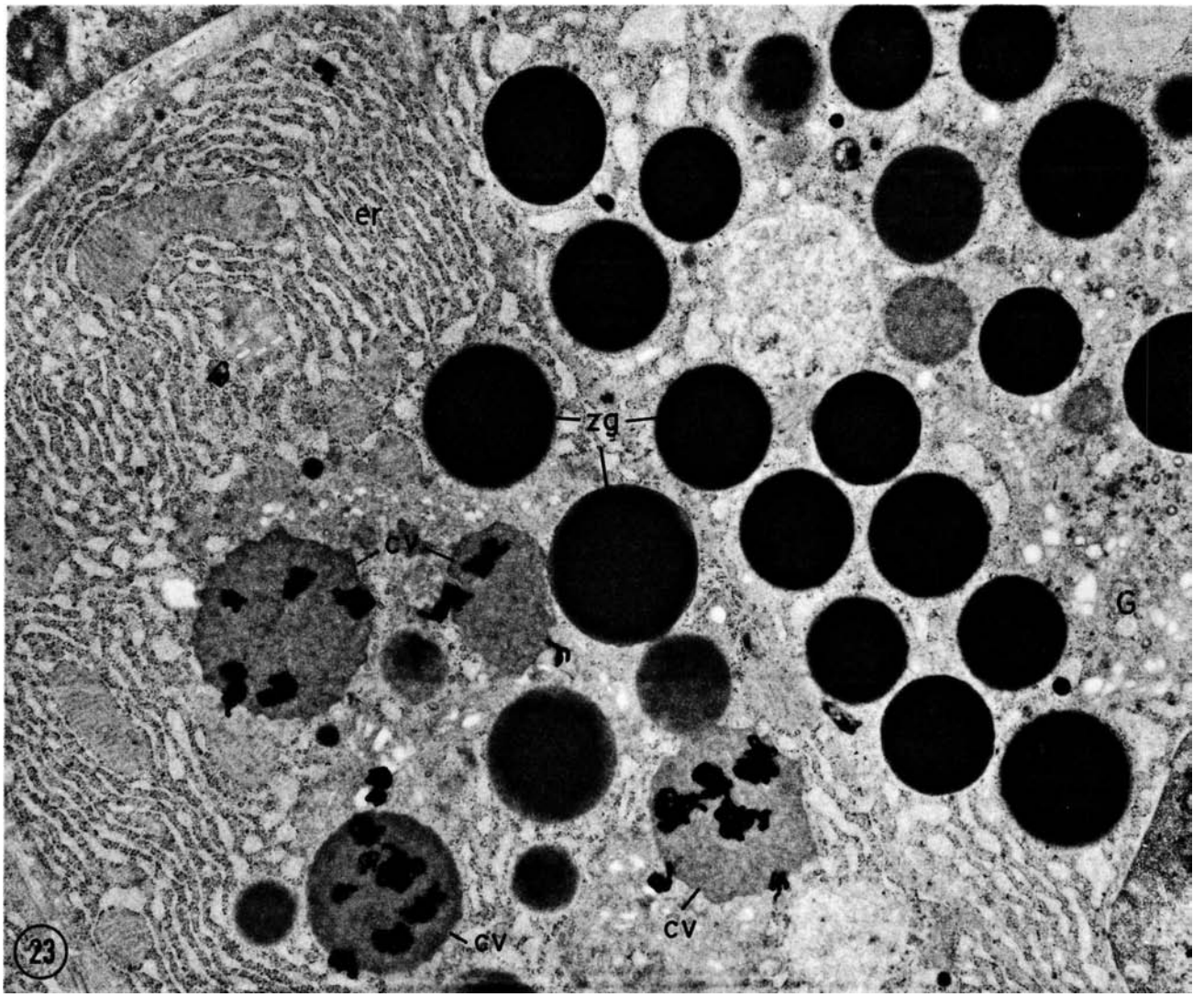


FIGURE 23 Autoradiogram of a pancreatic exocrine cell (guinea pig) pulse-labeled with [^{3}H]leucine in vitro and fixed at the end of a 20-min chase. Grains over condensing vacuoles (cv) are much more numerous than over the rough ER (er) or Golgi elements (G) at this time point. The mature zymogen granules (zg) are not labeled; their peak of radioactivity is reached later (60–80 min) postpulse. × 16,000. From Jamieson and Palade (54).

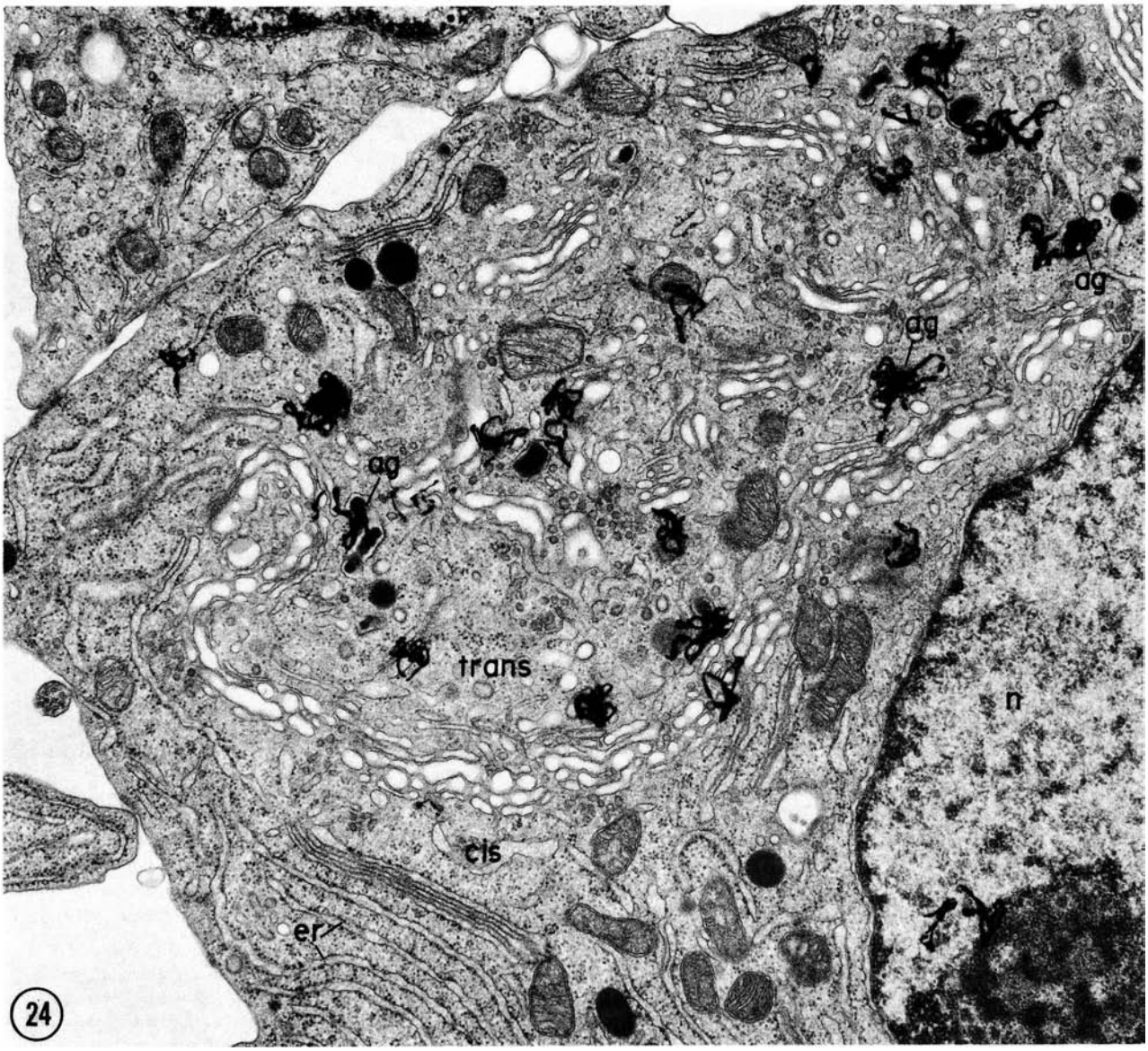
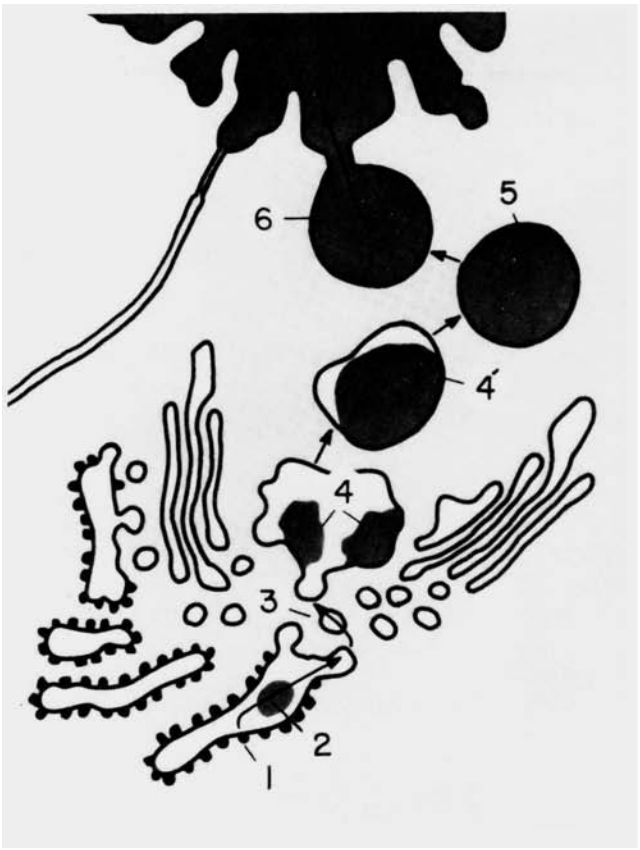


FIGURE 24 Autoradiogram of a prolactin cell (rat anterior pituitary) from a dissociated cell preparation pulsed in vitro for 5 min with [^{3}H]leucine and fixed after a 30-min chase. Grains are concentrated over immature or aggregating granules (ag) located on the trans side of Golgi stacks. When corrected for radiation spread, the grain density (grains/unit area) of the immature granules at peak labeling is 50–200 times that of the rough ER, indicating that the secretory product (> 85% prolactin) undergoes a ~ 200-fold concentration (56). × 24,000. From Farquhar et al. (55).

secretory products are transported to condensing vacuoles located on the trans side of the Golgi stacks (Fig. 23). As already mentioned, in most other cell types concentration normally takes place in the distended rims of the transmost cisternae (Fig. 24), which are the equivalent of condensing vacuoles. The same pattern was found in hyperstimulated pancreatic exocrine cells (58). Transport out of the ER to the Golgi was shown to be vectorial and energy-dependent, as it was arrested by inhibitors or uncouplers of oxidative phosphorylation (antimycin A, DNP). Subsequently it has become clear that, while in transit between the ER and forming granules, secretory proteins may undergo modifications such as glycosylation, sulfation, and proteolytic processing (described in subsequent sections), as well as concentration.

The general applicability of this model to a wide variety of cell types has been well documented and reviewed elsewhere (51, 52, 57). As far as Golgi involvement is concerned, the best studied cell types, are the parotid cell (52), the fibroblast (59 and Hay, this volume), the odontoblast (60), the β-cell of the pancreatic islets (61), the hepatocyte (42, 62), the thyroid cell (63), the mammotroph or prolactin cell of the anterior pituitary (64, 65) (Fig. 26), and leukocytes (53). There is no documented example of a cell in which the secretory product bypasses the Golgi. At one time it was suggested that collagen secretion by fibroblasts and immunoglobulin secretion by plasma cells might represent exceptions to the accepted scheme, and that in these cells at least part of the secretory product might be discharged directly from the ER, thus bypassing the Golgi. Autoradiographic studies carried out by several investigators (66, 67) were interpreted as supporting this contention. However, subsequent immunocytochemical results (Fig. 27 and Fig. 7 in Hay, this volume) have demonstrated the presence of the appropriate product (procollagen [59], and immunogloblins [68, 69]) in Golgi cisternae, thus confirming that in these cells



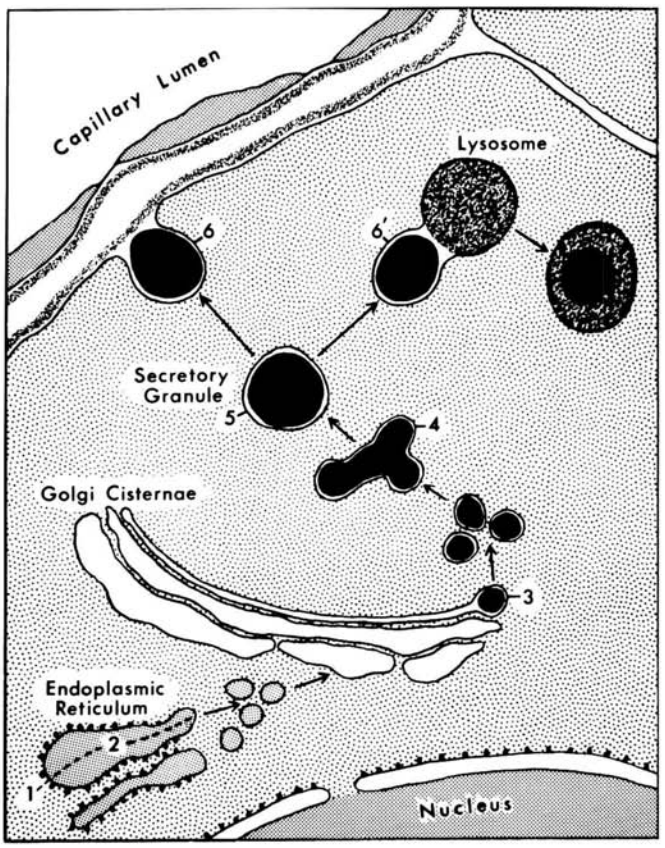


FIGURE 25 (left panel) Diagram of an exocrine pancreatic cell (guinea pig) showing the steps worked out by Jamieson and Palade for the synthesis and intracellular transport of digestive enzymes. The secretory proteins are (1) synthesized exclusively on polyribosomes which attach to the membranes of the rough ER, and are cotranslationally transferred across these membranes to be segregated (2) within the cisternal space of the rough ER. They are then transported (3) via small vesicles from the rough ER to condensing vacuoles located in the Golgi region where concentration (4) and (4′) takes place. The concentrated product is then stored (5) in secretion granules until discharged (6) by exocytosis, or fusion of the granule membrane with the plasmalemma at the apical cell surface.

FIGURE 26 (right panel) Diagram of events in the secretory process of the prolactin cell or mammotroph in the anterior pituitary of the rat from the work of Farquhar and co-workers. Prolactin is synthesized on attached ribosomes (1), segregated in the rough ER (2), transported to, and concentrated within granules in the Golgi complex. Small granules arising within the inner Golgi cisterna (3) aggregate (4) to form mature secretory granules (5). During active secretion, the latter fuse with the cell membrane (6) and are discharged into the perivascular spaces by exocytosis. When secretory activity is suppressed and the cell must dispose of excess stored hormone, some granules fuse with lysosomes (6′) and are degraded. This scheme is basically similar to that which takes place in the pancreatic exocrine cell (Fig. 25) except that (*a*) concentration begins in the stacked Golgi cisternae (instead of in specialized condensing vacuoles) and continues away from the complex in structures analogous to condensing vacuoles, and (*b*) there is a discharge option whereby the granules can be discharged either extracellularly (into perivascular spaces) or intracellularly into lysosomes by crinophagy. From Smith and Farquhar (154).

too, the secretory proteins follow the Golgi complex route. It is now clear that the earlier confusion and the inconclusive autoradiographic results can be explained by the fact that these cell types represent a special variant of the model in which the secretory products do not undergo concentration as a prerequisite for storage, and hence no secretion granules are formed. They are packaged in the Golgi complex in the usual manner and discharged by exocytosis in the usual manner, but the carrier consists of a fluid-filled vesicle instead of a dense granule (52, 69, 70).

The fact that concentration commonly takes place in the dilated ends of the Golgi cisternae raised the intriguing question of how concentration is brought about in the dilated ends of a continuous compartment. The first information on this problem came from the experiments of Jamieson and Palade (71), who showed that concentration in both condensing vacuoles and zymogen granules was maintained *in situ* in the absence of ATP synthesis. The findings led to the conclusion that concentration is *not* dependent on continuous expenditure of energy, as expected if the operation depended on an ion pumping mechanism. Instead, concentration apparently results from the formation of osmotically inactive aggregates, which is accomplished either by crystal formation (blood eosinophil [53] and pancreatic β-cell [52]), or by electrostatic interaction between secretory products and other molecules of opposite charge—especially protein-polysaccharide complexes: e.g., mast cell heparin with a basic polypeptide (72), cationic lysosomal enzymes (73) or cationic pancreatic proteins (74) with sulfated glycosaminoglycans (GAGs), prolactin with sulfated GAGs and glycopeptides (65). There is also evidence that calcium is present in certain secretion granules (i.e., those of pancreatic β-cells [75] and exocrine pancreatic cells [76]) where

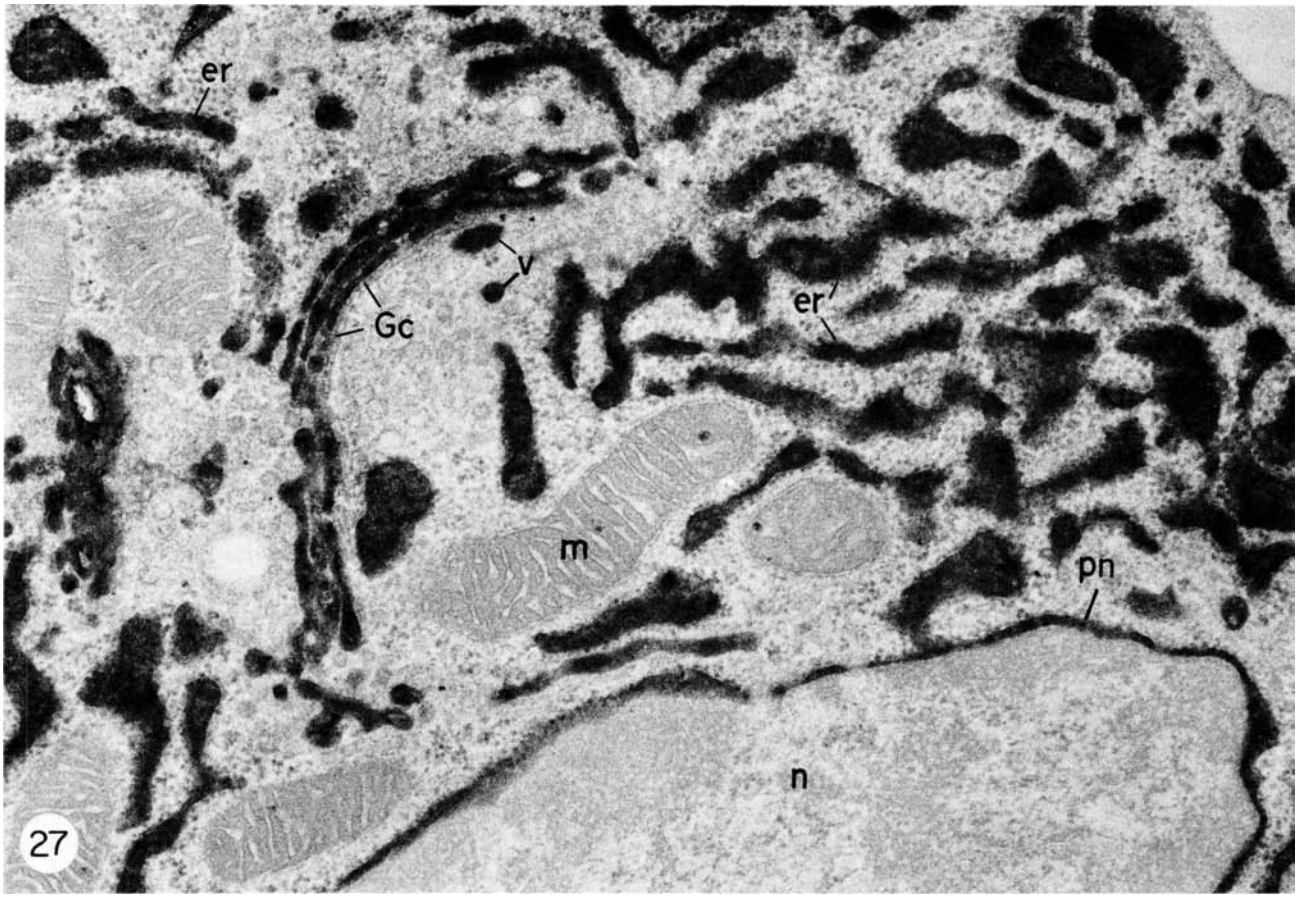


FIGURE 27 Immunocytochemical localization of immunoglobulins in the secretory compartments of a plasma cell from the spleen (rat). Spleen cells were harvested from a rat immunized against horseradish peroxidase (HRP), and lightly fixed; cryostat sections were incubated with HRP and subsequently reacted with diaminobenzine (DAB). Reaction product, indicating sites of localization of anti-HRP immunoglobulins, is seen throughout the rough ER (er), including the perinuclear cisterna (pn), and in the stacked Golgi cisternae (Gc) and associated secretory vesicles and vacuoles (v). × 22,000. From Ottosen et al. (69).

it is concentrated along the inner surface of their limiting membranes. This raises the possibility that calcium may participate in the ionic interactions that take place during concentration (76). In a few cases it has been shown that a constant ratio exists between the packaged products, e.g., in the adrenal medulla (ATP/catacholamines = 4/1) (77) and neurohypophysis (neurophysin/oxytocin or vasopressin) (78). In other cell types such as pancreatic acinar cells and prolactin cells of the anterior pituitary, the presumptive packaging molecules (sulfated polyanions) represent a relatively minor constituent of the contents and may serve to initiate aggregate formation (65, 74). Many secretory granules are insensitive to the osmolality of the medium even after isolation (58, 65), but are extremely sensitive to pH changes, presumably because the aggregates are stabile only at certain pHs.

Because one of the main functions of the Golgi complex in the packaging operation is to provide a membrane container that is competent for exocytosis of the secretory product, one would like to know the nature of this membrane and how its composition compares with that of membranes of other cell structures, especially those with which it interacts during intracellular transport. There are only a few cases in which the secretory granule membranes have been isolated in pure enough form (free from content proteins) to permit analysis of their protein composition. In such cases, e.g., the membranes of chromaffin granules, parotid granules, zymogen granules of the exocrine pancreas (reviewed in 79), it has been shown that the protein composition is different from, and generally simpler than that of the membranes of other cell compartments (ER, Golgi, plasmalemma).

In summary, it is clear that passage of secretory products through the Golgi complex is obligatory, and involves extensive modification and transfer to a membrane container which is competent to fuse with the plasmalemma at the time of exocytosis. It is in this Golgi-derived membrane container that concentration of secretory products is accomplished, but concentration is not an obligatory operation. When it does occur, which is in the majority of secretory cells, it often involves the complexing of secretory products leading to the formation of macromolecular aggregates which are insoluble under *in situ* conditions. Further details about the nature of the membrane containers and the factors that affect or control concentration mechanisms need to be obtained.

GLYCOSYLATION OF GLYCOPROTEINS: It is now clear that one of the major functions of the Golgi apparatus pertains to the posttranslational modification of glycoproteins. The apparatus is exclusively responsible for the attachment of terminal or capping sugars (*N*-acetylglucosamine, galactose, fucose and sialic acid) to the oligosaccharide chains that are *N*-glycosidically-linked to glycoproteins in the rough ER (63, 80, 81). Less is known about the site of addition of oligosaccharide chains O-glycosidically linked to serine, threonine, and tyrosine resi-

dues of mucin-type glycoproteins, but the biochemical information available (81) and the autoradiographic findings summarized below suggest that it also takes place in the Golgi complex.

Progress in understanding the biochemical events in glycoprotein synthesis and the intracellular localization of these sequential biosynthetic steps has been so rapid that one must pause and recall that the first evidence of a role for the Golgi apparatus in glycoprotein synthesis was obtained barely 15 years ago. That evidence was provided by the findings of Neutra and Leblond (80); they showed, by autoradiography in animals sacrificed 5–15 min after administration in vivo of radiolabeled hexose ([^{3}H]glucose and [^{3}H]galactose), that the vast majority of the autoradiographic grains were localized over the Golgi region of intestinal goblet cells and many other cell types (80). The grains were localized directly over Golgi cisternae by electron-microscope (EM) autoradiography. A few years later, using a similar LM and EM autoradiographic approach to study glycoprotein synthesis in the thyroid epithelial cell, Leblond and co-workers (63, 82, 83) demonstrated that the addition of core sugars ([^{3}H]mannose) to the peptide backbone of thyroglobulin takes place in the rough ER, whereas the addition of terminal sugars (galactose, fucose, and, more recently, sialic acid [63]) takes place in the Golgi apparatus. Thus, autoradiography proved to be very useful for identifying the initial cellular site of incorporation of various monosaccharide precursors. As used by Leblond and his associates, it has not only provided the first indication of the role of the Golgi complex in glycoprotein synthesis, but also has yielded the first evidence for intracellular separation of labor—between the rough ER and the Golgi complex—in the proximal and distal glycosylation of complex glycoproteins.

The localization of hexose incorporation to the Golgi complex by autoradiography took place well before the discovery that the glycosyltransferases responsible for the addition of terminal hexoses are associated exclusively within Golgi fractions. The next key event in the development of the glycoprotein story was the discovery in 1969, by B. Fleischer, S. Fleischer, and H. Ozawa (34), that a galactosyltransferase activity with the ability to transfer radioactive galactose to exogenous receptors (from UDP-gal to *N*-acetylglucosamine) was concentrated (80×) in Golgi fractions from bovine liver. Subsequent studies by the Fleischers and others, notably Morré (36), and Schachter and Roseman and their co-workers (37, 38), confirmed the presence of galactosyltransferase activity in Golgi fractions. This provided the first biochemical evidence for the involvement of Golgi membranes in the addition of terminal hexose residues to glycoproteins. Subsequently, fucosyl and sialyl transferases were also shown to be characteristic Golgi enzymes (84), but to this day, galactosyltransferase remains the main marker enzyme for the Golgi complex. Recently, B. Fleischer (85) has established that both galactosyltransferases and sialyltransferases are membrane proteins with active sites located on the luminal side of the Golgi cisternae.

It should be noted that, although galactosyltransferase activities are found inside most cells bound to Golgi membranes, they also occur in soluble form (84) (e.g., in milk, serum, and epididymal [86] fluids), and in milk globule membranes (usually assumed to be derivatives of the plasmalemma of the mammary epithelium [87]).

As information increased about the existence of different types of oligosaccharide chains in glycoproteins and the steps involved in their biosynthesis, it became apparent that many secretory and membrane proteins contain *N*-glycosidically-linked, complex-type oligosaccharides which are first synthesized (from dolichol intermediates [88]) in the ER as mannose-rich precursors with extra glucose and mannose residues. These residues are subsequently trimmed, with removal of all of the glucose and some (six) of the mannose residues, before addition of the terminal hexoses (89). The trimming of mannosyl residues was localized indirectly to the Golgi apparatus by the discovery in Golgi fractions of an α-D-mannosidase activity, which is capable of processing asparagine-linked oligosaccharides and is distinct from the mannosidases of the cytosol and lysosomes (89, 90).

Recently, Kornfeld and his associates (91, 92) have delineated a role for the Golgi complex in the trimming and glycosylation of lysosomal enzymes. They found that the biosynthesis of lysosomal enzymes involves the transfer of an *N*-acetylglucosamine phosphate to mannose residues of the enzymes. These glucosamine residues are then removed to expose the mannose-6-phosphate, which is believed to be the recognition marker for lysosomal enzymes (see Bainton, this volume). Kornfeld's group has shown also that both the transferase activity (*N*-acetylglucosamine 1-phosphotransferase) and the trimming enzyme (α-*N*-acetyl glucosaminyl phosphodiesterase) are concentrated in Golgi fractions.[1]

An important but still unresolved question is where, in the heterogeneous Golgi complex, do glycosylation and trimming take place? The question has not yet been answered because the transferases were found to be equally distributed in Golgi subfractions (93). However, that there may be a restricted or specialized distribution is suggested by the results obtained by a new affinity separation technique, which showed that galactosyltransferase and NADPH-cytochrome P-450 reductase are associated with different, morphologically recognizable Golgi elements (43).

Two other important questions are the subject of current research by B. Fleischer and her associates: How are the nucleotide sugars that serve as substrates for the transferases (and which are synthesized elsewhere in the cell) transported across the Golgi membranes? And how are the products of the transferase reaction (UDP and CMP) removed? Regarding the latter, Brandon and Fleischer (94) have shown that UDP formed in intact Golgi vesicles during galactosylation is rapidly broken down by nucleoside diphosphatases (NDPases) present in the lumen of Golgi vesicles. It is tempting to suggest that the neutral NDPase activity, as well as the acid phosphatase activity (which can be demonstrated using a variety of substrates including CMP) found by cytochemical localization in certain Golgi membranes, may be involved in these operations. To address the first question, Fleischer (95) recently has studied the nucleotide profile of rat liver Golgi by high-pressure liquid chromatography and found major peaks associated with several nucleotides: UDP, AMP, UMP, and CMP. The fact that there is a selective distribution of nucleotides, together with the finding that UDP is selectively retained after osmotic shock, led Fleischer to suggest (96) that the Golgi is not freely permeable to these molecules, and that a selective transport system or binding protein exists for the uptake or exclusion of specific nucleotides from this organelle.

GLYCOSYLATION OF GLYCOLIPDS: There is also evidence (97, 98) that, in addition to glycosylation of glycoproteins, the Golgi apparatus is involved in glycosylation of at least some glycolipids, especially those that contain terminal galactose and

[1] Kornfeld, S. Personal communication.

sialic acid residues, i.e., cerebrosides and gangliosides. As far as is known, the glycolipids are present in tissues exclusively as membrane constituents, but their concentration differs from one tissue to another (i.e., high in brain and kidney and low in liver). B. Fleischer (95) has shown that a number of glycosyltransferases (as well as a sulfotransferase) (98) which function in the addition of hexose residues to glycolipids are localized in Golgi fractions isolated from kidney homogenates, and Richardson et al. (97) have found the same in Golgi fractions from rat liver.

SULFATION: As in the case of glycosylation, the first indication that the Golgi complex functions in sulfation was obtained by autoradiography. In 1964, Lane et al. (99) and Godman and Lane (100), working with L. Caro, who, with R. van Tuburgen, had just introduced techniques for EM autoradiography a few years before, demonstrated that immediately after administration of radioactive sulfate in vivo, exposed grains were concentrated over Golgi cisternae and vacuoles in goblet cells and cartilage cells. Much later, Young (101) surveyed a variety of cells and found uptake of radioactive sulfate by the Golgi complex in 14 additional cell types, e.g., leukocytes, Schwann cells, endothelial cells, keratinocytes, fibroblasts, and follicular cells of the ovary. The uptake of sulfate by cartilage and goblet cells was to be expected, because these cells are known to produce high levels of sulfated proteoglycans (chondroitin sulfate) and sulfated glycoproteins (mucins), respectively. More surprising at the time was the finding of sulfate incorporation into the other cell types mentioned. Since then, however, it has become clear that many cells (leukocytes, endothelial cells, fibroblasts, and ovarian cells) synthesize sulfated proteoglycans which can either be deposited in the extracellular matrix, retained intracellularly (e.g., in secretion granules), or remain associated with the cell surface. It has also become clear that many other classes of molecules, such as glycolipids (98), glycoproteins (99), and steroid hormones can be sulfated (101). Apparently all these reactions occur in the Golgi, for in all cases initial incorporation has been localized to this organelle by autoradiography. However, retention of sulfated steroids in such experiments remains to be proven.

In sulfation, as in glycosylation, the sulfate is activated by binding to a nucleotide from which it is transferred to an appropriate receptor molecule by a specific sulfotransferase (98, 101). Less is known about the location of the enzymes involved in sulfation than about that of glycosyltransferases, but the information available indicates that the a number of sulfotransferases are Golgi-associated enzymes (98, 102–104). The first sulfotransferase to be localized in Golgi fractions and to be solubilized and characterized (103), is a cerebroside sulfotransferase, present in rat kidney, which converts cerebroside to sulfatide (a sulfated glycosphingolipid). This enzyme, like the glycosyltransferases, appears to be an intrinsic membrane protein (103). Sulfotransferase activity has also been localized in Golgi-enriched fractions from liver (102) and mast cells (104), but the enzymes involved have not yet been characterized. Evidence has been presented that the mast cell enzyme is involved in proteoglycan synthesis (104).

In summary, autoradiographic findings and information obtained on Golgi fractions indicate that sulfation, like terminal glycosylation, is exclusively a Golgi function, but supporting biochemical data derived from cell fractionation are still quite limited.

PROTEOLYTIC PROCESSING OF PROPROTEINS: Over the past few years it has become evident that most secretory and membrane proteins undergo one or more intracellular proteolytic processing steps during biosynthesis. Examples are the cleavage of presecretory and prosecretory proteins, and cleavages that occur during the assembly of macromolecular structures such as virus capsids and membrane associated enzyme complexes (see Steiner et al. [105] for a recent review). The processing event that usually occurs in the Golgi complex involves the conversion of proproteins to secretory proteins. Many small peptide hormones (proinsulin, proparathormone, proopicocortin) as well as other secretory proteins (proalbumin) undergo processing of this type to yield their mature discharged form. The association between proprotein processing and the Golgi complex was made initially by Steiner and his collaborators (106) shortly after the discovery of proinsulin, and was based on the finding that when intracellular transport from the ER to the Golgi complex was stopped (by treatment with inhibitors of ATP synthesis such as antimycin A), no processing of proinsulin to insulin occurred. This finding demonstrated that transport out of the ER to the Golgi area was necessary for the processing of proinsulin to occur. The kinetics of the processing, which revealed an initial delay of 10–20 min followed by continued activity for up to 1 h, supported that conclusion. Similar findings were also obtained for the conversion of proparathormone to parathormone (107). The fact that conversion continued for up to 1 h, whereas in most systems, transport to the Golgi is assumed to be virtually completed by 30 min, suggested that processing might continue in secretion granules after packaging (105). Findings by Gainer and his associates (78) on the kinetics of processing of propressophysin (the common precursor of neurophysin and vasopressin) in neurosecretory neurons indicated that this is the case. In these cells, the precursor is packaged in the usual manner into neurosecretory granules in the Golgi complexes of the neuronal cell bodies, which are located in the supraoptic nuclei of the hypothalamus. After packaging, the granules migrate (by axonal flow) down the axons in the pituitary stalk to reach the posterior lobe of the pituitary, where storage takes place. When the products obtained from the hypothalamus, the stalk, and the posterior lobe were compared, it became evident that processing was more complete in the stalk and posterior lobe than in the hypothalamus. Gainer et al. concluded (78) that progressive processing takes place in the granules while they are in transit down the stalk. By implication, the granules must contain the enzyme(s) involved in processing.

This brings us to a consideration of what is known concerning the enzymes involved in proteolytic processing within the Golgi complex and/or secretion granules. Work from Steiner's laboratory (105) demonstrated that conversion of proinsulin to insulin can be accomplished in vitro by the combined action of an endopeptidase (pancreatic cationic trypsin) and an exopeptidase (carboxypeptidase B). Habener and associates (107) found the same situation to apply to the processing of proparathormone to parathormone. However, the nature of the endogenous activity that accomplishes the conversion is still problematical. Over the years, there have been claims that the zymogen forms of trypsin and chymotrypsin, cathepsins, kalikreins, or plasminogen activator (among others) are the proprotein processing enzymes (105). According to Steiner (personal communication), all of these alternatives have been challenged, and the actual identity of the proteolytic activity remains an open question. It does appear that there is a fundamentally similar processing enzyme for all proproteins, for they all contain paired basic residues at the sites the enzyme recognizes for cleavage; however, Golgi proteases have not

been purified and characterized, and their precise intra-Golgi location is entirely unknown.

In short, the available evidence indicates that the proteolytic processing of proproteins is a post-ER step which requires transport to the Golgi complex and continues after the secretory product is packaged into granules. The precise nature of the processing enzyme(s) is unknown, as it has not yet been isolated and characterized. Indirect evidence suggests that it may be acquired at the time of formation of the secretion granules. Its mode of delivery to the granules—whether it is acquired with the Golgi membrane during packaging or by membrane fusion after packaging—is also unknown.

LIPOPROTEIN PACKAGING: It has been assumed that the Golgi complex plays a role in lipid metabolism since the electron microscope studies in the 1950s of Palay and Karlin describing the presence of lipid droplets in the Golgi cisternae of intestinal absorptive cells (108). Observations were soon extended to physiologically defined conditions in an attempt to correlate the presence of lipid droplets within the Golgi complex either to lipid absorption in the intestinal epithelium (109) or to lipoprotein secretion in hepatocytes (110–112). Moreover, lipoprotein particles were isolated from Golgi fractions and found to contain particles comparable to serum VLDL (111, 112). More recent work on this topic has been extensively reviewed (113).

At present it is assumed that the ER is the site of synthesis of both the apoproteins and lipids (triacylglycerols, cholesteryl esters, and phospholipids) of hepatic lipoproteins. The assembly of these different components is thought to take place in the cisternal space of the ER as suggested by the appearance of osmiophilic (lipid) droplets of appropriate dimensions in that space, especially within the smooth ER (110). The pathway taken thereafter is the same as for other secretory products, that is, ER → Golgi cisternae → condensing secretory vacuoles, which are discharged by exocytosis at either the vascular (hepatocyte) or lateral (enterocyte) front of the cell. Thus far, the only functions established for the Golgi complex in lipoprotein secretion are terminal glycosylation of the appropriate apoproteins, all of which are glycoproteins (113), and packaging. Evidence obtained over the last few years indicates that the hepatocytes produce only VLDL and HDL; however, recent work by Howell and Palade (114) on lipoprotein particles isolated from hepatic Golgi fractions revealed extensive heterogeneity in particle size and biochemical composition. These findings suggest that most Golgi lipoprotein particles are immature products that require extensive modification in their lipid composition before release by exocytosis as either VLDL or HDL.

Traffic Through the Golgi Complex

At present it is clear that there is extensive traffic from more than one direction into and through the Golgi complex. This traffic is connected with membrane biogenesis, discharge of secretory proteins, membrane recycling, and uptake (interiorization) of informational molecules. In this section we will review the available evidence on the nature and direction of that traffic, as well as the ways in which the evidence was obtained.

TRAFFIC OF SECRETORY PRODUCTS: The general route taken by secretory proteins through the cell—from rough ER → transitional elements at the periphery of the Golgi complex → condensing vacuoles → secretion granules → discharge by exocytosis—was established as a result of the work on the exocrine pancreatic cell by Palade and his associates, primarily Jamieson and Palade. Still uncertain, however, is the route taken by secretory products through the Golgi complex itself as they move from the transitional elements of the rough ER to condensing granules or vacuoles (reviewed in 115).

For more than 20 years, the prevailing idea has been that secretory products move sequentially across the Golgi stack from the cis to the trans side, traverse the cisternae one by one, and undergo packaging on the trans face (see references 15, 38, and 63). The Golgi cisternae were thought to be formed on the cis face and used up in packaging on the trans face. The origin of this concept can be traced to Grassé, who in 1957, based on EM findings, proposed that the continuous formation of peripheral (cis) Golgi saccules (cisternae) balances the conversion of central (trans) saccules into secretion granules (116). Inherent in this formulation was the idea that membrane and contents move in synchrony from one side to the other of the stack, the products remaining in the same cisterna throughout the process. Subsequent morphologic, autoradiographic, and cell fractionation data were, for the most part, interpreted as supporting this scheme. In this section evidence that pertains directly to the pathway taken by secretory products is considered.

In their early autoradiographic studies, which involved the use of [^{3}H]hexose labeling, Neutra and Leblond (80) found grains associated at early time points with Golgi cisternae and at later time points with mucous granules of intestinal goblet cells. They interpreted these findings as support for the cis-to-trans flow diagram, and they and others estimated a turnover time for a cisterna of ~2 min. Jamieson and Palade (54), using autoradiography to investigate the route taken by secretory proteins in the pancreas, found no evidence for the direct involvement of the stacked cisternae in transport, as indicated by the absence or low density of grains over the Golgi stacks after a pulse-chase experiment with [^{3}H]leucine. However, they and others subsequently found autoradiographic grains over Golgi cisternae in other cell types, i.e., parotid cells (117) and pituitary prolactin cells (55), as well as in hyperstimulated pancreatic acinar cells (58). In none of these autoradiographic studies was the route and direction of movement of label *within* the stacks studied in detail.

These morphological findings, primarily the autoradiographic data of Neutra and Leblond, are the basis for the widespread belief that secretory products enter the Golgi at the cis side and emerge on the trans side. Indeed, this traffic pattern is implied in the naming of the two faces of the Golgi: i.e., the entry or immature face vs. the exit or mature face. Other work, e.g., the study of Bergeron et al. (42), on Golgi subfractions, in which it was shown that [^{3}H]leucine-labeled secretory proteins peak first in heavy Golgi fractions (believed to be derived primarily from cis Golgi cisternae) and a few minutes later in light Golgi fractions (believed to consist largely of secretory vacuoles from the trans side), was in keeping with this view. Moreover, EM studies on the assembly of scales in certain algae (118) supported the view that individual cisternae, in which scales are progressively assembled, move in the trans direction across Golgi stacks, while new scale components are added at each 'station.' As a result, the concept of cis-to-trans flow across the Golgi stacks became almost a dogma, and was the framework in which most investigators interpreted their findings without questioning the validity of the 'dogma.'

Studies in which secretory products have been localized within Golgi cisternae by cytochemical procedures also have contributed information concerning traffic through the Golgi

complex. In the first of these studies, endogenous peroxidases were localized in eosinophils (119) and parotid cells (120), and were found to be present in all the cisternae in the Golgi stacks (Fig. 28). Similar but less striking findings have also been obtained when secretory products were localized by immunocytochemistry in other cell types, i.e., pancreatic enzymes in pancreatic exocrine cells (121), procollagen in fibroblasts (59; see Fig. 7 in Hay, this volume), and IgG in plasma cells (68, 69; Fig. 27). These observations suggested that all the stacked cisternae are involved in the transport and processing of secretory products, and hence, were compatible with the cis-to-trans flow diagram. However, they did not give any *direct* information on the route followed.

Thus, the evidence available is compatible with the assumption that all Golgi cisternae are involved in the transport and/or processing of secretory products. The data are also compatible with the view that secretory products move sequentially across the Golgi in the direction cis-to-trans, but with the evidence at hand, other possibilities cannot be ruled out (115).

SEGREGATION OF MULTIPLE SECRETORY PRODUCTS: Because the Golgi complex is responsible for concentration of secretory products, the question arises, how does the organelle handle the processing and packaging of multiple secretory products? In many if not most cases (e.g., the exocrine pancreas), the problem is resolved by avoiding segregation, and the Golgi complex packages the secretory proteins as a mixed cocktail in the same container (121). In one case, that of the PMN leukocyte which has two granule populations of different composition (one lysosomal, one not) (53), the problem is solved by making the two sets of granules in two waves of protein synthesis, which are well separated in time in the maturation process of the cells; one set is completed before the other is started. Interestingly enough, opposite sides of the Golgi complex are used for the packaging of the two sets of products (122).

LYSOSOMAL ENZYMES: The major problem in segregation faced by all secretory cells concerns the handling of lysosomal enzymes and their separation from secretory proteins. There is now large body of circumstantial evidence (see Bainton, this volume) which supports the hypothesis that mannosyl phosphate residues (mannose-6-phosphate) on lysosomal enzymes constitute a special recognition marker that serves to direct lysosomal enzymes to lysosomes. There is also evidence that coated vesicles are involved in the transport of lysosomal

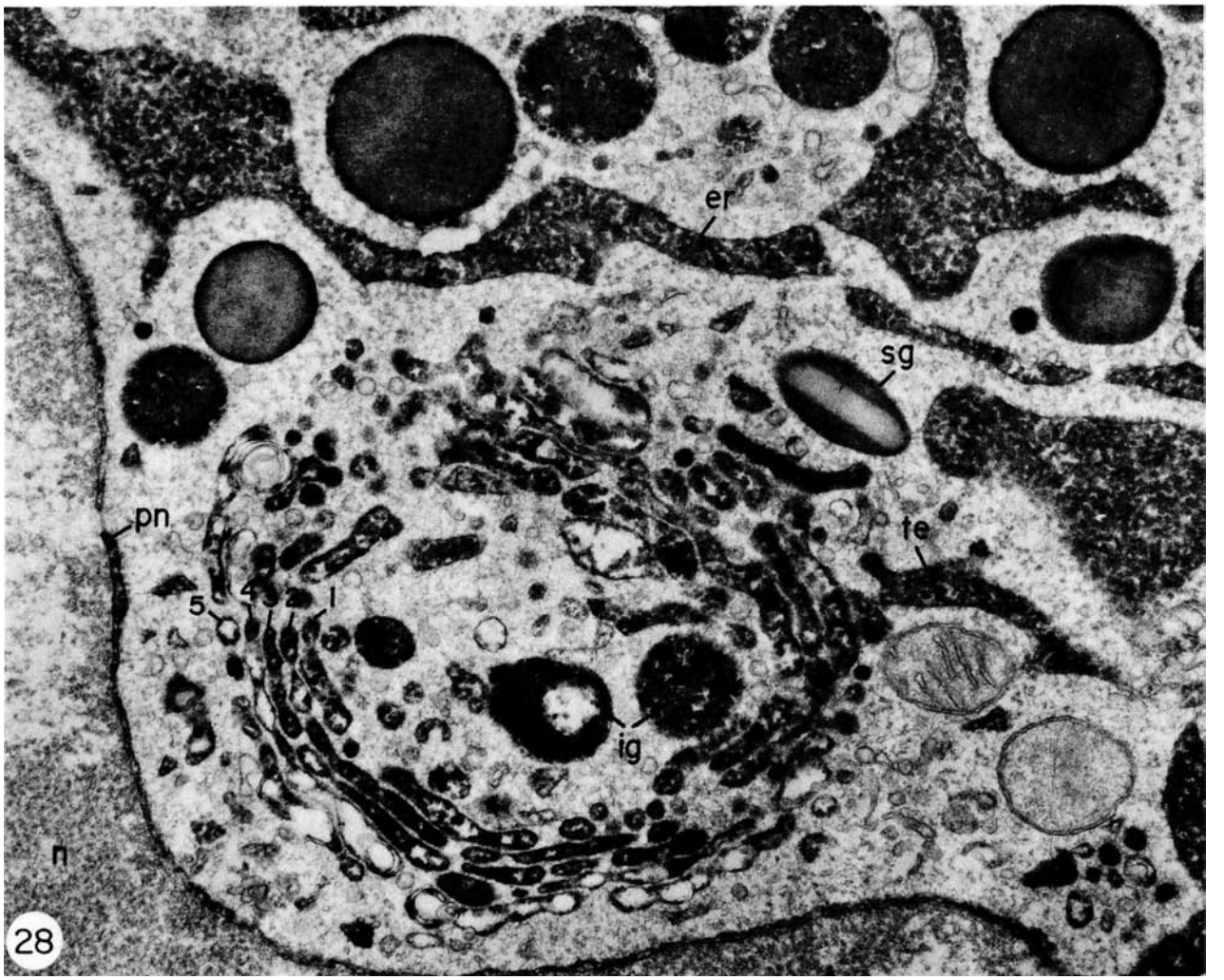


FIGURE 28 Developing eosinophilic leukocyte from rat bone marrow (myelocyte stage) incubated for endogenous peroxidase activity. During this stage, peroxidase is synthesized and packaged into eosinophil secretion granules. Here the peroxidase reaction product is seen throughout all the secretory compartments—rough ER (er), transitional elements of the ER (te), Golgi cisternae (1–5) and associated vesicles, and immature granules (ig). Note that all five cisternae in the Golgi stack are reactive. sg = mature secretion granule with crystalline inclusion. × 50,000. From Bainton and Farquhar (119).

enzymes from the Golgi complex to lysosomes (18, 18a). However, it is not known where in the Golgi complex the sorting of lysosomal enzymes and secretory proteins takes place.

In 1964, Novikoff and co-workers presented evidence (reviewed earlier) that in nerve cells acid phosphatase (AcPase) was present in a special ER cisterna, which Novikoff called GERL. Based on this finding, he proposed that lysosomal enzymes were synthesized in the associated ER and packaged in GERL, thus bypassing the stacked Golgi cisternae. Later, to explain the presence of AcPase and secretory granules in the same cisterna (see Fig. 15), he expanded the GERL concept to include condensing granules and condensing vacuoles (11, 19). Inherent in the earlier formulation was the assumption that lysosomal enzymes and secretory products remained separated from one another; however in the revised concept, the idea is that lysosomal enzymes and secretory products are segregated together and sorted out within the same (GERL) cisterna or condensing vacuole by an unknown mechanism. The revised concept also assumes that both secretory products and lysosomal enzymes move from the rough ER to the GERL, *without* passing through the rest of the Golgi complex, an assumption that does not fit with the autoradiographic findings (referred to earlier) in which exposed grains were located over the stacked Golgi cisternae in several secretory cell types.

At present there is no clear understanding of the GERL concept; some investigators consider GERL as a separate entity, distinct from Golgi cisternae, whereas others consider it part—often the granule forming part—of the Golgi complex. Several of the reasons for this situation were discussed in the preceding paragraph; another reason is the variability in the morphological properties, cytochemical staining, and detectable functions of the cisternae on the trans side of the Golgi stack. In many cells concentration of secretory products can be seen not only in cisternae that correspond morphologically to Novikoff's description of GERL (rigid cisternae, often separated from the stack, limited by thick membranes), but also in one or more of the transmost cisternae in the regular Golgi stack of the same cell type (See Figs. 1–4, 13, 15). Another problem is that sometimes AcPase is found in the transmost cisterna of the Golgi stack, rather than exclusively in structures which correspond morphologically to GERL (122a). Also, since multiple AcPases have been found in several cell types (see 115) AcPase may not be exclusively a lysosomal marker enzyme.

In summary, in view of the absence of criteria other than morphological ones for distinguishing GERL from Golgi cisternae, the frequent overlap between their cytochemical straining properties and functions, and the absence of supporting data for the assumption that traffic from the ER is directed to GERL bypassing the Golgi complex, there seems to be little justification for setting GERL aside as a distinct entity from the rest of the Golgi complex. It is clear that a major function of the Golgi complex is to sort secretory and certain membrane (see below) proteins and to direct them to their correct intracellular and extracellular distinations. It is also clear that the trans side of the Golgi complex is where a great deal of the biosynthetic and other traffic converges. Certainly, however, much more work is needed in order to disentangle the traffic lanes, to identify the site of sorting of lysosomal enzymes and secretory products, and to understand "fractionation" mechanisms in the Golgi complex.

MEMBRANE BIOGENESIS: The earliest concept of biogenesis of membrane constituents, proposed in the late 1950s, was that the traffic of cell membranes was accomplished by the movement in concert of the membrane and content (secretory) proteins from the rough ER (or nuclear envelope) → cis Golgi face → trans Golgi face → plasmalemma, with delivery occurring by exocytosis upon fusion of the granule membranes with the plasmalemma. What was envisaged was a movement of membranes in bulk. The term 'membrane flow' was introduced in 1956 by Bennett (123); he placed the main emphasis on what was then the new concept of incoming vesicle flow (pinocytosis), but he also clearly envisaged an outbound flow, at least from the nuclear envelope to the rough ER. Bennett did not consider the Golgi complex in his formulation, for its existence was still questioned at the time. The membrane flow concept lay dormant until 1971, when it was introduced again by Franke, Morré, and their co-workers (124) to designate the physical transfer of membrane from one compartment to another in sequential fashion. The concept was based on radiolabeling experiments in which they found sequential labeling of ER, Golgi, and plasmalemma. Subsequently, Morré and his associates (13, 15) developed this concept further and coupled membrane flow and membrane differentiation to account for the origin of the complex system of internal membranes or 'endomembranes' in eukaryotic cells. Inherent in this concept was the idea that membrane flow is *undirectional* and *coupled* to the flow of secretory products. The route taken through the Golgi was also addressed; and in the case of the hepatocyte three pathways were envisaged and depicted in diagrammatic form: (1) New membrane was believed to arise as primary vesicles which, as in the case of the pancreatic secretory model, pinch off transitional elements and fuse with Golgi cisternae on the cis side of the complex, after which they were assumed to undergo progressive transformation across the stack to be used in packaging at the forming face and eventually reach the plasmalemma. The secretory products (lipoproteins in this case) were believed to be transported either (2) by direct connections at the periphery of the Golgi, called the 'boulevard periphérique,' or (3) by a direct pathway from the smooth ER to the cell surface thus bypassing the Golgi. The last alternative can be seriously questioned and essentially ruled out because, as stated before, there is no evidence supporting direct discharge of secretory products from the ER, in general, and for lipoprotein particles, in particular. In the latter case, obligatory passage through the Golgi is expected by most workers, because the apolipoproteins are glycoproteins (113). The second pathway—i.e., the existence of direct connections between ER and Golgi—has also been questioned on the grounds that because such connections are visualized primarily in unfixed, negatively stained preparations, are not seen with comparable frequency in fixed, negatively stained preparations, and are rarely seen in thin sections (see, however, Claude [125]), they may arise from artifactual fusions that occur during negative staining procedures. The first pathway, as already stated, was generally accepted among the proponents of the membrane flow theory.

In the last few years a number of new developments have forced a reconsideration of this concept which, as originally proposed (15) and usually understood (38, 63, 87), oversimplifies intracellular conditions. (*a*) First, it is now clear (see reference 79) that membrane proteins turn over at much slower rates than the transit time of secretory proteins; hence, concurrent synthesis of membrane and content is no longer tenable. (*b*) Additional findings (summarized below), difficult to reconcile with the membrane flow diagrams, concern the extensive, membrane reutilization or membrane recycling that takes

place in most cells—i.e., recycling of membranes of secretory granules, synaptic vesicles, and transitional and endocytic vesicles (see below). Therefore, the traffic of recycling membrane must be considered together with the biogenetic traffic. (*c*) It has also been established that although some membrane proteins, especially glycoproteins, follow the postulated membrane flow route, others, namely peripheral membrane proteins and their viral equivalents (such as the 'M' protein of the vesicular stomatitis virus [VSV]), are inserted directly from the cytoplasmic matrix without the involvement of either the ER or the Golgi complex (126). (*d*) Furthermore, the existence of cytochemical specialization among Golgi cisternae and within the same cisterna is difficult to reconcile with the concept of progressive transformation across the stack. (*e*) Finally, the realization that membranes on different domains of the cell surface differ in their composition and that secretory granules fuse preferentially with one domain (usually the apical domain in exocrine cells), leads to the conclusion that secretory granule membranes cannot serve as vehicles for all the domains of the plasmalemma.

Current Information on Involvement of Golgi in Biogenesis of Membrane Components

At present it is assumed that the Golgi complex is involved in the biogenesis of at least some membrane components, primarily intrinsic transmembrane proteins, which are found not only in the plasmalemma but also in other membranes of the secretory (and perhaps the endocytic) pathway. The rationale is that all transmembrane proteins studied so far have oligosaccharide chains of the type that is produced or completed in the Golgi complex. Examples of terminal glycosylation occurring at the cell surface are limited (84), and their general applicability is questioned. Hence, transit through the Golgi is expected to effect terminal glycosylation. A similar situation applies to glycosylation and sulfation in the case of membrane glycolipids (96–98).

The information available is compatible with the involvement of the Golgi complex in biosynthesis of membrane glycoproteins, but the data are still limited. Leblond and coworkers (63, 127) have obtained autoradiographic evidence that in intestinal epithelial cells (considered nonsecretory), labeled fucose and sialic aid precursors (*N*-acetylmannosamine) are first incorporated into macromolecules in stacked Golgi cisternae. Later, the cognate autoradiographic grains are found over small vesicles, and later still, over the plasmalemma. These findings have been interpreted as indicating that plasmalemmal components (in this case, mainly fucosylated and sialated glycoproteins) are modified (terminally glycosylated) in the Golgi and then ferried to the cell surface via small vesicles. The data are certainly compatible with this interpretation, but due to the limitations of resolution of the autoradiographic method, transport by alternate routes cannot be ruled out. Moreover, although it is likely, in view of the biochemical data (128), that the labeled species are membrane proteins, their identity was not established.

The involvement of Golgi cisternae in plasma membrane biogenesis is also suggested by the demonstration of adenylate cyclase (21) and insulin receptors (129) in Golgi fractions and of acetylcholine receptors in the Golgi apparatus of chick skeletal muscle cells in culture (130). In the work on insulin receptors and adenylate cyclase, no distinction could be made between recycling and biogenetic traffic to explain the presence of these proteins in the Golgi complex, for there was no way of knowing whether they appear first in the plasma membrane or the Golgi complex. In the case of chick muscle, suggestive evidence was obtained for the appearance of at least some (~10%) of the acetylcholine receptors in the Golgi complex prior to their insertion into the plasma membrane, but interpretation of the data was complicated by the presence of a large number of receptors in structures other than the Golgi complex.

The best and only direct evidence on the involvement of the Golgi complex in biogenesis of membrane proteins comes from work on the viral envelope spike or 'G' protein of VSV. Biosynthetic studies have established that this glycoprotein is synthesized on membrane-bound polyribosomes and partially glycosylated in the ER, and then further glycosylated as it is transported within a smooth membrane fraction before it is delivered to the plasma membrane (126), apparently via coated vesicles (131).

Indirect evidence for glycosylation of the VSV-'G' protein by Golgi membranes was recently brought forward by Rothman and Fries (132) in experiments in vitro in which they mixed a crude extract obtained from a CHO cell mutant incapable of carrying out terminal glycosylation with either a crude membrane fraction from wild-type CHO cells or a Golgi fraction prepared from rat liver. In this heterogeneous, reconstituted system, they obtained terminal glycosylation of the G protein. More direct evidence for the participation of the Golgi in the biogenesis of the G protein was obtained recently by Bergman, Tokuyasu, and Singer (133). They used immunochemical procedures to demonstrate the sequential appearance of the G protein in ER, Golgi, and plasmalemma, and took advantage of the availability of a temperature-sensitive mutant and a shift-down step to synchronize the release of the protein from the ER. The authors also observed that initially (before 11 min) only cis cisternae were labeled, but within 11 min after the temperature shift, labeling was seen throughout the Golgi complex. These findings represent the first *direct* evidence for the involvement of the Golgi complex in the biogenesis of a membrane protein. The authors plan to use this approach to study the route taken through the Golgi complex and to the plasmalemma.

The existing evidence is compatible with the view that transmembrane proteins are transported to their site of final function already assembled in a membrane carrier. The assumption is in keeping with the asymmetry of their assembly and with the progressive glycosylation of their cisternal domains, which would make other ways of transport highly improbable thermodynamically. Retention of membrane specificity implies that the traffic of these carriers is regulated so as to allow each one to arrive (and be accepted) at the appropriate destination.

MEMBRANE RETRIEVAL AND RECYCLING OF GRANULE MEMBRANES: The concept of membrane *retrieval* was actually suggested as early as 1959 (134), when exocytosis in secretory cells was first described. At that time, it was recognized that membrane must be removed from the cell surface to compensate for that added during exocytosis, in order to maintain a constant cell size. It was also suggested that the 'pile of cisternae in the centrosphere region' may represent the membrane depot of the cell. Thus, from the beginning it was suggested that endocytosis and exocytosis are coupled, and that the Golgi cisternae may be involved in these phenomena. Membrane reutilization or recycling was not mentioned.

A long period followed in which the recycling of granule

membranes was questioned and generally not favored (see 135, 136). This situation lasted until a few years ago. Evidence on the fate of granule membranes came from two sources: biochemical experiments in which the turnover rates of membrane proteins and content proteins were compared in secretion granule fractions, and experiments in which the fate of the membrane was followed using electron dense tracers. The results of early turnover experiments, which were carried out on membranes heavily contaminated with content proteins, revealed no differences in the turnover of proteins for the two sources; hence, it was erroneously concluded that the membranes are not reutilized but are destroyed after each exocytotic event.

The results of early tracer experiments that used content markers, mainly horseradish peroxidase and native ferritin, were also misleading. They clearly demonstrated that, after exocytosis, membrane is recovered intact by endocytosis; but they suggested that the membranes were subsequently destroyed, rather than reutilized, because, in the majority of these studies, the tracers were found early in endocytic vesicles and later in lysosomes.[2] More recent experiments have shown, however, that the content and the membranes of recycling vesicles do not necessarily follow the same pathway. Thus, both the early tracer and turnover data were erroneous and led to the conclusion that, after exocytosis, membrane is recovered by endocytosis and destroyed in lysosomes. This view was proposed in several reviews (87, 135, 136) that were published just a few years ago (1978–1979).

Three recent developments (reviewed in 137) have changed the situation and have led to a gradual acceptance of granule membrane recycling. The first was the publication of more reliable turnover data, based on the preparation of granule membranes free from content proteins, which demonstrated that the proteins of granule membranes turn over at a much slower rate than do content proteins. The second development came from new tracer experiments, primarily with dextrans and cationized ferritin, in which transport of exogenous tracers to multiple stacked Golgi cisternae was demonstrated in a variety of cell types: parotid and lacrimal gland cells (138); anterior pituitary cells (139; Figs. 29–31); thyroid epithelium (140); pancreatic endocrine (61) and exocrine (141) cells; plasma cells and myeloma cells (69), and macrophages (142). The demonstration of endogenous secretory product and exogenous tracer segregated together in newly formed secretion granules (or vesicles) was possible in a few instances—i.e., anterior pituitary (139; Figs. 29–31), exocrine pancreatic (141), and plasma cells (69). The third development was the demonstration of extensive membrane reutilization in other systems, especially in neurons and macrophages (see 115 and 135), which, together with the data on granule membranes, led to a gradual realization of the widespread occurrence and (often) surprising magnitude of membrane recycling and its importance for a wide variety of cell processes.

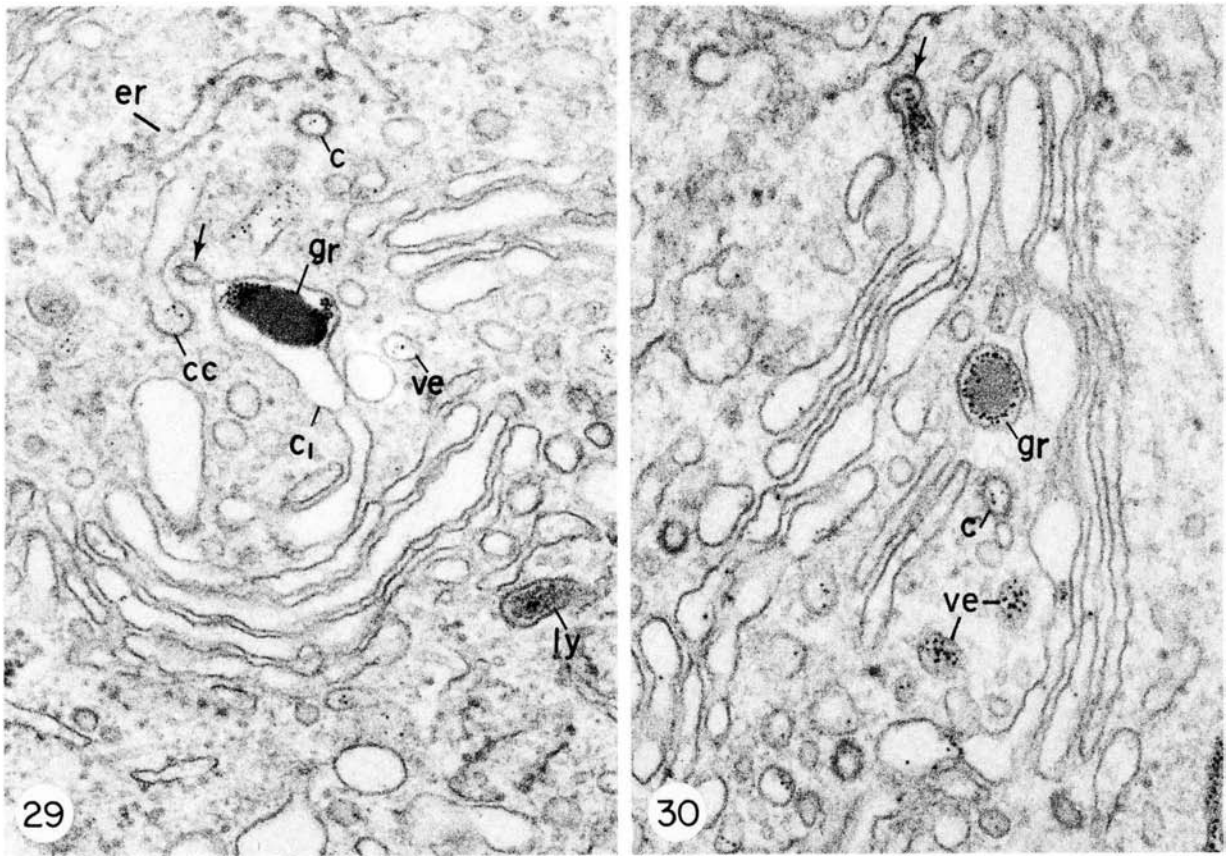
[2] In a few of these early studies, (e.g., those by Mata and David-Ferreira on the seminal vesicle, Pelletier and Farquhar and co-workers on anterior pituitary cells, Orci and associates on the β-cells of the endocrine pancreas and Gonatas on cultured neurones), tracers were also detected in Golgi elements or GERL (see reference 137). The amount of reaction product was either small or limited to certain Golgi elements. Much more attention was given to the presence of tracer in lysosomes so that at that time the latter were assumed to be the primary terminus of the incoming vesicular traffic involving membrane recovered from the cell surface.

RECYCLING OF OTHER GOLGI MEMBRANES: Secretion granule membranes, as well as synaptic and endocytic vesicle membranes, represent particularly favorable objects for following the fate of retrieved and recycled membrane, thanks to the large quantities involved and the fact that, at least in the first two cases, relocation of membrane can be controlled experimentally. In many other cases in which membrane is relocated intracellularly—e.g., vesicles that transport secretory products from ER to Golgi, or lysosomal enzymes from Golgi to lysosomes—a similar recycling mechanism seems likely, but pertinent data are much more difficult to obtain in the absence of appropriate tracers and well-established membrane markers.

USE OF AGENTS WHICH PERTURB GOLGI TRAFFIC: Various agents, including uncouplers of oxidative phosphorylation, inhibitors of tubulin polymerization into microtubules, amines, and ionophores have been used in attempts to gain information on Golgi traffic and functions; some of these agents disrupt the architecture of the Golgi complex (see [143] for a recent review). In the few cases in which specific agents were available, and their effects were investigated by combined morphological and biochemical procedures (e.g., intracellular transport of secretory proteins [52]), this approach has provided useful and clearly interpretable information. In other cases, however, the use of traffic perturbants has produced results that are difficult to interpret, primarily because these agents have multiple effects that are not limited to the Golgi complex. For example, at appropriate concentrations inhibitors of tubulin polymerization prevent microtubule assembly anywhere in the cell, not just in the Golgi region. Acidic ionophores (nigericin, X537A, and monensin) as well as weak bases (e.g., methylamine, ethylamine, and chloroquine) are "lysosomotropic agents," so-called because they accumulate in lysosomes, thereby causing an increase in the intralysosomal pH (from 4.5 to 6.0) (144), which prevents or retards intralysosomal digestion. Amines also have been shown to block endocytosis (145, 146). Many of these agents cause an increase in size of the lysosomal compartments, probably by interfering with membrane recycling along the endocytic pathway. However, they may also disturb membrane traffic at other intracellular sites. Of particular interest is the recent finding (147) that monesin as well as nigeracin, now commonly used as Golgi-perturbing agents, perturb recycling of LDL receptors and inhibit lysosomal digestion of LDL (presumably by raising the intralysosomal pH).

The use of agents that perturb Golgi traffic is potentially an interesting and promising approach for unraveling traffic patterns into and out of the Golgi complex, but the interpretation of the results obtained require caution (as well as additional work) because of the problems outlined above and the inherent and still poorly understood complexity of the traffic through the Golgi apparatus.

IMPLICATIONS OF MEMBRANE TRAFFIC FROM THE CELL SURFACE TO THE GOLGI COMPLEX: The existence of a pathway along which membrane from the cell surface can reach Golgi cisternae has broad implications: it provides a means by which cell surface molecules can be brought back to a biosynthetic compartment. Thus, a mechanism exists whereby—in principle—surface membrane components such as receptors, enzymes, and other membrane proteins could be modified or repaired (e.g., reglycosylated, sulfated, phosphorylated) while in transit through the Golgi complex during recycling. To date no specific examples of this type of phenomenon are available, but there is no reason why it could not take



FIGURES 29 and 30 Golgi complexes from pituitary prolactin cells incubated with cationized ferritin (CF) for 60 min to trace the fate of membrane internalized at the cell surface. Fig. 29 shows that, when the CF binds to the cell surface, it is taken up by endocytosis and the incoming vesicles carrying CF fuse preferentially with the trans Golgi elements. Here the tracer is particularly concentrated around a forming secretory granule (gr) within the transmost Golgi cisterna (C_1). Note that the CF is concentrated at the periphery of the forming granule adhering to its dense content. An empty coated vesicle appears to be in the process of budding from (or fusing with) the cisterna (arrow). CF is also seen within several vesicles (ve) one of which is coated (c) in the Golgi region, within another cisterna with a coated rim (cc), and within a lysosome (ly). Fig. 30 shows CF within multiple (4–5) stacked Golgi cisternae and around a forming granule (gr). One of the CF-marked cisternae has a coated tip (arrow), suggesting that a coated vesicle loaded with CF has just fused with it. CF is also seen within numerous vesicles (ve) adjacent to the stacks; some of these vesicles are coated (c). Fig. 29—× 70,000; Fig. 30—× 87,000. From Farquhar (139).

place if the molecules are brought into contact with the proper Golgi compartments or subcompartments. The route from the cell surface to the Golgi complex also provides a pathway whereby various informational molecules from the extracellular environment (peptide hormones, catecholamines and other agents) can reach the Golgi complex where they may undergo modifications as described for surface components and from where they may influence certain intracellular events. It is now quite clear that many peptide hormones are internalized by endocytosis and can reach lysosomes (148). Work primarily by Bergeron, Posner, and their associates demonstrates that several hormones (prolactin, insulin) reach bona fide Golgi elements (149, 150) upon internalization.

It is not yet known whether this uptake is connected with specific modifications of metabolic events, with the removal and degradation of hormones, with the regulation of receptor distribution, or simply with constituitive (continuous) membrane recycling. The physiological meaning of hormone internalization into Golgi elements, lysosomes, and perhaps other cell compartments is a most intriguing problem yet to be resolved.

Summary and Conclusions

We have related herein the major developments of the last 25 years that have brought us to our current level of understanding of the structure and function of the Golgi apparatus. Thirty years ago, when only light microscopes were used in cell research, the very existence of the Golgi apparatus was questioned, but electron microscope observations demonstrated that the complex is a cytological reality and its acceptance was rapid and general. The conclusion was based primarily on the reproducible demonstration by relatively simple procedures of an ubiquitous, characteristic structure.

Initially, our concepts of the functions of the Golgi complex and traffic within it were rather simplistic. The organelle was seen as a kind of 'bottling station', which existed primarily or solely for the packaging of secretory products. The cisternae were believed to move undirectionally from one face to another across the stack, as on a conveyor-belt in a factory assembly line, being used up for granule packaging on the trans (then called mature) face and reformed by new membrane assembly on the cis (immature) face. As information accumulated, it became apparent that the complex had the exclusive capability to modify secretory products by terminal glycosylation, sulfation, and proteolytic processing of proproteins. Similar modifying activities were detected for membrane proteins. The conveyor-belt or assembly line concept was retained, and new data concerning the role of the Golgi complex in the production and

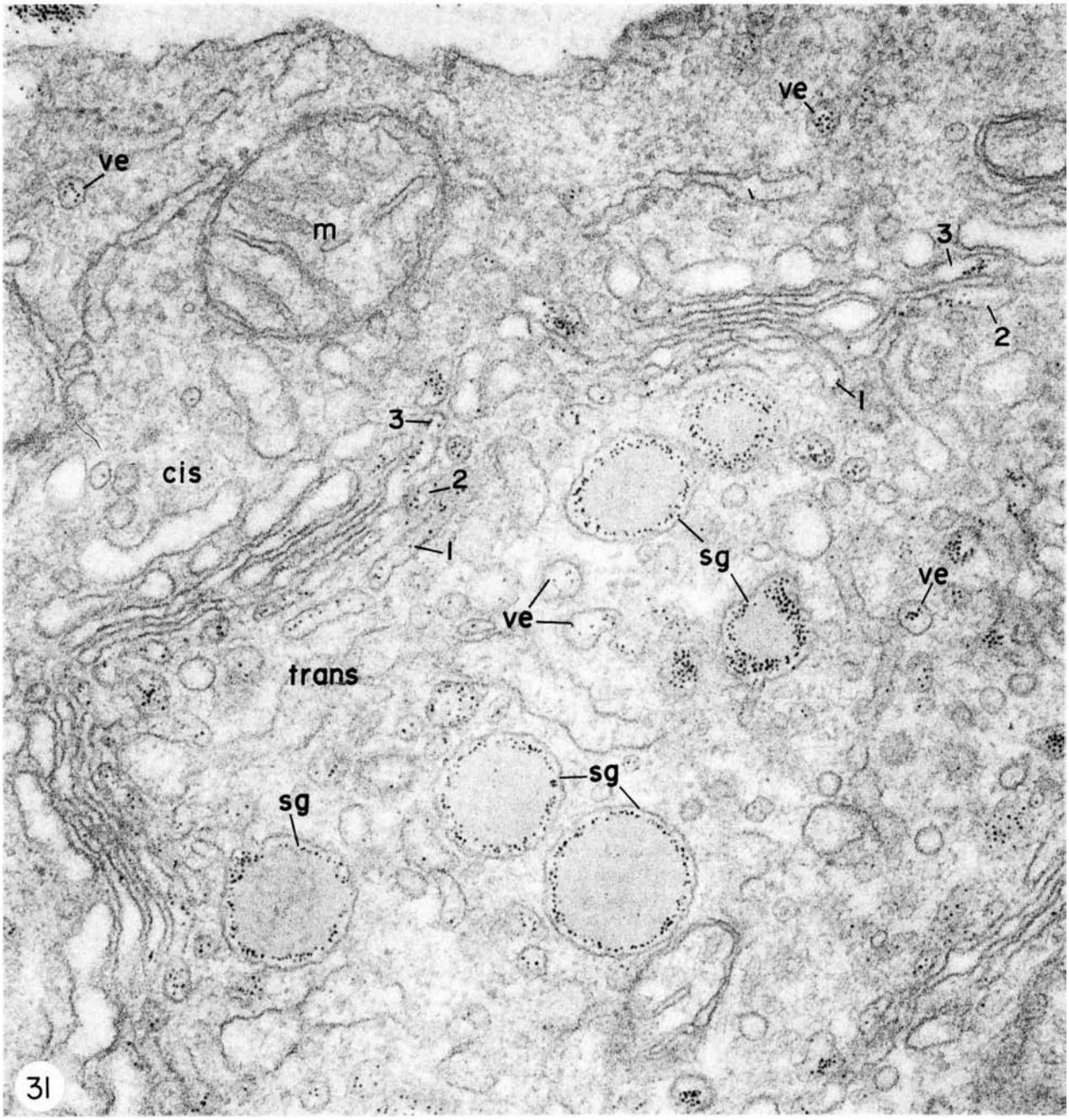


FIGURE 31 Somatotroph or growth hormone secreting cell from a male rat incubated 60 min in CF (0.05 mg/ml), illustrating uptake of CF into multiple Golgi cisternae and secretion granules (sg). CF molecules are also present within multiple vesicles (ve) in the Golgi region. Note that the CF is most abundant in the three cisternae (1–3) on the trans side of the Golgi stack. The incoming vesicles carrying the CF appear to fuse preferentially with the trans cisternae. The tracer is packaged along with growth hormone into the forming granules where it is located between the granule membrane and its dense contents. × 85,000. From Farquhar (139).

processing of secretory products or membrane components were interpreted within the framework of this concept. Diagrams that reflected this concept of the flow of secretory products and membranes to and through the Golgi complex were published repeatedly (13, 15, 38, 63).

Data acquired more recently, however, are not compatible with these ideas about traffic through the Golgi complex, and especially do not support a simple cis-to-trans flow diagram.[3] One set of data concerns the composition of the Golgi membranes themselves. Originally the membrane flow hypothesis proposed that each Golgi cisterna is immature as it enters the stack and is progressively modified (matures) in transit across the stack. However, cytochemical and biochemical data now available attest to the existence of considerable heterogeneity among Golgi membranes, and documentation has been pro-

[3] At present the only case in which a cis-trans movement of Golgi cisternae appears to be established is that of scale-producing algae (118). However, this may represent a rare formula connected with the unusual geometry and size of the product: a whole cisterna is needed to accommodate each scale under construction.

vided for the existence of specific compositional differences between adjacent cisternae within a given stack and within the same cisterna. Also, data on the recycling of secretion granule membranes (137) (diagramed in Fig. 32) are not in agreement with the old flow diagrams. These diagrams should be revised to take into account the multiple formulae that are now known to be involved in biogenesis and transport of membrane constituents, as well as recent data concerning the mechanisms and pathways for transport of secretory products and lysosomal enzymes. Although the new data are not compatible with a simple cis-to-trans flow diagram, they are in agreement with the following conclusions:

(*a*) Individual Golgi cisternae, like other cell components, retain the specificity of their membranes.

(*b*) Transport of both secretory products and membrane components is largely effected by vesicular carriers, which interact (fuse) preferentially with the dilated rims of the cisternae.

(*c*) Each Golgi cisterna is a mosaic in which differentiated domains are maintained in the plane of the membrane by means so far unknown.

(*d*) The dilated rims of the Golgi cisternae represent a special subcategory of Golgi membranes that differ from the rest in their protein composition and enzymic activities.

(*e*) The main flow of both the secretory products from the rough ER and the secretion granule membrane recycled from the cell surface is to the dilated rims of multiple Golgi cisternae.

(*f*) In cells that concentrate their secretory products, traffic from the cell surface is heaviest to the transmost Golgi cisternae where concentration takes place.

These conclusions (137), which are based on information about the three major types of traffic on which we have some, albeit limited, knowledge—i.e., secretory proteins, recycled granule membranes, and intrinsic membrane proteins—*are* accommodated by the diagram depicted in Fig. 33. The pathway taken by other types of membranes and products that are known to pass through the Golgi complex, such as lysosomal enzymes and lysosomal shuttles, cannot yet be drawn with certainty.

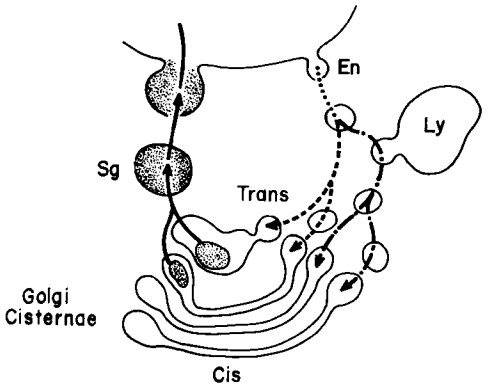


FIGURE 32 Diagram showing routes which can be taken by surface membrane to reach the stacked Golgi cisternae in secretory cells. Following exocytosis of secretory granules (——), patches of surface membrane are recovered by endocytosis (· · ·) and fuse with the dilated rims of multiple stacked Golgi cisternae or with lysosomes. The recovered membrane may fuse first with the membrane of lysosomes (—·—) and then with that of Golgi cisternae or may reach the latter directly (– – –). The available evidence suggests that both routes are used in different cell types. From Farquhar (137).

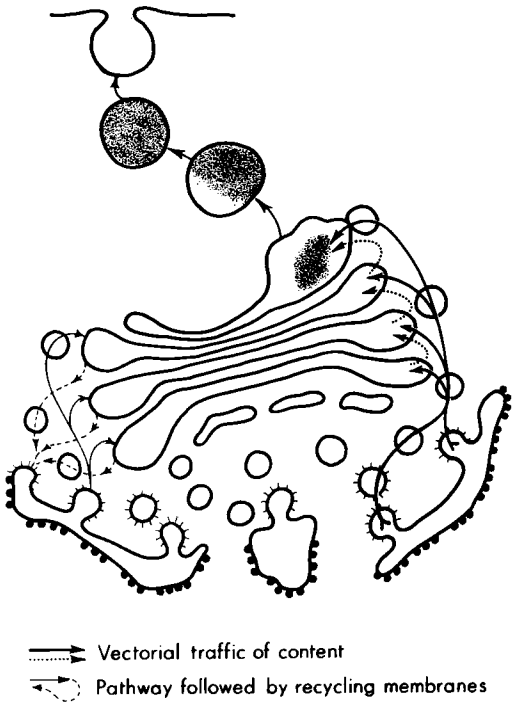


FIGURE 33 Flow diagram illustrating the proposed routes taken by membranes (left) and secretory products (right) to and through the Golgi complex. The available evidence suggests that small transport vesicles which bud from the transitional elements of the rough ER and fuse with the dilated ends of multiple Golgi cisternae are involved in this operation. The secretory proteins (shown to the right) move vectorially and become concentrated in the trans Golgi cisternae where they are packaged into granules. The dotted line indicates that secretory proteins may be moved sequentially from cisterna to cisterna. The membrane vesicles (shown to the left) which serve to ferry secretory proteins, pinch off and return—i.e., recycle, back to the transitional elements of the rough ER.

It is clear that a major function of the Golgi complex is to sort secretory, lysosomal, and certain membrane proteins and to direct them to their correct intracellular or extracellular destinations. Fig. 34 is a tentative interpretation of sorting mechanisms in a Golgi cisterna seen *en face*. The position of the sorting devices in the Golgi stack is unknown. They may be present in different or in the same cisternae.

In revising the membrane flow-differentiation hypothesis the most important point to take into account is that each cell inherits at birth a complete set of differentiated membranes from its mother (151)—it does not have to differentiate ER membrane into either Golgi membrane or plasmalemma, but it must retain the biochemical specificity of each of these membranes. This appears to be achieved, not by converting one type of membrane into another, but by controlling the traffic from the sites of synthesis to the sites of final assembly for either protein molecules or membrane vesicles, so that only appropriate components are assembled in each membrane (152). Retention of biochemical specificity also requires nonrandom removal of membrane carriers from at least one of the interacting compartments. The only 'differentiation' established so far for membrane proteins that move down the secretory pathway does not exceed the usual posttranslational modifications—i.e., terminal glycosylation and partial proteolysis undergone by many proteins and most glycoproteins that pass through the Golgi complex.

Although the proposed formulations accommodate our cur-

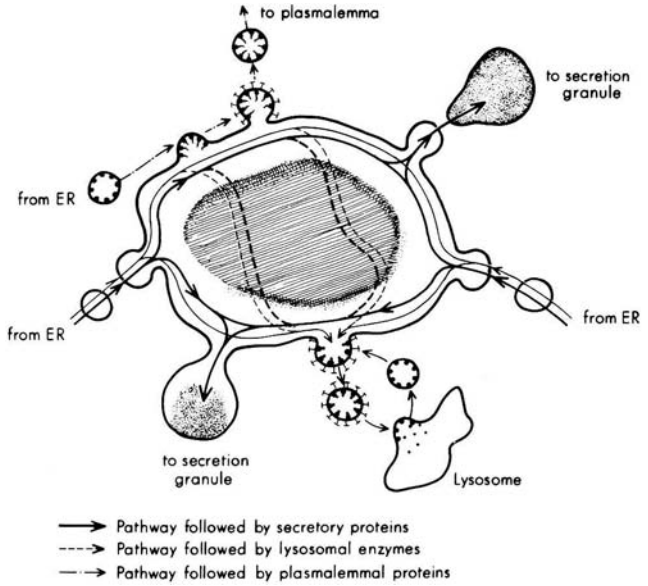


FIGURE 34 Diagram of a Golgi cisterna viewed *en face* showing the presumed routing of the biosynthetic traffic of membranes and secretory products along its dilated rims. Four types of traffic are depicted: (1) ER → Golgi; (2) Golgi → lysosomes; (3) Golgi → condensing granules or vacuoles; and (4) Golgi → plasmalemma. In all cases, transport is assumed to be effected by vesicular carriers which must possess specific receptors for transported species on their inner (cisternal) surfaces and appropriate recognition signals for the receiving compartment on their outer surfaces. In two cases (types 2 and 3) there is evidence that coated vesicles are involved. In only one case (type 2) is the specific recognition marker (mannose-6-phosphate) known. The large dots attached to the membrane represent the receptor and the small dots the lysosomal enzymes. Most of the traffic is assumed to move along the dilated periphery of the cisterna (solid lines) rather than through its flattened central region (dotted lines, shaded area).

rent knowledge, it can be safely anticipated that in another 25 years—or even sooner—they, too, will prove to be far too simplistic. The reasons should be clear: first, the biological sciences, especially cell biology, are advancing at a remarkably rapid rate. In addition, information on the complexity of the Golgi complex, which appears to be the hub of intracellular traffic, and the multiplicity of its connections continues to accumulate as a result of new findings. In the meantime, however, these new formulations may provide a useful framework to be tested and validated or modified by further experiments.

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III. Motility and Stability Mechanisms

Cilia and Flagella of Eukaryotes

I. R. GIBBONS

> The simple description that cilia are "contractile protoplasm in its simplest form" (Dellinger, 1909) has fallen away as a meaningless phrase . . . A cilium is manifestly a highly complex and compound organ, and . . . morphological description is clearly only a beginning.
>
> Irene Manton, 1952

As recognized by Irene Manton (1) at the time that the basic 9 + 2 structural uniformity of cilia and most eukaryotic flagella was first becoming recognized, these organelles are sufficiently complex that knowledge of their structure, no matter how detailed, cannot provide an understanding of their mechanisms of growth and function. In our understanding of these mechanisms, the substantial advances of the intervening 28 years have, for the most part, resulted from experiments in which it has been possible either to correlate changes in structure with the changes in waveform that occur during a normal beat cycle, or to make experimental changes in the structure, chemical properties, or mechanical loading of the organelle, and then relate these to the consequent changes in its motility. Research has thus tended to concentrate on organisms in which the cilia or flagella are suitable for study by as many techniques as possible.

The number of organisms that readily yield sufficient cilia or flagella for protein chemistry is relatively small. Among these, the flagella of sea urchin spermatozoa have proved the most favorable for many purposes because their length of 40–50 μm is sufficient for detailed measurement of wave parameters, and they are rugged enough for the demembranated flagella to be reactivated easily with adenosine triphosphate (ATP). The availability of mutants with altered flagella makes *Chlamydomonas* also a highly favorable material, although observation of the detailed pattern of motility is more difficult because the flagella are only 12–15 μm long. Cilia of *Tetrahymena* have been used as a source of ciliary proteins, and cilia of molluscan gills can also be obtained in good quantity, but, in both cases, observations of their motility are difficult to achieve, and have been useful mainly for investigating conditions that induce ciliary reversal or arrest. For experiments that do not involve protein chemistry, a broader choice of organisms is available, including protozoa with a wide range of motility patterns and metazoa whose sperm flagellar structure differs substantially from the usual 9 + 2 organization.

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Early Developments

Among the most notable steps in the history of early studies on cilia and flagella were the initial light microscope observations of beating cilia on ciliated protozoa by Anton van Leeuwenhoek in 1675; the hypothesis proposed by W. Sharpey in 1835 that cilia and flagella are active organelles moved by contractile material distributed along their length rather than passive structures moved by cytoplasmic flow or other contractile activity within the cell body; and the observation in 1888–1890 by E. Ballowitz (2) that sperm flagella contain a substructure of about 9–11 fine fibrils which are continuous along the length of the flagellum (Fig. 1). More detailed accounts with full references to this early work and to other studies before 1948 can be found in the monographs of Sir James Gray (3) and Michael Sleigh (4). Several of the observations and hypotheses that are often regarded as recent were anticipated in this early work. However, it is fair to note that it is only in retrospect that the significance of these findings becomes apparent, and that they were by no means generally accepted at the time because the techniques required to confirm and extend them were, of course, not then available.

The foundations for many aspects of the more recent work on cilia and flagella were established, largely independently of each other, in the period 1949–1955. The use of dark-field light microscopy to study sperm motility was initiated by Lord Rothschild and Michael Swann in 1949 (5), and was extended in 1955 to photographic recording of the waveforms of the single flagellum of sea urchin spermatozoa by Sir James Gray (6). Formulations for calculating the hydrodynamic forces resulting from different flagellar waveforms were developed in 1951 by Sir Geoffrey Taylor (7), and were further developed and shown to account for the translational velocity of sea urchin spermatozoa by Gray and G. Hancock (8). The basic cylindrical 9 + 2 structural organization of the axoneme was first deduced in 1949 by G. Grigg and Allan Hodge from studies of splayed flagella of cock sperm (9). In 1952 the widespread uniformity of this 9 + 2 organization in cilia and eukaryotic flagella was recognized for algae and lower plants by Irene Manton and G. Clarke (10), and for cilia of invertebrate and vertebrate animals by Don Fawcett and Keith Porter (11); within a year, however, the occurrence of variations in this basic pattern in the sperm flagella of some animals had been shown by C. Challice (12) and by John Bradfield (13). The work of Hartmut Hoffmann-Berling (14) in 1955 showed that it was possible to reactivate flagellar motility by addition of exogenous ATP to cells in which the selective permeability

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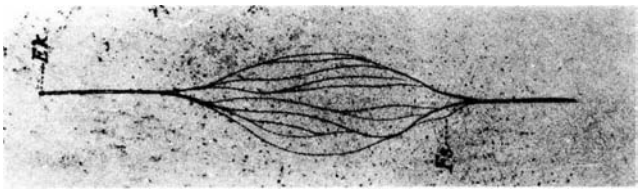

FIGURE 1 Drawing showing the substructure of the flagellar axoneme in a spermatozoon of the chaffinch, *Fringilla caelabs*. 11 "elementary fibrils" (Fs) are depicted in the frayed axoneme and presumably correspond to the 9 + 2 tubules known today. Additional density may have been contributed by the nine peripheral fibers associated with the nine doublet tubules in sperm of this species. The basal body (EK) is shown at the proximal end of the axoneme. The preparation was made by maceration of unfixed spermatozoa and stained with Gentian violet. Ballowitz, 1888 (2).

of the membrane has been destroyed by treatment with 50% glycerol. Studies of *Chlamydomonas* by Ralph Lewin in 1952–1954 (15–17), showed that mutants could be obtained with paralyzed flagella and laid the basis of complementation experiments in which the motility of the paralyzed flagella is rescued in the dikaryon formed by mating the mutant cells with those of wild type. In related studies at approximately the same time, Lewin demonstrated that uniflagellate cells of *Chlamydomonas* attached to a glass surface would glide steadily across the surface, flagellum leading, apparently because of interactions between the flagellar membrane and the axoneme within it. This work also provided a basis for the study of flagellar regeneration by showing that *Chlamydomonas* that had resorbed their flagella would grow new flagella within 90 min of being transferred to liquid medium in the light.

Knowledge of the principal proteins responsible for motility in cilia and flagella lagged behind the developments mentioned above by about 10 years. Several workers in the 1940s and 1950s, including V. Engelhardt (18), S. Burnasheva (19), Leonard Nelson (20), Hideo Mohri (21), Jack Tibbs (22), Frank Child (23), and Sir John Randall and co-workers (24), developed procedures for isolating flagella from spermatozoa of various animals and cilia from *Tetrahymena*, and demonstrated the presence of ATPase activity in the isolated organelles. However, attempts to characterize the axonemal proteins were hindered by the assumption that they were closely related to actomyosin from muscle, and by their apparent insolubility under mild conditions. In 1963, Ian Gibbons (25), using cilia isolated from *Tetrahymena*, found that this apparent insolubility was due to the ciliary membrane surrounding the axoneme. After the membrane had been removed with digitonin, the ciliary adenosine triphosphatase (ATPase) protein was extracted selectively and found to have properties very different from those of the muscle ATPase, myosin.

Knowledge of the physiology and biochemistry of cilia and flagella prior to the early 1950s is well summarized in the classic monographs of Sir James Gray (3) and of Thaddeus Mann (26). Among the many surveys of subsequent work are the 1962 monograph of Michael Sleigh (4) and its successor volume of review articles published in 1974 (27), the second edition of Mann's monograph (28), and the valuable reviews of sperm motility by David Bishop (29) and those of structure and function by Keith Porter (30), Don Fawcett (31), and Peter Satir (32). Detailed reviews of more recent work on ciliary and flagellar motility include those of John Blake and Sleigh (33), Michael Holwill (34), and Joseph Blum and Michael Hines (35).

Structure

To a large extent, the increasing knowledge of the structure of cilia and flagella during the past 28 years is the result of continued improvements in the techniques of specimen preparation for electron microscopy and for analysis of the resultant images. Although careful interpretation enabled a correct reconstruction of the basic axonemal structure of a cylinder of nine doublet tubules surrounding two central tubules from shadow-cast samples of splayed axonemes (Fig. 2) (1, 9, 10), this structural organization was more directly apparent to Fawcett and Porter in 1954 (11, 37) when they used the newly developed technique of thin sectioning to examine various ciliated epithelia. In addition to observing the 9 + 2 organization, they were able to say that only the nine outer tubules were doublets, whereas the two central tubules were singlets, and also to determine that the plane of beat was perpendicular to the plane of the central tubules (Fig. 3 *a,b*)

The enhanced contrast obtained by heavy-metal staining substantially increased the amount of structural detail visible in the axoneme. In 1959, Bjorn Afzelius (38) used a 40% solution of OsO_4 in CCl_4 to fix sea urchin spermatozoa, and was able to visualize an irregular double row of arms along one side of each outer doublet tubule in the axoneme, as well as sets of radial spokes that linked the arm-bearing component of each doublet to an undefined structure in the central region of the axoneme. Afzelius noted that the asymmetrical position of the arms made it possible to number the outer doublets in an unamibiguous manner (Fig. 3 *c,d*)

In the following year, Gibbons and A. V. Grimstone (42) obtained a further improvement in preservation and contrast by using epoxy resin, as developed by Audrey and Richard Glauert, to replace methacrylate as an embedding medium, and by staining the cut sections on a solution of uranyl acetate in 50% ethanol. Application of this procedure to flagellated protozoa confirmed the presence of most of the additional axonemal structures reported by Afzelius. In addition, it disclosed a more regular double row of arms along one side of each outer doublet tubule, a region of increased density near the middle of each of the nine radial spokes that was interpreted as a cross section through one of a set of longitudinally oriented "secondary fibers," and the presence of a central sheath, considered possibly helical, enveloping the two central tubules as reported earlier by Manton (1, 10, 36), but not previously observed in sectioned material. The large number of flagella in these protozoa and the regular arrangement of their attachments to the cell body made it possible to give a substantially more detailed account of both the structure of the basal body at the cytoplasmic end of each flagellum, and the transition zone between the basal body and the shaft of the flagellum. The basal body consists of a cylinder of nine triplet tubules, with the plane of each triplet skewed in toward the center of the basal body, and a cartwheel-like structure in the lumen of the proximal portion of the basal body. The three component tubules of each triplet in the basal body were designated as A, B, and C (Fig. 4 *a,b*). In the transition zone between basal body and flagellum, the C tubules terminate, whereas the A and B tubules continue into the flagellar shaft where the A tubule of each doublet acquires the double row of arms. The two central tubules of the flagellum terminate in the upper portion of the transitional region, and do not continue into the basal body.

A study of the gill cilia of the lamellibranch *Anodonta* (39) showed that the structural organization of the cilia, transition regions, and basal bodies was generally similar to that in the

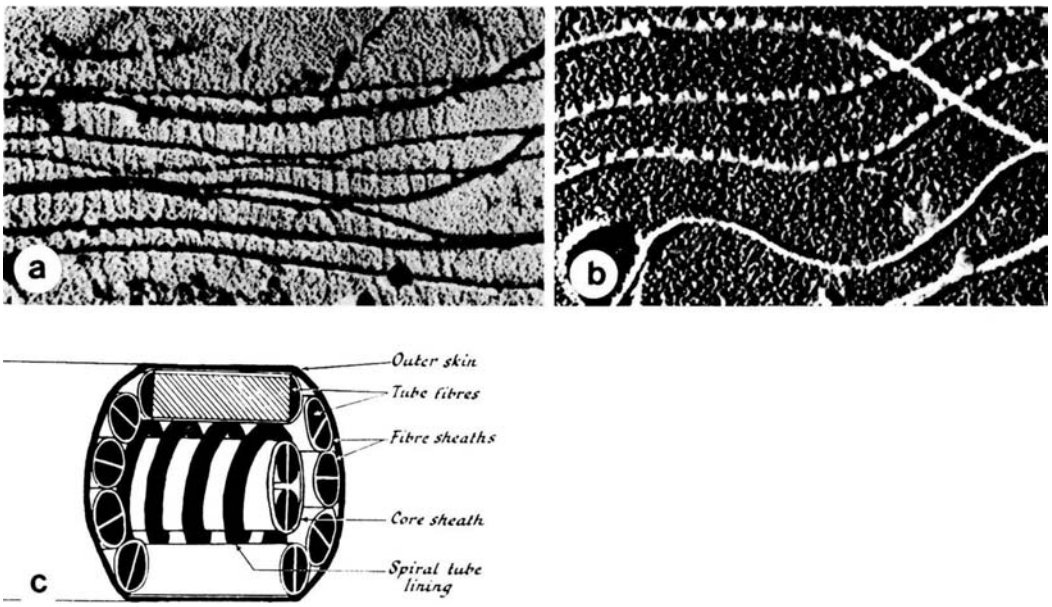

FIGURE 2 (*a*) Disintegrated flagellum of seaweed *Pylaiella*. Shadow-cast preparation showing "ladders" between doublet tubules, now interpreted as stretched nexin links. Manton, 1954 (36). (*b*) Same, with flagellum of moss *Sphagnum*, showing battlements on doublet tubules, now interpreted as paired radial spokes. Manton and Clarke, 1952 (10). (*c*) Diagrammatic reconstruction of flagellum of *Sphagnum* spermatozoid, prepared on the basis of *b*, and other contemporary micrographs. Manton and Clarke, 1952 (10).

flagellated protozoa; however, there were some differences in the pattern of linkages in the transition region; in the presence of a dense basal plate that ran across the lumen of the cylinder of doublets out to a constriction of the ciliary membrane, appearing to seal off the intraciliary matrix from the general cell cytoplasm; and in the presence of paired cross bridges spanning the gap between the central tubules in the cilia. The cilia also had a more definite structural polarity (Fig. 3 *e,f*), as indicated by a cross bridge between one particular pair of doublets (nos. 5 and 6) and a cross-striated, conical "foot" projecting from one side at the basal body. In all four types of ciliated cell on the gill epithelium, the direction of effective stroke in the ciliary beat cycle was toward the 5–6 bridge in the cilia and the foot on the basal body, with the plane of beat perpendicular to the plane of the two central tubules as reported previously by Fawcett and Porter (37).

The position of the arms on one side of the doublet tubules in cilia and flagella and the inward skew of triplet tubules in basal bodies give the structure an enantiomorphic asymmetry. In the studies of flagellate protozoa and gill cilia discussed above, as well as in a variety of other organisms surveyed (42, 43), the arms on the doublets have been found always to point clockwise, and, correspondingly, the triplets of a basal body are always skewed inward passing clockwise. (All orientations of structure and movement in this review are given as they would be seen by an observer looking outward along the organelle from its basal end.)

Application of the negative contrasting procedure, first described by Cecil Hall in 1955 (44) and developed for viruses by Sidney Brenner and Robert Horne, to the study of cilia and flagella enabled Jean André and Jean-Paul Thiéry (45) and Daniel Pease (46) to determine that the walls of flagellar tubules consist of longitudinally oriented protofilaments that were about 4 nm wide, and had a periodicity of about 8 nm along their length. More detailed information was obtained by Grimstone and Aaron Klug (47), who used optical diffraction of electron microscope images to analyze the arrangement of subunits in the walls; they reported that the surface lattice had a basic repeat of 4.0 × 5.0 nm, with a displacement in the relative radial positions of alternate subunits giving the actual repeat of 8.0 nm. Further development of the optical diffraction procedure by David DeRosier and Klug permitted three-dimensional image reconstruction by computer analysis of the digitized image of an object with helical symmetry. Application of this procedure to the singlet tubule portion of the doublets near their tip by Linda Amos and Klug (48) indicated that the wall of the singlet contained 13 protofilaments, and that dimers in neighboring protofilaments formed a staggered arrangement, equivalent to the lattice with 8-nm periodicity reported earlier. These dimers are believed to correspond to the α- and β-subunits of the tubulin molecule. Reconstruction of the B-tubule lattice from optically filtered images showed that the B tubule is also made up of 8.0-nm dimers but differs from the A tubule in that the dimers are lined up obliquely at a shallow angle, rather than in a staggered array. X-ray diffraction studies of tubules have been invaluable in providing a calibration indicating that the basic longitudinal periodicity is 4.0 nm in hydrated tubules (49). The number of protofilaments in the walls of the doublet tubules can be counted in thin sections of favorable material, and it was shown by Lewis Tilney and coworkers (41) that this substructure appears particularly clearly with negative contrast in sections of material fixed with a mixture of glutaraldehyde and tannic acid. These observations clearly illustrated that the A component of the doublet is a complete tubule with 13 protofilaments, whereas the B component is an incomplete tubule with 10 or 11 protofilaments (Fig. 3 *i*).

In addition to information about the flagellar tubules, negative contrasting has also provided much information about the other structures of the axoneme, particularly about the radial spokes and the appendages associated with the central tubules. The radial spokes appear to be rigid structures attached

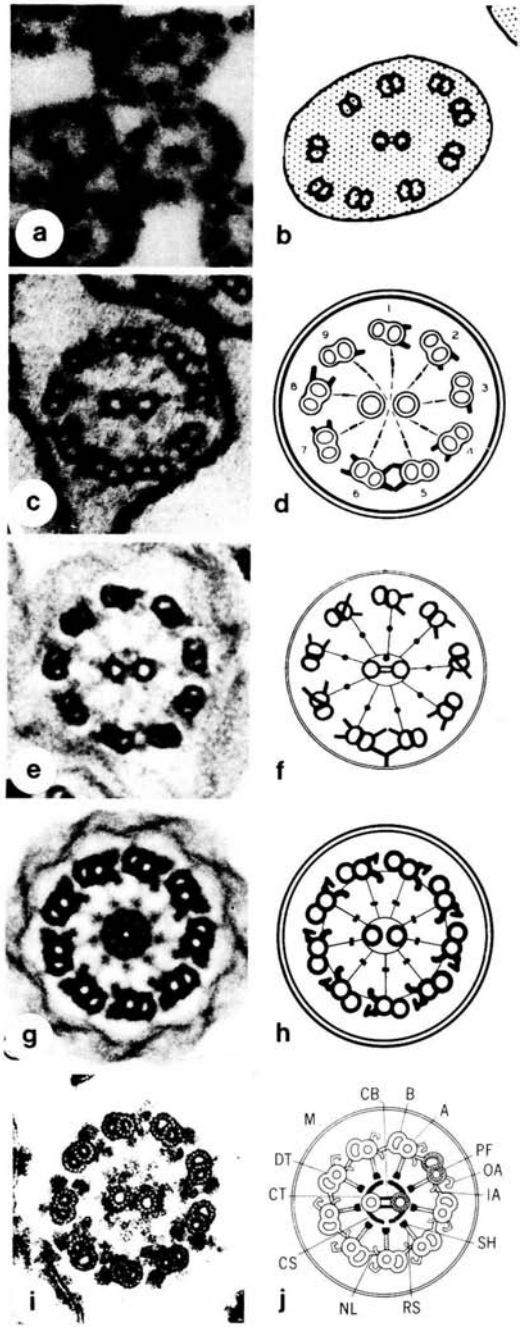

FIGURE 3 Increasing knowledge of flagellar and ciliary structure as depicted by electron micrographs of cross sections with contemporary diagrammatic interpretations. (*a*, *b*) Cilia on pharyngeal epithelium of frog. Plane of beat is vertical in figure. Fawcett and Porter, 1954 (37). (*c*, *d*) Flagellum of sea urchin spermatozoon. Afzelius, 1959 (38). (*e*, *f*) Lateral cilium of lamellibranch gill epithelium. Plane of beat is vertical in figure, with effective stroke toward bottom of page. Gibbons, 1961 (39). (*g*, *h*) Cilium of *Tetrahymena*, printed with ninefold Markham rotational translation. Structure of doublets and arms is reinforced. Structure of central tubules and central sheath which do not have ninefold symmetry is lost. Diagrammatic interpretation shows structure without rotation. Allen, 1968 (40). (*i*) Isolated axoneme from sea urchin sperm flagellum. Fixation with tannic acid and glutaraldehyde reveals protofilaments in tubule walls with negative contrast. Tilney and co-workers, 1973 (41). (*j*) Axonemal structures as currently known. M, membrane; DT, doublet tubule; A, A tubule of doublet; B, B tubule of doublet; OA, outer arm; IA, inner arm; PF, protofilament; CT, central tubule; CS, central sheath; CB, central cross bridge; NL, nexin link; RS, radial spoke; SH, spoke head. Modified from Holwill, 1977 (34). (With permission. Copyright by Academic Press [London] Ltd.)

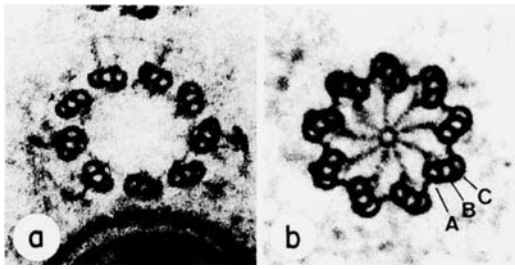

FIGURE 4 Cross sections of flagellar basal bodies in *Trichonympha*; (*a*) distal region; (*b*) proximal region. Gibbons and Grimstone, 1960 (42).

perpendicularly to the A tubule, and they are usually easily visible in axonemes where the tubules have splayed apart. In such specimens, the structures interpreted earlier as "secondary fibers" (39, 42) can be seen to consist of longitudinally oriented heads about 20 nm long, located at the centripetal end of each spoke. Although the heads on adjacent spokes sometimes appear to be in contact or to be joined by a thin fiber (50), the structures do not appear to have a general continuity along the length of the flagellum, and they are better described by the term "spoke heads." The studies of David Chasey, John Hopkins, Fred Warner, and others have shown that the spoke periodicity, originally given as about 27 nm (39), is in fact more complex and appears based on an overall repeat of 96 nm. In *Sphagnum* and *Chlamydomonas*, the spokes occur in pairs with alternate spacings of about 32 nm between members of a pair and 64 nm between adjacent pairs (10, 51, 52), whereas in *Tetrahymena* cilia, lamellibranch cilia, and rat sperm flagella, the spokes occur in groups of three, with spacings, passing from base to tip, of 32 and 20 nm between adjacent spokes, and 24 nm between adjacent triplets (Fig. 5*a*) (53–56). Regardless of whether the spokes on an individual doublet tubule occur as groups of two or of three, the spoke groups on the nine doublet tubules of the intact axoneme occur as a helix with a repeat of 96 nm. The structure in flagella of *Sphagnum* spermatozoids that Manton and Clarke (10) interpreted as a continuous helix, with a repeat of about 100 nm, situated between the outer doublets and the central tubules (Fig. 2*c*), is now interpretable as the discontinuous helix formed by paired radial spokes.

Recent studies of sectioned and negatively contrasted material have modified the description of the central tubule complex given by Gibbons in 1961 (39). The presence of paired, central bridges connecting the two central tubules at their nearest points, like double rungs on a ladder, has been confirmed in various cilia and flagella by Warner and others (56, 57). However, the helical structure described for the "central sheath" to which the radial spoke heads attach is incorrect, and from work originated by Chasey (55, 56), it is now clear that this structure is composed of two rows of projections arising from each central-pair tubule, with the projections on one tubule in close apposition to those on the adjacent tubule (52, 58).

The multiple photographic exposure procedure developed in 1963 by Roy Markham, S. Frey, and G. Hills (59) to enhance the visibility of periodic structures in electron micrographs has often proved useful in the study of cilia and flagella, particularly where the nature of the structure did not permit use of optical diffraction. This procedure was used with a ninefold rotation by Richard Allen in 1968 (40) to obtain substantially clearer images of the arms on the doublet tubules of *Tetrahymena* cilia. The resultant images showed that the outer and inner arms on each doublet had different profiles, with the

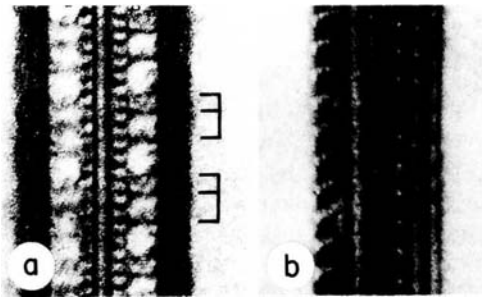

FIGURE 5 (*a*) Medial longitudinal section of lateral cilium of lamellibranch gill, showing triplet groups of radial spokes. Multiple-exposure Markham print with linear translation. Periodicity given as 86 nm at the time, but now believed to be ca. 96 nm. Brackets on right indicate two groups of triplets. Orientation is with ciliary basal end downward. Warner and Satir, 1974 (53). (*b*) Group of doublet tubules in ATP-disintegrated cilium of *Tetrahymena*, negatively contrasted with uranyl acetate. Note the basal tilt of the free arms on the doublet at left. Two-exposure Markham print with linear translation of 24 nm. Warner and Mitchell, 1978 (60).

outer arms extending out about 20 nm toward the membrane and then hooking sharply back toward the center of the axoneme, whereas the inner arm curved gently inward and had a small knob of increased density on its terminal end (Fig. 3 *g,h*); similar arm structures have been seen since then in cilia and flagella of many other species. The rotated images of *Tetrahymena* cilia also emphasized the presence of frequent connections between the doublet tubules and the ciliary membrane.

The arms on the doublets tend to become disrupted upon negative staining, and have been difficult to study with this procedure. Although in early studies the arms were reported to have a longitudinal periodicity of 13–16 nm (39, 42), most recent studies have found a periodicity of about 24 nm (51, 53, 60), and it has been suggested that the lower values obtained earlier may have been the result of superimposition of inner and outer arms staggered in their attachment by about half a period (55). However, the inner arms in *Chlamydomonas* have recently been reported to have a periodicity of 45 nm (61), whereas the structures thought to correspond to rows of detached outer arms from cilia of *Tetrahymena* had a periodicity of only 15 nm (62). The extent to which this confusion may be due to differences among different species is not yet clear. In the best-preserved preparations, the outer arms usually appear to be tilted relative to the longitudinal axis of the doublet (Fig. 5 *b*) (51, 60). The appearance of the arms changes with the angle from which they are looked at, and it may also vary depending on the presence or absence of ATP (60, 63).

In micrographs of thin sections of cilia and flagella fixed with most fixatives based on glutaraldehyde and/or OsO_4, the arms on the A tubule of each doublet extend only part way toward the B tubule of the adjacent doublet (Fig. 3). However, Nina Zanetti, David Mitchell, and Warner (64) have recently shown that, when fixation is performed in HEPES buffer with 5–10 mM Mg^{2+}, the arms appear to bridge completely the gap between the pairs of the doublet tubules. The relationship of these cross bridges to the transient cross bridges between doublets that are presumed to occur during normal movement (see below) is not yet clear.

In addition to the radial spokes and the arms, the axoneme is held together by a set of circumferential linkages that join the centripetal side of each doublet tubule to that of the next. These linkages are difficult to see in intact axonemes, and they were first noted in 1963 by Gibbons (25) in preparations of *Tetrahymena* cilia from which the arms, central tubules, and spokes had all been removed by chemical extraction (Fig. 6 *a*). Their presence was confirmed in intact cilia of *Tetrahymena* by Allen (40), and in sea urchin sperm flagella by Raymond Stephens (65), who tentatively identified them with a 160,000-dalton electrophoretic band and gave them the name "nexin." Studies on negative-contrasted material by Romano Dallai, F. Bernini, and Falco Giusti (66) and by Warner (57) showed that the nexin links are highly elastic, and that although their normal length is about 30 nm, they can be stretched to as much as 250 nm without breaking (57, 67). The longitudinal periodicity of the nexin links is about 96 nm, and, in retrospect, they can be seen clearly in the micrographs of shadowed flagella from *Pylaiella* spermatozoids published by Manton in 1954 (Fig. 2 *a*) (36).

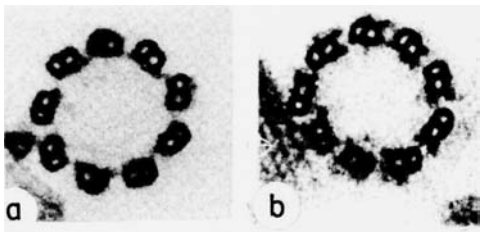

FIGURE 6 Cilia isolated from *Tetrahymena* and demembranated with digitonin. (*a*) Insoluble fraction after dialysis against 0.1 mM EDTA, 1 mM Tris/HCl, pH 8. Nexin links can be seen joining adjacent doublet tubules and are believed responsible for maintaining the integrity of the axonemal cylinder of nine doublet tubules after the central tubules, radial spokes, and dynein arms have been removed. (*b*) Same preparation after recombination with soluble ATPase fraction in presence of 2 mM Mg^{2+}; note reappearance of arms. Gibbons, 1963 (25).

The widespread uniformity of the 9 + 2 structural organization in cilia and flagella of different species is well known, and a current concept of the components visible in cross sections is presented in Fig. 3 *j*. However, variations do occur. The most common consist of additions exterior to the 9 + 2 structure, such as the mastigonemes found on many algal flagella (36), and the linkages that join the multiple axonemes of compound cilia (39, 68), rather than changes in the 9 + 2 structure itself. The sperm flagella of mammals, gastropods, and many insects contain an additional set of nine peripheral fibers situated centrifugal to the usual 9 + 2 axonemal core as well as an extension of modified mitochondria along much of their length. Whether the nine peripheral fibers are passive structures whose function is to strengthen the flagellum, or whether they contribute actively to flagellar motility is controversial. The studies of David Phillips (69) show that, among mammals, the sperm flagella of species in which the peripheral fibers are thickest have a relatively low amplitude of beating, suggesting that these fibers are major factors in flagellar stiffness; analysis of the isolated fibers by Baccio Baccetti, Vitaliano Pallini, and Anna Burrini (70) has shown that they are composed of a keratin-like protein with no detectable ATPase activity. The structure of the peripheral fibers, however, in electron micrographs appears similar to that of the single fiber in the undulating membrane of toad spermatozoa, and the work of Mario Burgos and Fawcett (71) has shown that this undulating membrane is motile with a beat frequency different from that of the flagellum proper. Similarly, the sperm flagella of several species of insect have been shown to propagate simultaneous bending waves of two different frequencies (72), suggesting the presence of two distinct motile mechanisms.

Variations in the basic 9 + 2 organization itself are less

widespread, although fairly numerous examples have been described (72, 73). Patterns reported in motile sperm flagella include 9 + 0, 9 + 1, 9 + 3, and 9 + 7; patterns of 12 + 0 and 14 + 0 have also been reported, but in these cases the doublets lack arms, and the spermatozoa are nonmotile. The 9 + 0 flagella in eel spermatozoa (74), and the 6 + 0 and 3 + 0 flagella in sporozoan gametes (75–77) are of particular functional interest (see below).

Composition

The first unequivocal characterization of the major protein components of cilia and flagella was performed by Gibbons and collaborators using cilia isolated from *Tetrahymena*. In a series of studies between 1963 and 1968, procedures were developed for isolation and characterization of the two major axonemal proteins, the ATPase protein responsible for mechanochemical energy transduction and the principal structural protein of the ciliary tubules, which account for about 15% and 70% of the total axonemal protein, respectively. A principal reason for the success of these experiments in opening up the field of ciliary and flagellar proteins for study was that the isolation of the cilia and the effects of successive extractions were monitored by the high-resolution electron microscopy permitted by heavy-metal staining of thin sections. This enabled the conditions to be adjusted to optimize the structural preservation of the cilia during their isolation and the selective removal of particular components during the successive extractions. After the ciliary membranes had been removed with digitonin, the proteins of the ciliary axonemes could be fractionated by dialysis against EDTA at low ionic strength, which solubilized almost all of the axonemal ATPase activity but only about 30% of the protein (25). Examination of the insoluble residue showed that it consisted of the outer doublet tubules alone, still largely arranged in cylinders of nine; the other structural components, including the arms, central tubules, and radial spokes, were almost completely removed (Fig. 6*a*). The axonemal structure could be partially reconstituted by restoring Mg^{2+} to the dialyzed preparation, which resulted in about half of the solubilized protein and ATPase activity becoming rebound to the doublet tubules. Electron microscopy showed that a high percentage of the arms had been restored to their original positions on the doublet tubules (Fig. 6*b*). This correlation of the presence or absence of ATPase activity with the presence or absence of the arms was taken to indicate that at least part of the axonemal ATPase was located in the arms.

Study of the physicochemical properties of the solubilized axonemal ATPase by Gibbons and Arthur Rowe (62) confirmed that its properties were quite distinct from those of the muscle ATPase, myosin; the name "dynein" (after dyne = a unit of force) was proposed for the axonemal ATPases and other related ATPases associated with microtubule systems. The dynein from *Tetrahymena* cilia occurred in two forms with sedimentation coefficients ($s^0_{20,w}$) of 14S and 30S, and average molecular weights of 600,000 and 5,400,000, respectively. Electron microscopy of shadow-cast particles showed that the 14S dynein consisted of globular particles measuring about 14 × 9 × 9 nm, whereas the 30S dynein consisted of rodlike particles of variable length, with a globular substructure repeating at a period of about 14 nm. Detailed examination of the recombination of the two forms of dynein to extracted axonemes indicated that only the 30S dynein was capable of rebinding and restoring the arms on the doublets, and that little 14S dynein became bound under the same conditions (78).

Extensive further studies have been performed on the enzymic properties of the two forms of dynein from *Tetrahymena*. The reports of Gibbons, of Blum and collaborators (35), and of Issei Mabuchi, Takashi Shimizu, and Ichiro Kimura (79) have shown that the ATPase activity of 30S dynein can be activated two- to sixfold by any of a number of treatments including high concentrations of salt, mild heating, acetone, SH reagents, and amino reagents, whereas the same reagents applied to 14S dynein usually cause only inhibition. Although 30S dynein can be broken down to 14S particles by sonication or by brief treatment with trypsin, the properties of these particles are not the same as those of the 14S dynein obtained directly by extraction at low ionic strength (35).

The development by A. Shapiro, E. Viñuela, and J. Maizel (80) of the technique of electrophoresis in polyacrylamide gels containing Na dodecyl SO_4, which enables easy analysis of the number and size of distinct polypeptides in a sample, had a major impact on studies of the composition of cilia and flagella. In 1970–1973, Richard Linck applied this technique to the study of axonemes and semipurified axonemal extracts containing 14S dynein ATPase from gill cilia and sperm flagella of the lamellibranch mollusc *Aequipecten*, and found that the presence of dynein ATPase activity was correlated with the presence of the upper of a closely spaced pair of slowly migrating electrophoretic bands with apparent molecular weights of 450,000–500,000 daltons (81). Subsequent studies have confirmed that at least a major part of the dynein ATPase copurifies with the slower migrating band, but have reported somewhat smaller values for the high molecular-weight polypeptides, in the range 300,000–350,000 daltons (82). The presence of one or more polypeptides in the 300,000–350,000 dalton range appears to be a characteristic property of dynein that distinguishes it from myosin and other ATPases.

Improvements in electrophoretic techniques, like improvements in electron microscopy, have revealed additional layers of complexity (Fig. 7). In 1976, Gibbons and colleagues (85) reexamined sea urchin sperm axonemes and were able to resolve four high molecular-weight bands in the same region as the two bands observed earlier. A further improvement in resolution was obtained by using the discontinuous-pH Na dodecyl SO_4 procedure of Ulrich Laemmli (86), and in 1979 Christopher Bell, Earl Fronk, and Gibbons (83) resolved as many as eight distinct high molecular-weight bands in this same region (Fig. 7*b*). A similar electrophoresis procedure applied to axonemes of *Chlamydomonas* flagella by Gianni Piperno and David Luck (84) resolved 10 bands with apparent weights between 300,000 and 330,000 daltons. Analysis of axonemes from spermatozoa of species in which the axonemal structure is simplified have shown a roughly parallel decrease in the number of high molecular-weight bands present (74). In these very high-resolution gel systems, the relative mobilities of different high molecular-weight bands vary even between closely related species, and it has not yet been possible to identify which bands correspond to functionally equivalent polypeptides in axonemes from different species.

Fractionation of the axonemal proteins containing the various high molecular-weight polypeptides, either by differential extraction (81, 85) or by chromatography on hydroxyapatite as used by Kazuo Ogawa and Mohri (89, 91, 92), has indicated that several, although probably not all, of these proteins have ATPase activity. Gibbons and colleagues have distinguished dynein 1 and dynein 2 as two electrophoretically distinct isoenzymic forms from sea urchin sperm axonemes (85, 88). Dynein 1, which constitutes the outer arms, can be solubilized

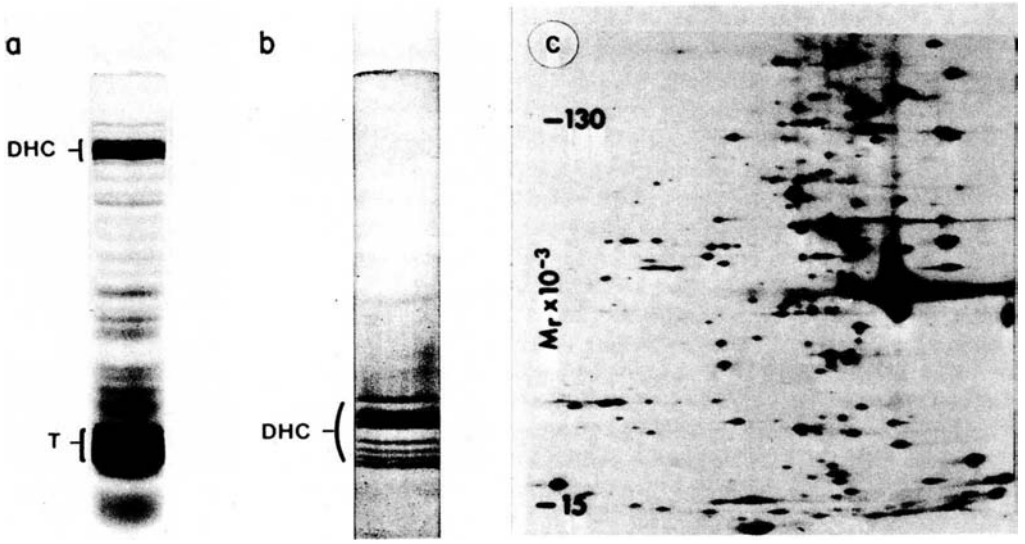

FIGURE 7 (*a*) Electrophoresis of axonemes from sea-urchin sperm flagella on 4% polyacrylamide gel in presence of 0.1% Na dodecyl SO_4, 50 mM phosphate buffer, pH 7.0. DHC indicates the group of dynein heavy chains migrating at a position corresponding to ca. 330,000 molecular weight; T indicates tubulin migrating at 55,000 molecular weight. Gibbons and Fronk, 1975 (unpublished). (*b*) Similar sample electrophoresed on Laemmli discontinuous pH Tris-Cl/Na dodecyl SO_4 system. Run was continued for twice time required for dye front to reach bottom of gel. Complex of high molecular weight bands containing dynein heavy chains now shows 8 bands. Bell, Fronk, and Gibbons, 1980 (83). (*c*) Autoradiogram of polyacrylamide slab gel used for two-dimensional separation of polypeptides in whole axonemes of *Chlamydomonas*. Horizontal separation between spots derives from isoelectric focusing (first dimension), with the more basic polypeptides lying on the right. Vertical separation derives from electrophoresis in the presence of Na dodecyl SO_4, and corresponds to differences in molecular weight. Only a portion of the original gel is shown, comprising the molecular weight range between 130,000 and 15,000 (see marker on left). The large streak is formed by the tubulin polypeptides, which are heavily overloaded in order to detect minor axonemal components. Piperno and Luck, 1979 (84).

as a 21S particle of 1,250,000 daltons with a complex polypeptide composition, and retains functional capability to rebind and restore the beat frequency of dynein-depleted sperm flagella (87, 90). This 21S form of dynein 1 can be dissociated into smaller particles that retain ATPase activity, but have lost the ability for functional recombination (87). Ogawa (91) has used trypsin digestion to isolate a 400,000 dalton fragment of dynein 1 that retains ATPase activity, but has lost the ability to rebind to dynein-depleted axonemes. An antibody prepared against this tryptic fragment inhibits the ATPase activity of dynein 1, but not that of dynein 2 (88, 92). The 14S and 30S dyneins from *Tetrahymena* cilia are also isoenzymic forms with electrophoretically distinct heavy chains (79). Dynein extracted from *Chlamydomonas* flagella by Takahiko Watanabe and Martin Flavin showed two forms sedimenting at 13S and 18S (93), and further chromatographic separation by Piperno and Luck (84) has indicated the presence of at least three isoenzymic forms of dynein with electrophoretically distinct heavy chains. In two *Chlamydomonas* mutants lacking outer arms, pf13 and pf22, the 13S and 18S ATPases are both missing, suggesting that the outer arms in these flagella contain two ATPase proteins. The set of polypeptides missing in a mutant lacking inner arms, pf23, shows no overlap with those missing in the outer arm mutants (61).

A characterization of the protein constituting the walls of ciliary tubules was begun in 1963 by Gibbons (25) and extended in 1966–1968 by Fernando Renaud, Rowe, and Gibbons (94, 95), who employed both acetone powders of whole cilia from *Tetrahymena*, and preparations of doublet tubules isolated by selective solubilization. The tubule protein was found to migrate as two closely spaced bands of equal intensity upon electrophoresis in polyacrylamide gels containing 8 M urea, to possess an amino acid composition resembling that of actin, and to exist as a 6.0S dimer of 108,000 daltons at low ionic strength and as a monomer of approximately 55,000 daltons in 8 M urea or in 5 M guanidine-HCl. Studies by Stephens, Renaud, and Gibbons (96) also showed that the dimer of tubule protein from cilia and sperm flagella contained 2 mol of mixed guanine derivatives, half of which were tightly bound. At this time, the tubule protein appeared to have many properties resembling actin, but shortly afterward the generally accepted weight of actin was revised sharply downward from 57,000 to 46,000 daltons (97), and it became clear that the two proteins were distinct.

At approximately the same time as the above work on cilia and flagella, Gary Borisy and Edwin Taylor (98) were studying the properties of a 6S colchicine-binding protein found in tissue culture cells and in several types of tissue containing high densities of microtubules, and they proposed that this protein was a subunit of microtubules. In a related study, Michael Shelanski and Taylor (99) used brief dialysis in the usual Gibbons fractionation procedure to isolate a 6S colchicine-binding protein from sea urchin sperm flagella, and identified it as the protein of the central tubules.

On the basis of its distinct amino acid composition, the protein of flagellar tubules was given the generic name "tubulin" by Mohri (100). Attempts to repolymerize tubulin from *Tetrahymena* cilia and from sperm flagella by Renaud et al. (94) and by Stephens (101) yielded only fibers and ribbons of protofilaments. Repolymerization of tubulin into intact tubules that had the same properties as naturally occurring tubules was first achieved with brain tubulin incubated in the presence of GTP at 37°C by Richard Weisenberg in 1972 (102). This discovery made it possible to purify tubulin by cyclic assembly/disassembly of tubules. An equivalent repolymerization of tubulin from cilia or sperm flagella was not achieved until

1976, when Ryoko Kuriyama (103) showed that tubulin solubilized from flagella by sonication would repolymerize under the standard conditions used to polymerize brain tubulin. The resulting tubules are singlets, and their stability is more like that of labile brain microtubules than that of flagellar doublets.

Although earlier workers had noted differences in the relative stability of different types of microtubules, these differences were first systematized by Olav Behnke and Arthur Forer (104), who distinguished four classes—cytoplasmic, ciliary or flagellar central pair, B tubule, and A tubule, in order of increasing stability. The subunit composition of the tubulin dimer was for a time confused with differences among these stability classes, but in 1971 several groups of workers independently showed that cytoplasmic tubules from a single source gave rise to two closely spaced bands of equal intensity when electrophoresed under appropriate conditions, and concluded that the 110,000 dalton 6.0S tubulin molecule was probably a heterodimer composed of α- and β-subunits (105). These α- and β-subunits were isolated electrophoretically from tubulin of chick embryo and of sea urchin spermatozoa by Richard Ludueña and Don Woodward (106), who then used cyanogen bromide peptide-mapping and a partial amino acid sequence to show that the α- and β-subunits were similar but distinct polypeptides, and that the sequence of each had been highly conserved during evolution. Microheterogeneity has been reported in the α- and β-chains of tubulins from different types of tubules and organelles (107), but it is not yet clear whether this results from multiple α- and β-tubulin genes or from posttranscriptional modifications.

In addition to the major components, dynein and tubulin, axonemes contain a large number of minor components present in relatively small quantity. The number of minor polypeptides is such that they can be resolved adequately only on a two-dimensional system (Fig. 7 *a*, *c*). The application of such techniques to flagella was pioneered by Piperno, Bessie Huang, and Luck (108), who analyzed ^{35}S-labeled axonemes from *Chlamydomonas* by an isoelectric-focusing/Na dodecyl SO_4-electrophoresis procedure modified from that developed by Patrick O'Farrell. In its present state of refinement, this procedure reveals as many as 180 polypeptides (Fig. 7 *c*) (84). Flagella from the paralyzed mutant pf14, which completely lacks radial spokes and spoke heads, are missing 12 polypeptides, whereas those from pf1, in which only the spoke heads are absent, lack 6 polypeptides that are a subset of the 12 missing in pf14. Subsequent study of flagella in which motility was rescued in the dikaryon formed by mating with wild type, together with analysis of UV-induced revertants, enabled identification of the two polypeptides that are the mutant gene products in pf1 and pf14 (109).

Piperno and Luck (110) have used chromatography on a DNase 1 affinity column to purify a component from *Chlamydomonas* flagella axonemes that appears identical with β-actin. It constitutes about 1.5% of the axonemal protein, corresponding to a molar ratio of 1:40 relative to tubulin and may be associated with one of the high molecular-weight polypeptides, but its significance in flagellar function is unknown. Other recent work by Gordon Jamieson, Thomas Vanaman, and Blum (111) has shown that chromatography on a chlorpromazine affinity column can be used to isolate calmodulin from *Tetrahymena* cilia. Calmodulin occurs partly associated with the 14S dynein fraction and is presumably involved in the mechanisms by which Ca^{2+} regulates the direction of beating.

Wave Parameters and the Hydrodynamics of Propulsion

As discovered by Gray in 1955 (6), the flagellar beating of marine invertebrate spermatozoa, such as those of sea urchin, is nearly planar and almost ideal for waveform analysis. When these spermatozoa encounter an obstructing surface, such as the bottom of an observation dish, they become trapped by it and, without actually being tethered, they swim in repeated circles, with their plane of flagellar beat parallel and close to the surface, and so remain constantly within the plane of focus. This circling movement makes possible extended observation of the wave parameters of an individual sperm flagellum; the degree of constraint on beating is much less than if the sperm head were tethered to the surface. Gray reported that the flagella propagated planar bending waves along their lengths at a beat frequency of 30–40 Hz, and that at certain stages of the beat cycle the flagellum had the form of a sine curve (Fig. 8 *a*), although there was an overall asymmetry in the degree of bending on the two sides of the flagellum. A subsequent analysis of sea urchin sperm flagellar movement by Charles Brokaw (113), who used a 100-μs flash to achieve improved spatial resolution, indicated that the waveform at any instant could be represented more accurately by a series of circular arcs joined by short, straight segments than by a sine curve; the departure of the flagella from a sinusoidal waveform is particularly evident in spermatozoa with tethered heads (Fig. 8 *b*, *d*) (112, 113). This "arc-line" waveform has been accepted by most subsequent workers as being a reasonably close approximation to actual flagellar waveforms, although it has often been noted that the curvature of bends at certain stages of the beat cycle is noticeably nonuniform (114, 115). An important basic parameter of the arc-line waveform is the total angle of each bend, for in a sliding-tubule mechanism (see below), the amount of sliding displacement in a bend is proportional to its angle. In situations where the arc-line curve does not represent a flagellar waveform with sufficient accuracy, the likelihood of significant end effects suggests that it may be more helpful to analyze the waveform numerically rather than by attempting to fit more refined analytical curves relatable to the underlying mechanisms involved.

The obvious importance of the motility of mammalian spermatozoa in both human and veterinary medicine has led to fairly numerous studies of their flagellar waveforms. The initial

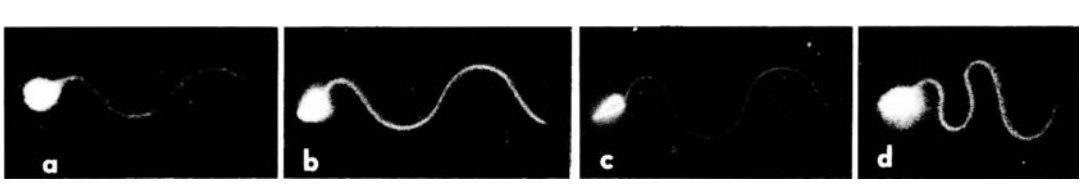

FIGURE 8 Flagellar waveforms of sea urchin spermatozoa. (*a*) Live spermatozoon in seawater. Species: *Psammechinus miliaris*. Gray, 1955 (6). (*b*) Live spermatozoon in seawater. Species: *Tripneustes gratilla*. Gibbons, 1974 (112). (*c*) Demembranated spermatozoon reactivated with 1 mM ATP. Species: *Tripneustes gratilla*. B. Gibbons, unpublished, 1980. (*d*) Live spermatozoon in seawater with head stuck to bottom of dish. Species: *Tripneustes gratilla*. Gibbons, 1974 (112).

studies of bull spermatozoa by Gray (116) and by Robert Rikmenspoel, G. van Herpen, and P. Eijkhout (117) showed that the amplitude of bending increases progressively along the tail toward the distal end, and that whereas the movement in the proximal region is planar, that of the distal region contains a significant three-dimensional component. More recent observations by David Phillips (69), and David Katz, R. Mills, and T. Pritchett (118) have shown that the pattern of flagellar movement in mammalian spermatozoa can undergo drastic change when they swim in close proximity to a surface or enter cervical mucus.

The motion of individual cilia on ciliated epithelia or on the surface of a ciliated protozoan is more difficult to visualize than that of flagella, partly because of their large number and close packing, and partly because the form of their beat is more complex. As a result of this difficulty, the early workers usually assumed that the motion of individual cilia was similar to that of the relatively large and easily observed compound cilia, which beat with a planar asymmetric movement composed of a rapid, effective stroke followed by a relatively slow recovery stroke. The first clear indication that this was not the case was obtained in an extended series of studies by Bela Párducz (119, 120). He modified the procedure for preserving ciliary waveforms by rapid fixation with a mixture of OsO_4 and $HgCl_2$ that had been developed in 1926–1927 by J. von Gelei, and used it to show that the beat of the body cilia in *Paramecium* is not planar, for, in the recovery stroke, the cilium sweeps out to the side and makes the overall beat cycle markedly three-dimensional, with the tip moving clockwise in an approximately semicircular path. This observation was not generally accepted at first because of the possibility that the rapid fixation might not be preserving accurately the waveform of the live cell. However, comparative studies of live and rapidly fixed waveforms have been made for *Opalina* by Sidney Tamm and Adrian Horridge (121), for the lateral cilia of *Elliptio* and of *Mytilus* by Peter Satir (122) and Edward Aiello and Michael Sleigh (123), and for *Paramecium* by Hans Machemer (124), and, in all cases, the three-dimensional waveforms seen in rapidly fixed preparations for light microscopy and scanning electron microscopy have appeared to be reasonably true representations of the waveforms in the live state. Somewhat surprisingly, the tips of the lateral cilia of *Mytilus* move in a counterclockwise direction (123), the mirror-image of that in *Paramecium*, in spite of the fact that structural enantiomorphism of the arms on the doublet tubules is clockwise in both cases (43). As emphasized by Párducz, the basic rotary motion of protozoan cilia is particularly evident in narcotized or moribund ciliates (e.g., *Paramecium*, *Colpidium*, and *Opalina*), in which, as the beat frequency falls to around 2 Hz, the cilia change from their normal beat pattern to a swiveling about their basal region in such a way that the ciliary shaft sweeps continuously around a wide-angled, conical envelope, moving clockwise (120).

Because of the close spacing of cilia on most ciliated cells, they need to move in a coordinated manner to work effectively. The coordination of cilia into propagated, metachronal waves used to be considered the result of a "neuroid" transmission process within the cell, but it is now generally thought to be the result of hydrodynamic forces acting on the autonomous beating of the individual cilia (124).

Sir Geoffrey Taylor appears to have been the first to realize that the propulsive forces of flagella and cilia result almost wholly from their viscous interaction with the medium (7), and that they cannot be modeled, even qualitatively, by the effect of the human arm in swimming in water. (Reynolds number, which is the ratio of inertial to viscous forces, has values of 10^{-4} to 10^{-6} for individual cilia and flagella, 10^{-2} to 10^{-1} for the body of ciliated protozoa, and of the order of 10^{4} for human swimming [33].) A rigorous treatment of the movement of flagellated microorganisms requires solution of Stokes equations with the appropriate boundary conditions. Equations appropriate for propulsion by flagellar waves of normal amplitude were developed by Hancock in 1953 (125), but the form of the equations is such that computation is not simple. This factor led Gray and Hancock (8) to develop a simplified computation based upon expressing the viscous force acting on a short element of flagellum in terms of normal and tangential coefficients of resistance, which is equivalent to assuming that the velocity field around the element is independent of the cell body and of the bending of other parts of the flagellum. They were then able to show that the forward velocity of the sperm computed from its observed flagellar waveform was in good agreement with that actually observed, and also to show that the viscous drag of the sperm head was small compared to that of the flagellum itself. This approach was extended by Brokaw (113, 126) to propulsion by flagella with nonsinusoidal waveforms of moderate asymmetry and showed that the computed time-averaged velocity and the angular velocity of yaw were both close to the actual measured values.

While the resistance coefficient model was being applied in this way, other workers were attempting to develop a more rigorously based hydrodynamic approach, and especially to consider the effect of the cell body on the fluid flow around the flagellum. A recent study of J. Higdon (127) used an iterative numerical procedure to consider the case of a spherical head propelled by planar sinusoidal waves and showed that minimal power consumption for locomotion of a given size head is obtained when the flageller length is 20–40 times the radius of the head, which agrees with the values found for actual spermatozoa. Comparison with results obtained using the resistive coefficient procedure indicated that the predicted swimming speed agreed within 10%, as was to be expected inasmuch as the predicted speed agrees with that of real sperm. However, the Gray-Hancock procedure appears to underestimate the power consumption by 30–50% for small cell bodies such as spermatozoa.

The hydrodynamic analysis of propulsion by large fields of cilia beating in metachronal rhythm requires a different approach from that of propulsion by a single flagellum. In the first approach to the problem by John Blake in 1971 (128), the ciliary motion was represented by a surface envelope containing the tips of the cilia, with the metachronal waves being modeled as nonsinusoidal undulations in this surface envelope. A second approach initiated by Blake (129) considers discrete cilia and involves calculating the velocity of fluid flow as a function of distance from the body surface, including both the ciliary sublayer and the exterior flow field. The calculated velocity profile for a spherical model of *Paramecium* is in reasonable agreement with the experimental observations of Theodore Jahn and J. Votta (130).

The substantial advances in hydrodynamic theory during the past few years have made it feasible to make detailed comparisons of the calculated and experimental propulsive velocities and flow fields associated with swimming and fluid propulsion by cilia and flagella in different organisms. Such comparisons may reveal something of the wide variety of ways that different organisms have exploited the basic uniformity of movement in cilia and flagella.

Theoretical Models

Various attempts have been made to create theoretical models that will reproduce the observed oscillatory beating of flagella and cilia as the result of balancing an active bending moment, M_a, which is dependent upon the parameters of bending, against the passive viscous and elastic resistances, M_v and M_e, according to the equation $M_a + M_v + M_e = 0$ at all locations along the length.

Initial work by Kenneth Machin (131) showed that waves generated by active bending moments located solely at the base of the flagellum would be highly damped by the viscous and elastic resistances distributed along the flagellar length, with the wave amplitude decreasing by 50% or more within half a wavelength of the proximal end. On the other hand, waveforms resembling those of real sperm flagella could be obtained by assuming generation of active bending moments by contractile elements distributed along the length of the flagellum, with these elements being activated by local bending after an appropriate time delay. Machin subsequently extended this work (132) to show that propagated bending waves could arise spontaneously on a flagellum, if changes in length of its contractile elements cause delayed changes in tension. The nonlinearities that must exist for the wave amplitude to remain finite were found to enable control of frequency and direction of propagation to be exercised from the proximal end, and indicated that two nearby flagella would tend to synchronize in frequency and phase.

The approach used by Rikmenspoel (133, 134) has been to balance the calculated external viscous resistance and the internal elastic bending resistance by an active moment specified as an arbitrary forcing function dependent upon time and position along the flagellum. A forcing function was found that reproduced the motion of a variety of cilia, but it required specifying two time constants as arbitrary parameters, as well as the observed velocity of bend propagation and the length of the bent region (133). In similar studies on the motion of flagella, Rikmenspoel reported that waves resembling those of sea urchin sperm flagella could be generated by a nonpropagated active moment varying sinusoidally with time, together with a propagated active moment of appropriate phase (133, 135).

Brokaw has developed several models of wave formation and propagation in flagella, most of which involve numerical solution of the equations of motion for a time-delayed active shear force proportional to curvature (136, 137). It was originally thought that four passive internal forces—viscous and elastic shear resistances and viscous and elastic bending resistances—were required to stabilize the motion, but more recent work has shown that the apparent need for viscous shear and bending resistances derived from problems with the numerical solution of the equations (35).

Investigations with these formal models have been useful in clarifying the constraints necessary for stable oscillations in a sliding-tubule system. However, more realistic models must consider the kinetic parameters of the cross bridges involved in producing sliding, and a thermodynamic framework for these parameters has been developed by Terrell Hill (138). The cooperative self-oscillating behavior of opposed cross-bridge systems is potentially interesting, because of the possibility of initiation and propagation of bending waves without need for control by a macroscopic variable such as curvature, but such models have so far been able to propagate bending waves only under conditions of high internal viscosity (139). More satisfactory results have been obtained with two-state cross-bridge models involving curvature-dependent rate functions, and Michael Hines and Joseph Blum (35, 140) have shown that such models will generate stable propagated waves with frequencies and amplitudes typical of sperm flagella. However, even the best of current models does not provide a completely satisfactory explanation of the mechanisms that control bending in flagella and cilia. They have particular difficulty in explaining the high curvature of developing bends at the basal end of flagella, and the observed independence between the waveform and the beat frequency.

Functional Mechanisms

The possible mechanisms by which the then-recently discovered, fine stuctural components of flagella and cilia might give rise to their motility were discussed in 1955 by Bradfield (141) and by Gray (6). On the strength of Gray's (1928) argument (3) that "a moving wave cannot provide the energy for propelling an organism and at the same time pass on with unreduced amplitude, unless the energy being lost is continually being replaced as the waves pass along," Bradfield concluded that the 9 + 2 tubule bundle plus its matrix and membrane produce much, if not all, of the force necessary for movement. On this basis, he advanced a hypothesis founded largely on the assumptions that the nine doublet tubules are capable of propagating active, localized contractions along their lengths, that the impulses producing contraction arise rhythmically at the basal end of one doublet, and that propagation of the contractile activity to the other doublets around the axonemal cylinder is unidirectional in cilia and bidirectional in flagella having a planar beat. In his 1955 study of beating in sea urchin sperm flagella, Gray supported his argument given above with experiments using celluloid models, and independently proposed a hypothesis explaining flagellar beating in terms of active localized contractions propagated along the doublet tubules. One of the most sriking features of these two reports is that they were almost wholly based upon localized contractions of tubules, an extension of William Astbury's well-established contractile fiber hypothesis (142), with little indication as to how the energy for repeated contractions might be supplied. The only mention of sliding (by Bradfield) was as a possible basis for tubule contraction with one component of each doublet tubule "sliding up on the other, without either shortening, in the manner suggested for muscle by Hanson and Huxley," thus reflecting the very tentative acceptance of what was then the radically new sliding-filament model for muscle.

By 1959, when Afzelius (38) described the arms and the radial spokes on the doublet tubules in sea urchin sperm flagella, the sliding-filament mechanism of muscle contraction was no longer a novelty and had gained widespread acceptance. The structural analogy between flagellar arms and the cross bridges on the thick filaments in muscle led Afzelius to suggest a sliding-tubule model in which flagellar bending was based upon relative sliding movement between adjacent doublets as a result of activity of the arms on the doublets. He calculated that a relative sliding movement of 0.23 μm would be sufficient to account for the observed bending, and noted that some pairs of doublets were better situated to produce bending than others.

More direct evidence for a sliding-tubule mechanism was obtained by Satir (122, 143, 144) in a series of electron microscope studies between 1963 and 1968. Using a modification of the rapid fixation procedure of Párducz to preserve the metachronal pattern in actively beating gill cilia of the lamelli-

branch, *Elliptio*, Satir focused attention on the structure of the tips of cilia fixed either at the end of their effective stroke or at the end of their recovery stroke, and found that in both cases, the tubules located on the inside of the bend in the cilium protruded beyond those on the outside of the bend, as would be expected if the tubules slide relative to one another, with their lengths remaining constant. In later work (144, 145), the amount of sliding displacement of each doublet was found quantitatively equal to that predicted by the geometry of a bend, on the assumption that the lengths of all doublets remain constant during bending, and that no sliding occurs at the basal end. This work provided the first experimental evidence for a sliding-tubule, as opposed to a contractile, mechanism of ciliary beating. Additional indirect support came from an observation of Brokaw on the movement of sea urchin spermatozoa in solutions containing thiourea (113).

The period 1955–1970 also saw the development of a powerful new approach to the functional mechanisms of flagella and cilia that was based upon removal of the membrane barrier so that the motile mechanism would be directly accessible to experimental manipulation. In 1955, Hoffmann-Berling (14) discovered that grasshopper sperm flagella, in which the selective permeability of the membrane had been destroyed by treatment with 50% glycerol, could be reactivated by addition of exogenous ATP. In spite of the limitation that the flagella in these preparations beat only rhythmically from side to side and did not propagate bending waves along their length, Hoffmann-Berling was able to demonstrate that beat frequency increased with ATP concentration up to about 1 mM, and that the presence of Mg^{2+} was essential for motility and could not be substituted for by Ca^{2+}.

Propagation of bends in reactivated flagella appears to have been first achieved by Brokaw in 1961, using glycerol-extracted flagella isolated from *Polytoma* (146). In similar preparations of glycerinated sea urchin spermatozoa (147), it was found that the rate of ATP hydrolysis by motile flagella was greater than that of the same flagella in which motility had been prevented by gentle homogenizing, and this difference in rates was termed the "movement-coupled ATPase activity" by Brokaw. In these reactivated preparations in which 25–50% of the flagella were motile, it amounted to about 40% of the total ATPase activity. However, measurement of O_2 uptake by live spermatozoa indicated that the fraction of motility-dependent metabolism was as high as 80% of the total metabolism (148).

In 1969, Barbara Gibbons and Ian Gibbons discovered that improved reactivation could be obtained by replacing glycerol with the nonionic detergent, Triton X-100 (polyoxyethylene isooctylphenol ether)—first tried at the suggestion of Raymond Stephens—which completely removed the membranes from the flagella of sea urchin spermatozoa (149, 150). The resulting demembranated spermatozoa became essentially 100% motile when subsequently reactivated with ATP, and their flagellar beat (Fig. 8*c*) was very similar to that of live spermatozoa. Probably because of their high motility, the percentage of motility-coupled ATPase activity in these preparations was found to be as high as 70–80% (150).

Shortly thereafter, conclusive evidence for the occurrence of active sliding between flagellar tubules was provided by the work of Keith Summers and Gibbons (151, 152), who isolated Triton-demembranated flagellar axonemes from sea urchin sperm and digested them briefly with trypsin. The subsequent addition of ATP caused a disintegration of the axoneme into separated microtubular doublets, and direct visual observation by dark-field light microscopy showed that this disintegration occurred by extrusion of tubules from the axoneme by a gradual sliding process (Fig. 9) and that the length, after disintegration was complete, ranged up to eight times that of the original axonemal fragment. The ATP requirement and divalent cation specificity for this sliding closely matched the requirements for normal beating in undigested axonemes. These observations also indicated that the presence of the centriole completely blocked the sliding of tubules at the basal end of the flagellum. Examination of the trypsin digestion as a function of time showed that the rate at which the axonemes were sensitized to disintegration by ATP paralleled the rate of disruption of the nexin links and the radial spokes, whereas the dynein arms and the tubules themselves were relatively resistant to disruption by trypsin (152). As a result, it was concluded that the dynein arms generate active shearing stress between adjacent doublet tubules, and that in the intact axoneme these shear stresses are coordinated and resisted by the radial spokes and the nexin links, leading to the formation and propagation of bending waves; whereas in trypsin-treated axonemes, in which the nexin links and radial spokes are disrupted, these shear stresses lead to unlimited sliding and the disintegration of the axoneme. In an extension of this work, Winfield Sale and Satir (153) used electron microscopy to study axonemes of *Tetrahymena* cilia that had undergone

FIGURE 9 Dark-field light micrographs of trypsin-treated axonemes reacting to ATP. The successive micrographs from left to right were taken at intervals of 10–30 s. A large group of tubules is shown sliding toward the bottom right of the fields, leaving behind a smaller, stationary group of a few tubules attached to the coverglass. The free, forward end of the sliding group coils around out of the plane of focus, and in the final micrograph, it has coiled completely around and come back into the focal plane. Summers and Gibbons 1971 (151).

sliding disintegration, and found that the direction of sliding was always such that the arms on tubule A of one doublet pushed the B tubule of the adjacent doublet toward the tip of the cilium. The renaissance of interest in dark-field light microscopy has since extended its use to observe the movement of individual bacterial flagella (154) and to measure the growth rates of polymerizing microtubules (155) and the rigidity of individual actin filaments decorated with heavy meromyosin (156).

Further studies by Gibbons and Gibbons have shown that extraction of the demembranated spermatozoa of the sea urchin *Colobocentrotus* with 0.5 M KCl (157) results in a selective removal of the outer arms from the doublet tubules (Fig. 10), and that when the resultant KCl-extracted spermatozoa are reactivated with 1 mM ATP, their flagellar beat frequency is decreased in proportion to the number of arms removed while their waveform remains essentially unchanged; this suggests that the inner and outer arms on the doublet tubules are functionally equivalent and that the rate of sliding between doublets under these conditions is proportional to the total number of outer and inner arms present. A second type of experiment indicated that the sperm flagella could be set into stationary waveforms by reactivating them with 30 μM ATP and then rapidly diluting into a large volume of reactivating solution containing no ATP (158). By analogy to muscle in rigor mortis, these stationary flagellar waveforms have been termed "rigor waves." The rigor waves relax slowly (straighten) upon addition of 1–5 μM ATP, which is too low a concentration to support oscillatory bending, whereas higher concentrations of ATP cause resumption of normal beating. Qualitative study of the mechanical properties of flagella bent in rigor waves showed that they could easily be twisted by the viscous force of fluid flow, but that they are very resistant to straightening. These properties have been explained on the basis that the arms form fixed cross bridges between the doublet tubules in the absence of ATP. After appropriate fixation, these cross bridges can be visualized by electron microscopy (Fig. 11) (159). These two studies provided confirmation that the sliding between doublet tubules is produced by an ATP-driven cyclic interaction of the arms on the A tubule of the doublet with sites along the length of the B tubule of the adjacent doublet.

A local reactivation procedure has been used by Chikako Shingyoji, Akira Murakami, and Keiichi Takahashi (160) in an elegant confirmation of the sliding-tubule mechanism. These workers used iontophoresis from a micropipette to apply brief pulses of ATP to localized regions along the lengths of axonemes in demembranated sea urchin spermatozoa. Application of a pulse of ATP to the midregion of the axoneme caused formation of two bends of equal and opposite angle in the region where the ATP was applied, whereas the overall angle between the head and flagellar tip was unchanged. This result is exactly as would be predicted for a brief period of active sliding localized in the zone where the ATP was applied, with no sliding able to occur at the basal and tip ends where there was no significant ATP.

In 1974, Brokaw, R. Josslin, and Lynette Bobrow (161) showed that the asymmetry of the bending waves in reactivated sperm flagella is dependent upon Ca^{2+}. This effect of Ca^{2+} appears to involve two distinct processes, one being an apparently irreversible Ca^{2+}-dependent process that occurs during demembranation with Triton X-100, and the second, a reversible effect of Ca^{2+} concentration in the reactivating solution, with increased Ca^{2+} causing greater asymmetry. Recent extension of this work by Gibbons and Gibbons has shown that

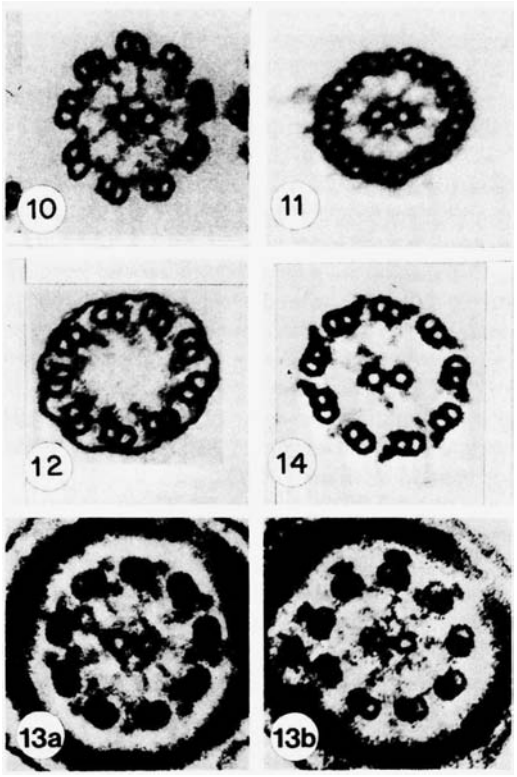

FIGURE 10 Axonemal cross section of sea urchin spermatozoon demembranated in 0.04% Triton X-100 containing 0.5 M KCl. Note absence of outer arms. Gibbons and Gibbons, 1973 (157).

FIGURE 11 Axonemal cross section of sea urchin spermatozoon fixed under conditions that preserve rigor waveforms. Gibbons, 1975 (159).

FIGURE 12 Cross section of 9 + 0 axoneme with only inner arms in spermatozoon of eel. Baccetti, Burrini, Dallai, and Pallini, 1979 (74).

FIGURE 13 Cross sections of principal piece of human sperm flagella. *(a)* Normal; *(b)* from patient with Kartagener's syndrome. Spermatozoa are nonmotile and appear to lack both inner and outer arms. Afzelius, 1976 (188). (Copyright 1976 by the American Association for the Advancement of Science.)

FIGURE 14 Cross section of isolated axoneme of *Chlamydomonas* mutant pf 23, which lacks inner arms. Huang, Piperno, and Luck, 1979 (61).

driving the sperm to an extreme degree of asymmetry causes them to become quiescent, with their flagella bent into a highly asymmetric cane-shaped form (67), and that the action of Ca^{2+} in causing asymmetrical bending and quiescence can be mimicked by low concentrations of methanol.[1]

A development of growing importance in the study of functional mechanisms has been the successive refinement of micromanipulation procedures for measuring the active bending moments produced by cilia and flagella and relating these to measured values of stiffness under different conditions. These procedures are based upon the early work of Haruo Kinoshita and Takeo Kamada with microneedles on the compound abfrontal cilium of *Mytilus* gill (162). In 1960, Mitsuki Yoneda (163) first succeeded in measuring the force exerted by this abfrontal cilium by the bending of a calibrated microneedle. Later work by Shoji Baba (164) measured the flexural rigidity and reported that, contrary to earlier ideas based largely upon

[1] Gibbons, B. H., and I. R. Gibbons. 1981. *Nature (Lond.)*. In press.

visual observation, the measured stiffness had an almost constant value irrespective of the stage of the beat cycle and of the direction of the force applied. A similar procedure was used by Charles Lindemann, W. Rudd, and R. Rikmenspoel (165) to determine the stiffness of individual bull sperm flagella. Extension of this work to the thinner flagella of echinoderm spermatozoa was achieved in 1979 by Makoto Okuno and Yukio Hiramoto (166), who showed that the stiffness of live flagella immobilized with CO_2 was only 5–10% of that of demembranated flagella in the absence of ATP. The stiffness of the immobilized, live flagella varied two- to three-fold when the spermatozoon was rotated about its long axis, whereas the stiffness of the demembranated rigor flagella was unaffected by such change in orientation. The change in stiffness of live flagella with orientation may reflect the relationship between the plane of the central tubules and the plane of bending. The high stiffness of rigor flagella presumably reflects the cross-bridges between doublets that are responsible for maintenance of rigor waves (158, 159).

In 1974, Warner and Satir (53) made a detailed study of the radial spokes in straight and bent regions of gill cilia fixed while beating. Their results indicated that the relative positions of the triplet spoke groups on any two doublet tubules remain constant in straight regions of the axoneme, either proximal or distal to a bend. However, in bent regions the positions of the spoke groups change systematically up to a maximum of 12 nm per group for tubules on opposite sides of the axoneme, whereas individual spokes tilted at angles up to 33° from their usual position roughly normal to the doublets. These observations confirmed Satir's earlier evidence that the lengths of the tubules remain constant during bending (143, 144), and provided direct evidence to support the earlier suggestions that the radial spokes play a major role in resisting sliding and converting it to bending (151). The limited range of tilt angles of the spokes in bent regions of the cilia suggested that the radial spokes are able to detach and reattach their connections to the projections of the central sheath. The factors influencing this cyclic detachment and reattachment of the radial spokes, and whether it is a passive process or an active process driven by ATP, remain unknown. The nexin links, on the other hand, appear to be elastic and to have permanent attachments to the tubules (53, 57). Their function may be to prevent the occurrence of excessive sliding displacement between tubules. When the sliding displacement between adjacent tubules attains a level of about 140 nm, the elastic limit of the nexin appears to be reached, and no further displacement occurs unless the flagellum is forced to such an extent that its structure is permanently damaged (67).

The hypothesis that the radial spokes and the central sheath complex to which they attach play a major role in coordinating sliding among the outer doublets and converting it into bending is supported by the work of George Witman, J. Plummer, and G. Sander (50), showing that the flagella of three mutants of *Chlamydomonas*, which lack either the radial spokes or the central tubules and sheath, are all paralyzed, although they are all capable of sliding disintegration after trypsin digestion, showing that the dynein arms remain capable of generating shear stress between doublets. Although the radial spokes and central sheath complex appear to be required for motility in *Chlamydomonas* flagella, it is nevertheless possible for flagella lacking these structures to show a simple form of oscillatory bending, as demonstrated by the slow helicoidal bending waves reported in the 9 + 0 flagella of eel spermatozoa by Baccetti and colleagues (Fig. 12) (74), and in the 6 + 0 and 3 + 0 flagella in male gametes of certain gregarine sporozoa by Joseph Schrével, Stuart Goldstein, and colleagues (75–77). There must be, therefore, a mechanism capable of coupling dynein ATPase activity to bending that exists even in these structurally reduced flagella. Perhaps the most likely hypothesis is that suggested by G. Douglas (167), which postulates that bending of a doublet microtubule causes a change in the subunit lattice that modulates the capability of the subunits to interact with the dynein arms. This conformational change could constitute the essence of a curvature control of dynein ATPase activity, of the type that appears required in theoretical models of flagellar bending. Support for this hypothesis is provided by the observation of Marie-Paule Cosson and Gibbons (168) that nonmotile sea urchin spermatozoa with their flagella fixed into normal waveforms by brief treatment with *N*-ethyl maleimide show an augmented ATPase activity that is lost when the flagella are homogenized.

Although a mechanism of this type appears capable of explaining slow helicoidal bending waves, it is evident that a more complex regulatory mechanism involving the central tubules and sheath, and the radial spokes is required to explain the more usual beat patterns of cilia and flagella. The work of Charlotte Omoto and Ching Kung (169), and of Robert Jarosch and Bernhard Fuchs (170) suggests that in some cases the pair of central tubules may rotate within the cylinder of nine doublets. Such an arrangement appears generally consistent with the semi-three-dimensional beat pattern of many cilia, and it might also explain the slow rotation of the plane of beat reported in flagella of some porifera by E. Kilian (171). On the other hand, the work of Tamm (172) has shown that no reorientation of either the cylinder of nine doublets or the pair of central tubules occurs during ciliary reversal in ctenophores. Moreover, the lack of bilateral symmetry in the axonemal structure, resulting from the arrangement of the dynein arms and their apparently unidirectional power stroke (43, 153, 158), make it difficult to envisage how such a pattern of activation would have sufficient torsional stability to generate the almost planar waveforms typical of echinoderm sperm, in which the nonplanar component is too small to be visualized and appears to have a propulsive effect of around 1% of the main force in the plane of bending (6, 173). It seems necessary to explore further the possibility that the arms in intact flagella may be capable of a bidirectional power stroke, for the unidirectional power stroke observed in disintegrating cilia and flagella might be due to a loss of normal regulation under these conditions.

Studies of reactivated cilia and flagella of other organisms have had particular value in illuminating the varied roles that Ca^{2+} plays in regulating movement. Reactivation of cilia of *Paramecium* was first achieved by Yutaka Naitoh (174) using a glycerol procedure. Improved results were achieved by Naitoh and H. Kaneko (175) who used a modification of the Triton X-100 procedure of Gibbons and Gibbons (149), in which the concentration of Triton was reduced to 0.001%, at which it destroys the selective permeability of the cell membrane system while leaving the structure of the cell cortex intact and the cilia still attached. With this system, Naitoh and Kaneko were the first to demonstrate a regulatory role for Ca^{2+} in reactivated cilia: at Ca^{2+} levels of 0.1 μM and below, the direction of swimming is forward, whereas at Ca^{2+} levels above 1 μM, the cells swim backward because of reversed beating of the reactivated cilia. This lent strong support to the hypothesis proposed by Roger Eckert (176) to explain the backward swimming induced by mechanical or electrical stimulation in *Paramecium*. In solutions containing ATP but no Mg^{2+}, the

cilia of Triton-treated cells do not beat, but they change their direction from pointing posterior at low-Ca^{2+} levels to pointing anterior at Ca^{2+} levels above 1 μM. This result was taken to suggest the presence of two motile components: one activated by $MgATP^{2-}$ responsible for cyclic beating, and a second, activated by $CaATP^{2-}$, that governs the orientation of the effective stroke (175).

An analogous regulation by Ca^{2+} in *Chlamydomonas* has been described for isolated pairs of flagella by Jeremy Hyams and Borisy (177), and for individual flagella by Matthew Bessen, Rose Fay, and Witman (178). The regulation of pairs of *Chlamydomonas* flagella is of particular interest because the beat cycle changes from a typical ciliary pattern at low Ca^{2+} to a typical flagellar pattern at higher Ca^{2+}, indicating that, at least in this case, a single organelle has the potential to produce both ciliary and flagellar beat patterns.

Studies by Holwill and collaborators (179, 180) on the motion of the trypanosomid *Crithidia* have shown that the flagellum has the unusual property that, during normal forward swimming of the organism, bends are formed near the flagellar tip and then propagate toward the base. During backward motion of the organism the direction of flagellar bend propagation reverses, so that bends propagate from base to tip. In demembranated preparations reactivated with ATP, tip-to-base propagation is observed at Ca^{2+} concentrations below 0.1 μM, while at higher concentrations base-to-tip propagation only is seen.

In most ciliated epithelia of invertebrates and vertebrates, nervous control appears to be limited to activation or arrest of beating. The studies of Tatsuo Motokawa, Murakami, and Takahashi (181) have shown that the arrest response of lateral cilia of live muscle gill is dependent on the presence of extracellular Ca^{2+}, suggesting that arrest is due to an increased level of intracellular Ca^{2+} as a result of opening of voltage-sensitive Ca^{2+} gates upon depolarization of the ciliary membrane. This hypothesis has been confirmed by Teizo Tsuchiya (182) and by Marika Walter and Satir (58) using reactivated cells as well as live cells treated with the divalent-cation ionophore A23187.

The above results make clear that Ca^{2+} exerts a regulatory role on the beating of cilia and flagella in many organisms. The detailed effect of Ca^{2+} on beating varies considerably from one organism to another, with a particularly striking instance of this variation being that increased Ca^{2+} causes increased asymmetry in sea urchin sperm and decreased asymmetry in *Chlamydomonas* flagella. The rule appears to be that, rather than having a single effect, it is always the low-Ca^{2+} form of beating that is the "normal" one for the organism, whereas the high-Ca^{2+} form occurs during taxis or an avoidance response, etc. The mechanism by which Ca^{2+} exerts its influence remains to be determined, but the recent discovery of calmodulin in *Tetrahymena* cilia (111) suggests strongly that this ubiquitous regulator will be somehow involved.

The basal bodies of each of the two pairs of flagella in *Platymonas* are anchored to the plasmalemma by a thick cross-striated fiber, and the recent work of J. Salisbury and G. Floyd (183) has shown that this fiber, which is about 2.2 μm long when the organism is fixed in the absence of Ca^{2+}, contracts to as little as 0.9 μm when fixed in the presence of 1 mM $CaCl_2$. The contraction of this fiber may be responsible for changes in angular orientation of the basal region of the flagella during swimming. A similar cross-striated fiber joins the two basal bodies in *Chlamydomonas* (184), which shows a decrease in the angle between the basal regions of the flagella upon addition of Ca^{2+} to either beating or nonbeating flagella pairs (177). Although the relationship of these apparently contractile fibers to other forms of cell motility remains to be clarified, their structural resemblance to the basal foot and to the cross-striated rootlet-type structures associated with basal bodies in other organisms has effectively reopened the whole question of a possible active role for these structures whose activity was much debated earlier, but that have recently been relegated to an inactive supporting role.

Since the discovery that, in many cases, demembranated flagella can be reactivated to apparently normal motility by supplying them with exogenous ATP, there has been a tendency to neglect the possible importance of flagellar and ciliary membranes. Recent work by William Dentler, Melanie Pratt, and Stephens (185) suggests that this may be an oversimplification. In 1977, Stephens (186) compared the compositions of the membrane fractions from gill cilia and sperm flagella of the scallop, *Aequipecten*, and found that a large fraction of the ciliary membrane protein appeared to be a glycosylated tubulin, whereas the flagellar membranes contained a major glycosylated protein of about 350,000 daltons with little or no tubulin. Similar differences in membrane composition appear to occur among protozoa and algae. These differences in composition may underlie some difference in membrane function, for photochemical cross-linking by the cleavable lipophilic agent 4,4′-dithiobisphenylazide in vivo causes inhibition of motility in cilia of *Aequipecten* and of *Tetrahymena* (185), whereas it has no apparent effect on the motility of *Aequipecten* sperm flagella.[2] Electron micrographs of partially disintegrated cilia suggest that the structural effect of the cross-linking is to stabilize the attachment of bridges between the doublet tubules and the membrane. The functional action of these bridges in untreated cilia is not clear, but the fact that their stabilization appears correlated with inhibition of ciliary motility suggests that ciliary membranes may in some cases play a more active role in overall function than the reactivation of motility in demembranated organelles might suggest.

A characteristic form of motility in the flagellar membrane of *Chlamydomonas* becomes apparent when a cell is attached to a solid substratum by just one of its two flagella. Under such conditions the cell glides continuously across the substratum, flagellum leading, at a speed of about 2 μm/s, and as noted by Lewin (15), gliding is particularly apparent in mutant strains with paralyzed flagella. The relationship of this gliding to the saltatory movements, at about the same speed, of particles attached to the flagellar membrane in *Chlamydomonas* described recently by Robert Bloodgood and co-workers (187) is not yet clear. It has long been known that the flagellar membranes in *Chlamydomonas* play an important active role in the pairing of cells during mating (15), and interest in these forms of flagellar membrane motility has been accentuated recently by their possible relationship to membrane-microtubule interactions in the cytoplasm (Haimo and Rosenbaum, this volume).

Largely as a result of the evidence summarized above, it is now widely accepted that the normal beating of flagella and cilia results from active sliding movements between adjacent doublets of the axoneme, with this sliding being powered by an ATP-driven mechanochemical cycle in which dynein arms on one doublet interact with successive binding sites along the B tubule of the adjacent doublet, and are coordinated and resisted by the radial spokes and nexin links that convert the sliding into bending.

The importance of flagellar and ciliary function in human

[2] Dentler, W. L., and R. E. Stephens. Personal communication.

medicine has become more apparent recently as the result of the discovery by Afzelius (188) and by Henning Pedersen and Heinrich Rebbe (189) that the respiratory difficulties and male infertility found in the hereditary defect known as Kartagener's syndrome are the result of immotile cilia and sperm flagella. This lack of motility is associated with lack of both inner and outer dynein arms on the doublet tubules of the flagellar and ciliary axonemes (Fig. 13*a*, *b*). Kartagener's syndrome appears to constitute one form of a broader immotile cilia syndrome, and a second form involving immotile cilia with defective radial spokes has been described by Jennifer Sturgess and colleagues (190).

Among other genetic variants are the mutants of *Chlamydomonas* having paralyzed flagella that lack either their inner or their outer arms (Fig. 14) (61). In view of the motility reported in two other instances in which the axonemal structure lacks outer arms (74, 157), the basis for the lack of motility in these *Chlamydomonas* flagella is not yet understood.

Evidence regarding the steps in the dynein cross bridge cycle is preliminary. In the absence of $MgATP^{2-}$, the arms appear to form fixed cross bridges between the doublet tubules, as indicated by the stability of flagellar rigor waves (158) and by the high stiffness of the flagellum under these conditions (166). The observations of Masami Takahashi and Yuji Tonomura (191) that 30S dynein from *Tetrahymena* cilia will bind to either the A or B tubules of isolated doublets, but that the addition of 1 μM ATP causes dissociation of the dynein from B tubules, while having no effect on the dynein bound to A tubules, suggests that $MgATP^{2-}$ causes detachment of the dynein cross bridges in intact axonemes. This is supported by the finding of Sale and Gibbons (192) that addition of $MgATP^{2-}$ to trypsin-treated axonemes in the presence of the inhibitor vanadate (193, 194) results in disintegration of the axonemes by a passive peeling apart of the doublets, rather than by the active sliding seen in the absence of vanadate, which suggests that vanadate does not interfere with the ATP-induced detachment of the arms but binds to the detached arm and inhibits reattachment. This conclusion is further supported by the finding of Okuno that the stiffness of axonemes in the presence of $MgATP^{2-}$ and vanadate is only about 5% of that in the rigor state (195). The presteady-state kinetics of the hydrolysis of ATP by dynein (196;[3]) indicate the occurrence of an early burst of ATP hydrolysis of around 1 mol per mol of active site, suggesting that the rate-limiting step in the overall reaction may be product release. These observations are consistent with an ATP-driven cross bridge cycle for dynein similar to that believed to occur in the myosin cross bridge cycle in muscle (197). However, the evidence supporting this mechanism for dynein ATPase is still quite limited, and the recent report suggesting the presence of two distinct ATPases in the outer arms of *Chlamydomonas* flagella (61) indicates the necessity for continued caution in drawing parallels between dynein and myosin.

The general stability of the oscillatory movements of flagella and cilia, as manifested by their capability to form and propagate uniform bending waves over a wide range of mechanical and chemical conditions and by their rapid recovery from transitory mechanical disturbances (e.g., collisions between sperm), indicates the presence of at least one feedback loop in the regulatory mechanisms. The report by Brokaw and Tom Simonick (198) of abrupt transitions between two oscillatory modes, one in which bends are propagated normally along the full length of the flagella and a second in which the amplitudes of the bending waves decrease rapidly as they propagate, suggests the presence of distinct feedback loops associated with bend initiation and bend propagation.

There are two general types of approach to the study of the regulatory mechanisms: one involves perturbing the beating flagellum with a wide variety of agents and then comparing their effects on the various wave parameters; the other involves study of flagella under nonoscillatory conditions in which the feedback loop has been opened to facilitate examination of its individual components. Survey of the effects of a wide variety of perturbing agents on the wave parameters of sea urchin sperm flagella by Gibbons (112) has suggested that two largely independent mechanisms are responsible for regulating the beat frequency and the waveform. The mechanism regulating beat frequency appears to be closely related to the mechanochemical cycle of dynein that causes active sliding between tubules and is relatively insensitive to the hydromechanical forces on the axoneme, whereas the mechanisms regulating waveform appear relatively more sensitive to the mechanical boundary conditions at the flagellar base and to the properties of the radial spokes, nexin links, and the tubules themselves, which are together presumed responsible for converting active sliding into a particular pattern of bending (199).

An example of the second approach of interrupting the feedback loop is the study of Summers and Gibbons in which digestion by trypsin was used to uncouple sliding from bending. More recent studies by Brokaw, Barbara Gibbons, Goldstein, and Flavin and their collaborators (67, 193, 194, 200, 201) have identified several agents—including Ca^{2+}, methanol, CO_2, decreased pH, and vanadate—that can be used to inhibit reversibly the normal oscillatory beating. The use of these agents makes it possible to study the bending of flagella that occurs upon addition of ATP to preparations in which oscillatory beating is inhibited. The preliminary reports by Goldstein (201) and by Gibbons and Gibbons (67) indicate that substantial amounts of active bending can occur in flagella inhibited by decreased pH, vanadate, or Ca^{2+}. This general approach of investigating the bending and straightening of demembranated flagella under nonoscillatory conditions may be a useful way to learn about the factors regulating the activity of dynein cross bridges at different positions on the flagellum as well as about the viscoelastic properties of the structural components that resist active sliding and convert it into bending.

Although, as indicated above, most evidence indicates that movement associated with microtubules occurs as a result of sliding, there are some indications that significant changes in microtubule length may occur in certain cases. Electron microscopic data suggesting that single microtubules in protozoan axostyles are capable of shortening by as much as 25% has been reported by Richard McIntosh (202), but more information is needed before the physiological significance of this finding can be interpreted.

Considerable evidence for small differences of the order of 1% in the lattice spacings of the A and B components of flagellar doublet tubules is provided by the work of Summers and Gibbons (151), Donald Costello (203), and Richard Zobel (204) showing that the doublets have a marked tendency to assume uniform helical forms as a result of bending approximately within the plane containing the centers of the A and B tubules, usually with the A tubule on the outside of the bend. Recent studies by Taiko Miki-Noumura and Ritsu Kamiya (205) have shown that small changes in pH or in Ca^{2+} concentration appear to cause discrete changes in pitch and diameter

[3] Evans, J., and I. R. Gibbons. Unpublished data.

of these tubule helices, and these factors, as well as organic solvents such as methanol are known to have substantial effects on the asymmetry of flagellar bending (205). It is possible that these agents may function by modifying the changes in lattice structure associated with a basic curvature-controlled regulation of dynein arm activity as discussed above. Since microtubules contain a variety of minor protein components in addition to tubulin, it is not clear whether the above factors act directly on the tubulin, in a manner analogous to the action of hydrodynamic stress, pH, and organic solvents in effecting transitions between the various polymorphic forms of flagellin in bacterial flagella (206–208), or indirectly through accessory proteins, as in the effect of Ca^{2+} on the structure of thin filaments in striated muscle (209), but the fact that mild trypsin digestion desensitizes the axonemes to Ca^{2+} (199) suggests that at least part of their action is indirect.

Growth Mechanisms

Investigation of flagellar and ciliary growth mechanisms is greatly facilitated by use of organisms in which the time of growth can be synchronous. For this reason, most studies have involved the regeneration of new flagella or cilia on cells from which the organelles have been either shed or resorbed, or the growth of flagella in cells that can be induced to undergo an amoeba-flagellate transition.

The early work of Lewin showed that *Chlamydomonas* that had resorbed most of their flagella as a result of being kept on agar in the dark, would regenerate full-length flagella within 1–2 h of being transferred to fluid medium. This work was extended by Malvine Hagen-Seyfferth (210), who showed that *Chlamydomonas*, after having been deflagellated completely by exposure to a pH shock or to ethanol, would regenerate new flagella within about 1 h.

Rosenbaum and Child (211) amputated flagella of *Euglena*, *Astasia*, and *Ochromonas* by mechanical agitation, and found that in all cases regeneration was characterized by an initial lag period, after which regeneration occurred at a rate that decelerated as the original length was approached. In these species, inhibition of protein synthesis by cycloheximide at the time of amputation resulted in almost complete inhibition of regeneration. However, *Chlamydomonas* flagella can regenerate up to one-third of their normal length (212), and cilia from the embryo of the sea urchin can regenerate to full normal lengths (213), both in the absence of protein synthesis, indicating the presence of significant pools of precursor proteins during normal growth in these cells. Using *Chlamydomonas* gametes that have a low basal level of protein synthesis, Paul Lefebvre and co-workers (214) have been able to detect deflagellation-induced synthesis of tubulin, dynein, and flagellar membrane protein, as well as of about 20 minor axonemal proteins. The factors responsible for triggering synthesis of flagellar proteins upon deflagellation are not clear, but it is notable that the same pattern of synthesis occurs upon induced resorption, even when assembly of the new protein into flagella is inhibited with colchicine.

As described originally by Schardinger in 1899 and more recently in greater detail by E. Willmer (215), the cells of *Naegleria gruberi* undergo transformation from an amoeboid form to a flagellated form upon being transferred from their growth environment to a nonnutrient buffer solution. This amoeba-flagellate transformation has been used in an extended series of studies of flagellar morphogenesis by Chandler Fulton and Alan Dingle and their collaborators (216, 217), who have shown that a burst of synthesis of new proteins precedes the appearance of visible flagella, and have suggested that a change in the compartmentalization of intracellular Ca^{2+} may be responsible for triggering the transformation.

The lengths of cilia and flagella are under close control by the cell, so that different cilia on a single cell may have greatly different lengths—as exemplified by the components of the compound laterofrontal cilium in lamellibranch gills, which range from 2 to 12 μm in length (39). This control by the cell permits even the resorption of certain flagella while others on the same cell are growing longer—examples are the studies by Tamm showing the resorption of the parental leading flagellum with simultaneous growth of the two new daughter leading flagella that occurs prior to cell division in *Peranema* (218) and the work of Rosenbaum (212) and of Randall (184) showing that in *Chlamydomonas* cells from which just one flagellum has been sheared off, the remaining old flagellum is partially resorbed at the same time as the new flagellum begins regenerating. The factors by which cells regulate the length of their flagella are not known, but several studies have shown that the presence of divalent-cation chelators in the medium causes partial or complete flagellar resorption in *Chlamydomonas*, and that this effect can be reversed by addition of Ca^{2+}, Sr^{2+}, or Mn^{2+} to the medium (219). Pulse labeling, followed by autoradiography, has shown that during flagellar growth in vivo most of the subunits are added to the distal region of the growing flagellum, although about 20% appear to be added within the proximal region (211, 212). Studies by Dentler and Rosenbaum (220) involving polymerization of brain tubulin onto partially disrupted flagella of *Chlamydomonas* have indicated that polymerization onto the outer doublet tubules occurs at their distal (+) ends, whereas polymerization onto the central tubules occurs at their proximal (−) ends, apparently because their distal (+) ends are blocked by a cap attached to the tip of the flagellar membrane. These results suggest that, during normal growth in vivo, the doublet tubules grow at their distal ends while the central tubules grow at their proximal ends. The full implications of this asymmetrical growth pattern are not yet clear, but it may be noted that in many organisms the proximal ends of the central tubules appear unattached—as perhaps they must be if they are to rotate in the way described for *Paramecium* and *Synura* (169, 170).

Knowledge of flagellar assembly mechanisms is still largely at a descriptive stage. Repolymerization of tubulin to form singlet tubules (103), and rebinding of dynein to extracted axonemes (78) are the only steps that have yet been accomplished in vitro. Study of the conditions under which mutants of *Chlamydomonas* with structurally defective flagella can be rescued as dikaryons may provide some more detailed information. However, if, as seems likely, the process of assembly for flagella is as complex as that of, for example, bacteriophage T2, then progress will be hard to come by until more of the assembly steps can be reproduced in vitro.

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Cilia, Flagella, and Microtubules

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In 1676 Leeuwenhoek sent to the Royal Society of London a letter describing his discovery of protozoa and their cilia and flagella. He wrote, "I also discovered a second sort of animalcules, whose figure was an oval, ... provided with diverse incredibly thin little feet, or little legs [cilia], which were moved very nimbly ..., and wherewith they brought off incredibly quick motions" (cf. translation of letter 18 [1]).

Cilia and flagella were observed on a variety of cells during the next two hundred years, and by the end of the 19th century several theories had been proposed to explain flagellar beating. For example, it was postulated that flagella were lifeless and were moved by elements within the cell body or were hollow structures into which fluid was injected and withdrawn. Alternatively, it was suggested that flagella contained a central contracting fiber or possessed a fibrillar substructure. This last theory had been formulated in 1868 by Engelmann, who proposed that the flagellum contained aligned fibrillar elements which shortened into a globular form during beating. Although Jensen in 1887 and Ballowitz in 1888 observed numerous fibrils in the fraying tips of sperm tails, the prevailing belief at the turn of the century was that the flagellum contained a solid contracting core (see reference 2).

In later studies, the fibrillar substructure of cilia and flagella was described in a variety of cells (2, 3), and these observations were confirmed with the development of the electron microscope (4–7). The number of fibrils was variously reported as between nine and twelve, and the diversity of ciliary and flagellar wave forms suggested no reason that the number of fibrils would be constant among species.

Axonemes: the 9 + 2 Pattern

Based on electron-microscope studies of plant sperm in a number of species (8–11), a diagrammatic reconstruction of the flagellum was proposed (10). Considering the resolution of the shadow-cast, whole-mount preparations used in these studies, the model was remarkable in its accuracy. During this same period, techniques were developed for embedding and sectioning biological material for electron microscopy, and in 1954 Fawcett and Porter published the first ultrastructural study of cilia (12). Regardless of origin, all cilia were observed to possess the same configuration, nine hollow, doublet fibrils equidistant from and radially surrounding a central pair of single fibrils. This structure was identical with that proposed for plant sperm flagella (10) and resulted in the recognition of the "9 + 2 axoneme" as the common structural organization of cilia and flagella. Later studies demonstrated, however, that there were notable departures from this format, particularly among the spermatazoa of insects (13, 14).

With the development of improved fixation and staining techniques, accessory structures within the axoneme could be visualized. By use of a high percent osmium solution to fix sea urchin sperm flagella, Afzelius (15) was first to describe the arms, later termed dynein (16), present in two rows along the larger fiber of each outer doublet. Noting their orientation toward the smaller fiber of the adjacent outer doublet, Afzelius (15) postulated that the dynein arms might be active in a sliding filament model of flagellar beating. The next 15 years were to prove this hypothesis correct. Afzelius also observed that the dynein arms imparted an asymmetry to the axoneme. Subsequently, Gibbons and Grimstone (17) demonstrated in several species that the arms always pointed clockwise around the axoneme when viewed from base to tip. They introduced the nomenclature terming the fiber bearing the dynein arms the A-subfiber, the other the B-subfiber.

Studies of axonemes in longitudinal sections demonstrated that the dynein arms, as well as the other major accessory structures, were regularly spaced along the length of the axoneme. The dynein arms in both the inner and outer rows had a center-to-center spacing of approximately 24 nm along the length of the A-subfiber (18–22). In addition, the inner and outer rows of arms were axially staggered with respect to each other (23, 24), and superimposition of the two rows of arms may have accounted for earlier reports of an arm spacing of 12–16 nm (17, 25).

Radial spokes were visualized in thin sections as projections from each A-subfiber to the central sheath (15). These spokes terminated in an enlarged head that was incorrectly identified as a fiber running the length of the axoneme (17). More recent studies verified the "spokelike" ultrastructure and revealed that the radial spokes occurred in pairs in *Chlamydomonas* (19, 22, 25, 26) or triplets in *Elliptio* gill and *Tetrahymena* cilia (18, 27), which were grouped at intervals of about 96 nm along the lengths of the A-subfibers.

The central sheath observed in whole-mount preparations by Manton and Clarke (10) and described in cross sections of cilia by Gibbons and Grimstone (17) consisted of two rows of projections spaced at 16-nm intervals along each of the two central fibrils (22, 25, 27, 28). The final axonemal structure to be described were the nexin links (29, 30), which connected the A-subfiber of one outer doublet to the adjacent outer doublet

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and were axially spaced at approximately 96-nm intervals (21, 22, 31).

Optical diffraction patterns of negatively stained axonemal fibers, microtubules, revealed the existence of a strong 4-nm as well as an 8-nm axial periodicity (23, 32–34) corresponding to the monomer and heterodimer subunit composition of the microtubules (33, 34). The relative intensity of the 8-nm repeat compared with the 4-nm repeat varied considerably with different types of microtubules, and suggested that accessory structures on microtubules might contribute to an amplification of the 8-nm diffraction pattern (23). The observations that the dynein arms, central pair projections, radial spokes, and nexin links, all bound to the flagellar microtubules at approximate multiples of 8 nm, supported this hypothesis, and it has been proposed that the axial spacing between adjacent binding sites on microtubules is 8 nm (20).

Dynein

Concurrent with a description of axonemal fine structure has been an elucidation of the molecular basis for ciliary and flagellar motility. Spermatozoa were shown to contain both measurable amounts of adenosine triphosphate (ATP) and ATPase activity (35), which later studies demonstrated was concentrated in the flagellum (36–38). Using techniques developed to study muscle contraction (39), Hoffman-Berling (40) discovered that addition of ATP to glycerinated sperm resulted in the reactivation of beating. The wave form resembled that of live sperm and indicated that the energy for motility was supplied by ATP hydrolysis. Reactivation specifically required ATP and Mg^{2+} and was inhibited by EDTA (38, 41, 42).

After the development of methods to isolate cilia in large quantities (43), Gibbons (29, 44) demonstrated by selective solubilization of *Tetrahymena* axonemes that the ATPase activity was localized in the two rows of arms on the A-subfiber. The enzyme was named "dynein," force protein, for its postulated role in the mechanochemical transduction of energy required for motility (16). Dynein specifically hydrolyzed ATP in preference to other nucleotides, required Mg^{2+} or Ca^{2+} for its activity, and was inhibited by EDTA, characteristics that correlated closely with those necessary for axonemal reactivation (45, 46). Although the inner and outer rows of dynein arms appeared to be functionally equivalent (47), they were morphologically distinct (48), had different solubilities (49), and were composed of different polypeptides (26, 50). The existence of more than one axonemal dynein has been demonstrated in a number of studies (29, 51–55).

A specific association between the dynein arms and the A-subfiber was indicated by the observations that the solubilized arms rebound to their original sites on the A-subfiber (29, 56). Recently, it was demonstrated that solubilized dynein arms also rebound to the B-subfiber, presumably to those sites with which the arms would normally interact during beating (57).

The role of dynein arms in motility, implicated both by their ATPase activity and orientation toward the adjacent outer doublet microtubule, was confirmed in studies that demonstrated that the beat frequency of reactivation was directly proportional to the number of dynein arms present within the axoneme (47, 56, 58). Furthermore, antibody prepared against dynein inhibited both its ATPase activity and axonemal reactivation (59–61). The existence of paralyzed flagellar mutants lacking dynein arms in both *Chlamydomonas* (26) and humans (62) also suggested a motile role for the dynein arms.

The Sliding Mechanism

Theoretical analysis of the mechanism generating the motive force within the flagellum indicated that shearing between adjacent outer doublets that resulted in microtubule sliding could account for the uniform propagation of waves along the axoneme (63, 64). Sliding between outer doublets was demonstrated first in sectioned axonemes (65, 66) and more directly by dark-field visualization of trypsin-digested axonemes supplied with ATP (67). These and later studies indicated that the sliding motions were mediated by dynein arms cyclically attaching to and causing a shearing force between adjacent outer doublet microtubules (68, 69) exerted toward the tip of the axoneme (70).

Dynein arms present on intact axonemes or reattached to dynein-extracted axonemes projected from the wall of the microtubules at an angle of approximately 55°. In *Chlamydomonas*, the arms tilted toward the tip of the flagellum (19), whereas in mollusc gill and *Tetrahymena* the dynein arms tilted toward the base of the cilium (57, 70–72). To exert a directional force resulting in sliding (70), it has been postulated that the orientation of the dynein arms changes during the cross-bridge cycle (69), and a recent study supports this suggestion (73). Such a change in the orientation might account for the above differences in arm-tilt direction.

Transient ATP-dependent bridging between adjacent outer doublets was predicted from the orientation of the dynein arms and by their role in sliding, but these bridges were preserved and, therefore, visualized only when axonemes were fixed in rigor (74). The existence of a rigor state had been suggested by observations that bull sperm flagella became plasticized after ATP addition (75). Subsequent studies demonstrated that removal of ATP from reactivating axonemes caused them to enter rigor, characterized by a wave form frozen at the time of ATP depletion and maintained by dynein arm cross-bridges between adjacent outer doublets (74, 76). Release of the B-subfiber of the adjacent outer doublet by dynein as manifested by relaxation of the rigor wave required ATP binding, whereas subsequent reactivation required ATP hydrolysis (69, 76, 77). In other studies, however, dynein cross-bridges between adjacent outer doublet microtubules could be produced by fixing axonemes in appropriate buffers containing divalent cations (78). Nevertheless, addition of ATP to these cation-induced rigor axonemes resulted in relaxation of dynein cross-bridges between particular outer doublets (79). Although these observations conflict with studies demonstrating that reactivating axonemes entered rigor when either the Mg^{2+} (80) or ATP (76) concentration of the reactivation medium was rapidly lowered, they indicate that the dynein arms do, in fact, cross-bridge the adjacent outer doublet microtubule, and that the cross-bridges are ATP dependent.

Microtubules

Based on the observations of Van Beneden that protoplasmic fibrillae existed within the spindle and of Ballowitz that sperm tails contained minute fibrils, Wilson (81) postulated that the substance and outgrowth of the flagellar fibrils were comparable with those of the fibrils within the spindle. Moreover, he suggested that the contractile behavior of the spindle fibers noted by Boveri might also apply to the flagellum. It was not until the 1960s, however, that the relationship between cytoplasmic and flagellar microtubules was to be established, and it has not yet been determined if motility associated with

cytoplasmic microtubules is elicited by a mechanism similar to that of the flagellum.

Although early researchers using the light microscope had observed fibers within the spindle, the periphery of red blood cells, and in neurites, the existence of these structures had been a matter of controversy that was finally resolved with the advent of electron microscopy. By use of osmium as a fixative, fibrous structures were observed in the mitotic apparatus (82, 83) and in nerve axons (84), but their widespread distribution was only fully appreciated after the development of glutaraldehyde fixation (85). In one of the earliest uses of this technique, the fibers were described as microtubules whose similarity in morphology to the fibrils within the axoneme was apparent (86, 87).

Ultrastructural analysis revealed the presence of 13 longitudinal protofilaments comprising the walls of both cytoplasmic and axonemal central pair and A-subfiber microtubules (88–91). Moreover, optical diffraction studies of cytoplasmic microtubules demonstrated that they have both a 4- and an 8-nm axial periodicity (92), as had been observed previously for flagellar microtubules. In addition, the subunit dimers were axially staggered in adjacent protofilaments in both the A-subfiber and cytoplasmic microtubules, indicating that they were structurally similar. On the other hand, in the B-subfiber the dimers were lined up in adjacent protofilaments, thereby indicating a distinct surface lattice (23).

The use of the drug colchicine has been basic to the understanding of the chemical composition of microtubules. Noting that colchicine caused a reversible loss in birefringence of spindle fibers, Inoué (93) postulated that the drug bound to the subunit of these fibers. Later, Taylor (94) demonstrated that colchicine was reversibly bound by a substance within the cell, and in subsequent studies a colchicine-binding protein was isolated and shown to be the subunit of both cytoplasmic (95, 96) and flagellar (97, 98) microtubules. Characterization of the purified colchicine-binding protein from brain tissue revealed it to bind 2 mol of guanosine triphosphate (GTP) per mol of protein and to have a sedimentation coefficient of 6S and a native molecular weight of about 110,000 daltons (99), properties identical with those of the colchicine-binding protein in the mitotic apparatus and axoneme. The protein was given the name "tubulin" (100). Electrophoretic analysis revealed tubulin to be composed of two closely migrating 55,000-dalton polypeptides present in equal amounts (101). Recent experimentation has shown that 6S tubulin is a heterodimer composed of these two components (102). Although similar in subunit composition and structure, flagellar microtubules differed from cytoplasmic microtubules in their ability to form doublets and in their relative stability; the biochemical basis for these differences has not been determined.

Assembly In Vitro: the Role of Accessory Proteins and Microtubule Polarity

With the discovery of conditions that permitted the in vitro assembly of tubulin into microtubules (103), it was possible to study their biochemistry. Microtubule assembly occurred at 37°C from homogenates of brain in buffers containing Mg^{2+}, GTP, and a calcium chelator. Tubulin has subsequently been assembled from a number of other sources, including flagellar outer doublet microtubules (104–106).

Electrophoretic analysis of the protein composition of brain microtubules assembled in vitro revealed the presence of several protein species in addition to tubulin (107–109). A prominent class of polypeptides having a high molecular weight (ca. 300,000 daltons) copurified stoichiometrically with tubulin through several cycles of assembly (110, 111). Separation of the 6S tubulin from these proteins inhibited its ability to polymerize into microtubules except at high protein concentration (112) or unphysiological solvent conditions (113–115). Readdition of the high molecular-weight MAPs (microtubule-associated proteins) to 6S tubulin stimulated both the rate and extent of polymerization by lowering the critical concentration of tubulin necessary for assembly (110, 116). MAPs stabilized the microtubules (117) by lowering the reverse rate constant for assembly (118). Another class of proteins that copurified with tubulin, termed tau, was also shown to stimulate microtubule assembly (109, 119).

By examining the incorporation of radioactive precursors into regenerating flagella, it was determined that flagella assembled principally at their distal tips (120, 121). Tubulin obtained from cytoplasmic microtubules also exhibited directional assembly. For example, addition of brain tubulin onto isolated basal bodies (122), centrioles (123–125), or axonemes (19, 126) resulted in microtubule polymerization predominantly onto the distal ends of these organelles. Similarly, addition of brain tubulin onto microtubule pieces resulted in preferential assembly onto one end of these pieces (108, 127). Recent studies of microtubule assembly in vitro have indicated that polymerization occurred at one end of the microtubule and depolymerization occurred at the opposite end (128). These studies suggest that the two ends of the microtubule have different critical concentrations for assembly, and at polymerization equilibrium the rate of tubulin addition onto one end of the microtubule would equal the rate off the other end. Other experimentation, however, has demonstrated that microtubules assembled from kinetochores or centrosomes polymerized and depolymerized at the same end (129).

The polarity of microtubules, as manifested in their directional polymerization, may permit them to function in directional intracellular movements. Of these movements, those exhibited during mitosis have generated the most interest and have been the subject of several different models (130–135). Both chromosomes (123, 136, 137) and centrosomes (124, 125, 136) served as nucleation sites for microtubule assembly in vitro. Studies of the direction of this assembly have indicated that both kinetechore (129, 138) and centriolar (129, 139) microtubules added tubulin subunits at the microtubule end distal to the organizing center. These observations suggest that each half-spindle of the mitotic apparatus is composed of microtubules present in an antiparallel array.[1]

While axonemes could be reactivated to beat in vitro and, accordingly, their movements analyzed biochemically, no equivalent assay has been developed to study the movements associated with cytoplasmic microtubules, although work on the reactivation of mitotic movements is progressing (140). Nevertheless, recent studies have provided some insight into the mechanism by which motility is elicited within the cytoplasm.

MAPs, Arms, and Movement

The high molecular-weight MAPs, which copurified with brain tubulin, had an electrophoretic mobility similar to that of flagellar dynein, leading to speculation that they might be

[1] Direct visualization of microtubule polarity has recently revealed the half-spindle to be composed of parallel microtubules (139*a*, 139*b*).

functionally equivalent (108). Moreover, a flagellar fraction containing dynein was shown to stimulate the assembly of brain tubulin (141) while, in other studies, in vitro assembly of flagellar outer-doublet tubulin was stimulated by the addition of brain MAPs (105). Early work indicated the presence of a low level of ATPase activity associated with brain microtubules assembled in vitro, although contamination by mitochondrial ATPases could not be precluded (142, 143). More recent studies have demonstrated the presence of a dyneinlike ATPase in the cytoplasm of unfertilized sea urchin eggs (144) and associated with the mitotic apparatus (145). In other studies, a protein that had properties similar to dynein was purified from brain microtubules (146), and the activity of an ATPase associated with brain microtubules was stimulated by addition of tubulin (147).

MAPs have been visualized as filamentous projections (110, 112) that exhibit an axial periodicity of 32 nm (148, 149) along microtubules assembled in vitro. Similar projections have been observed on brain microtubules *in situ* (150). The presence of arms on cytoplasmic microtubules from a number of sources has been well documented (see reference 20), and arms periodically cross-bridging microtubules have been observed within the mitotic apparatus of *Barbylonympha* (151). At present, however, it has not been determined if these arms have a motile function.

Other studies have suggested that a dyneinlike ATPase might be implicated in movements associated with cytoplasmic microtubules. Lysed mitotic mammalian cells were capable of continuing anaphase motions if ATP was present (152). These movements were blocked by vanadate, an inhibitor of ATPases, at concentrations that inhibited dynein, but not myosin, ATPase activity (140). In addition, antibodies prepared against sea urchin flagellar dynein prevented chromosome movements in isolated mitotic apparatuses, whereas those prepared against myosin had no effect on these movements (153). Moreover, fluorescently labeled antibody against dynein stained the mitotic apparatus (154). Recently, it has been demonstrated that cytoplasmic microtubules have the capacity to bind dynein (155). A specificity of flagellar dynein binding to brain microtubules assembled in vitro was indicated by the 24-nm axial periodicity of the bound arms along the microtubules. This spacing is identical with that of dynein arms present on axonemal microtubules. Furthermore, flagellar dynein caused brain microtubules to become cross-bridged together. Subsequent ATP addition dissociated these bridges. Together, these data suggested the possibility that ATP-dependent dynein cross-bridges between microtubules, which results in outer doublet sliding within the axoneme, may also be involved with movements occurring within the cytoplasm.

Flagellar dynein bound to in vitro assembled microtubules provided a direct means by which microtubule polarity could be determined (155). Application of this technique to microtubules within the cell would be analogous to the use of heavy meromyosin bound to actin filaments to determine their polarity (156, 157), and has the advantage that microtubule polarity could be determined in transverse, as well as in longitudinal, sections. By decorating the mitotic apparatus with dynein, for example, it could be determined if microtubules of opposite polarity are adjacent to each other during anaphase and, therefore, likely to interact during mitotic movements. Dynein, both in its role in motility and in its use as a probe for determining the polarity of microtubules, may therefore provide information about the mechanism by which movements occur in association with microtubules.

This brief presentation is not meant as a thorough review of the microtubule literature. The reader is directed to the recent review by Bloodgood and Kelleher (158), which lists most of the major reviews and books on microtubules up until 1976, beginning with Porter's initial review in 1966. In addition, several excellent and exhaustive reviews have appeared within the past three years (159–166). Rather, the foregoing account attempts to trace the overall direction that research on microtubules has taken over the past quarter-century. With the development of electron microscopy as a routine laboratory procedure came the ability to observe microtubules first in cilia and flagella and later in other organelles and in a great variety of cell types. Studies on the biochemistry of ciliary and flagellar microtubules and the colchicine-binding protein of brain and other tissues were soon followed by successes in the reactivation of microtubule-based motile systems, particularly the cilia and flagella and, more recently, the mitotic apparatus. Finally, the development of techniques to assemble microtubules and some of their associated proteins in vitro, and to visualize systems of microtubules and related cytoskeletal elements by labeled antibody procedures and by high-voltage electron microscopy, has provided the impetus for continued progress in this rapidly moving field.

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Cell Division and the Mitotic Spindle[1]

SHINYA INOUÉ

The study of cell division spans the past full century. Lately, the field has blossomed, and exciting advances have been made, especially at the molecular and fine-structural levels. Yet as we commemorate the centennial of Flemming's discovery[2] of "indirect" cell division, or mitosis, many basic questions still remain unanswered or incompletely explained.

The first half-century of study on cell division is synthesized in Wilson's (2) classic treatise "The Cell in Development and Heredity."[3] While laying a solid foundation for the cytology of the dividing cell and the genetic and developmental significance of mitosis and meiosis, Wilson (Chapter IX) also directs our attention to an important viewpoint regarding the structural basis of cell function. Thus he quotes Brücke:

"We must therefore ascribe to living cells, beyond the molecular structure of the organic compounds that they contain, still another structure of different type of complication; and it is this which we call by the name of organization."

It is this aspect of the dividing cell, its organization, especially in its dynamic attributes, that I shall stress in this brief historical sketch. In particular, I shall focus on the organization of the ephemeral mitotic spindle, which emerges cyclically at each cell division. With it, the replicated, condensed chromosomes are separated and positioned for inclusion into the (two) daughter cells.

The Mitotic Spindle

As we entered the early 1950s, evidence pointed to two mechanisms of anaphase chromosome movement (summarized by Schrader [6]). Chromosomes were pulled toward the spindle poles, via their kinetochores,[4] by shortening of a traction fiber or the chromosomal spindle fiber. In addition, chromosomes were "pushed" apart by the pole-to-pole lengthening of the central spindle to which the chromosomal fibers were anchored. The notion of a musclelike contraction for poleward chromosome motion had been propounded by Flemming in 1879 (1) and earlier workers, and questioned by Wilson (2) as not being consistent with the "dynamic nature of the cytoplasmic fibrillae" observed in living cells. As to the dual mechanism, Ris (8), in working with living grasshopper spermatocytes, was able to inhibit the pole-to-pole elongation without affecting chromosome-to-pole movement by exposing the cells to a solution containing a few tenths of a percent chloral hydrate.

Yet the nagging doubt, expressed by Wilson (e.g., pages 178–198 in reference 2) and others regarding the physical nature of the "achromatic" fibrous machinery of the mitotic spindle, which was believed to be responsible for chromosome movement, had not abated. Rather, the problems were compounded by the late 1940s despite, and partly because of, the wealth of studies that had been made on carefully fixed and stained cells and by the deductions drawn from observations of living cell behavior (6). In that atmosphere it was first necessary to learn whether the mitotic figures seen in fixed cells in fact represented, in living cells, a physically integral body capable of moving chromosomes or exerting force enough to deform cell shape.

ISOLATION OF THE MITOTIC SPINDLE: In 1952, Mazia and Dan [9] succeeded in developing a method for the mass isolation of "mitotic apparatuses," thereby identifying the mitotic spindle, chromosomes, and asters as a coherent physical body separable from the rest of the cell (Fig. 1). Although there were earlier reports of expelling the spindle out of an intact cell (e.g., Foot and Strobell [10] from earthworm eggs), the pioneering work of Mazia and Dan finally opened the way for the mass isolation and characterization of the mitotic apparatus. That same year, Carlson (11), in an extensive micromanipulation study on living grasshopper neuroblasts, demonstrated the integrity and mechanical anisotropy of the metaphase spindle, as well as the "liquefaction" of the spindle mid-zone observed during anaphase.

[1] Dedicated to Professor Kenneth W. Cooper, University of California, Riverside, whose continued friendship and advice have added immensely to my work.

[2] Translated and reproduced in 1965. *J. Cell Biol.* 25(1; part 2):1–69. Flemming saw that the nucleus did not divide directly into two, but formed chromatin threads (hence mitosis). The condensed chromatin threads, or chromosomes, were moved apart and placed into two new cells by a transient, fibrillar achromatic apparatus, the "nuclear spindle" (1) formed from the hyaline kinoplasm.

[3] Wilson's work is complemented in the botanical realm by Sharp (3). Bělař (4) provides a thorough, thought-provoking examination of achromatic spindle components and varying patterns of mitosis in protists. Morgan (5) illustrates and raises penetrating questions regarding the role of cell division in embryonic development and gene expression.

[4] Chromosomes commonly possess a single spindle fiber attachment point, or kinetochore. Some chromosomes have, or behave as though they have, diffuse kinetochores along the length of their chromosomes (6, 7). They are called holokinetic chromosomes.

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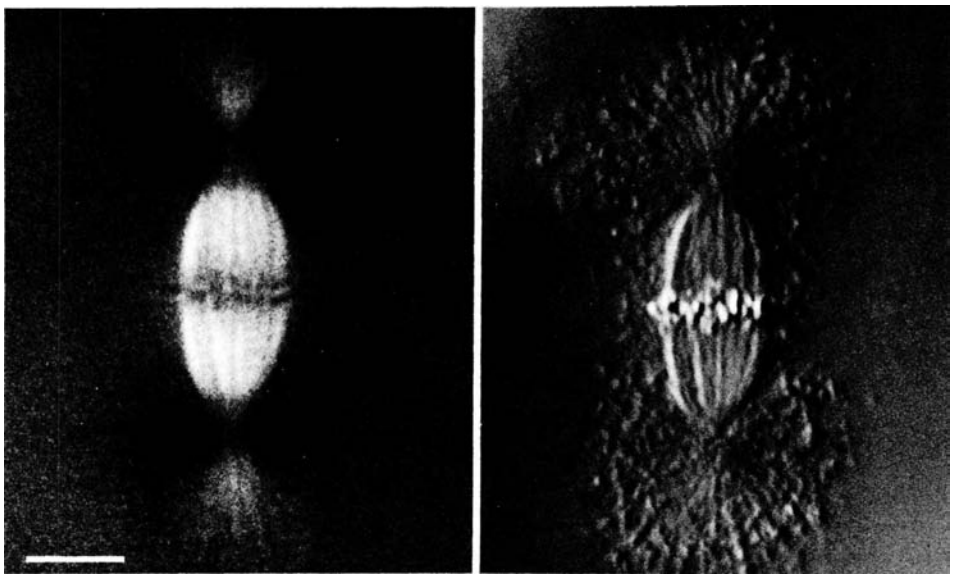

FIGURE 1 Mitotic spindle in metaphase isolated from the egg of a sea urchin, *Lytechinus variegatus*. (*Left*) Observed with a rectified polarizing microscope, spindle fibers and astral rays appear in light or dark contrast depending on their orientation. The weak birefringence (measuring a few nanometers in retardation) of the fibers produces the sharp contrast observed. Microtubular bundles are responsible for the (positive form) birefringence of the fibers. Chromosomes display little birefringence and appear as gray bodies at the equator of the spindle. (*Right*) The same spindle in Nomarsky differential interference contrast. Chromosomes show prominently. The microtubules in these clean spindles (isolated in a new medium devised by Salmon) depolymerize when exposed to *submicromolar* concentrations of calcium ions once glycerol is removed from the isolation medium. In these isolates, which lack vesicular components, the chromosomal fibers shorten as they are depolymerized by micromolar concentrations of calcium ions. Unpublished figures, courtesy of Dr. E. D. Salmon, University of North Carolina. Bar, 10 μm.

Many improvements were made on the basic isolation technique of Mazia and Dan. In particular, the work of Kane (12) that identified the pH and solute conditions (in effect, water activity) needed for mitotic apparatus isolation, helped shed light on the basic physicochemical parameters that delineated the functioning cytoplasm. On the other hand, early attempts at defining the chemical makeup of the mitotic spindle were less successful. In retrospect, that is not so surprising because the fibers of the spindle and aster are immersed in (and spun out from) the hyaline cytoplasm that permeates the cell. Large cytoplasmic granules are excluded from the spindle, but ribosomes and some membranes are not. Yolk and other granules also adhered to earlier isolates.

SPINDLE FIBERS IN VIVO: Whereas the isolated mitotic apparatus exhibited a physical coherence and clearly displayed spindle fibers, such fibers could have arisen by fixing or overstabilizing the cell, as was suggested by many investigators (see 6). Pollister (13), for example, argued that astral rays were not fibers in the living cell but rather were channels of flow of oriented molecules belonging to the hyaloplasm. Our own work, which paralleled that of Mazia and Dan, focused on the development of sensitive polarized light microscopy, with which we hoped to study directly in living cells the nature of the anisotropically arrayed molecules that made up the spindles and asters. From the 1930s to early 1950s, Schmidt (14), Hughes and Swann (15), Swann and Mitchison (16), Inoué and Dan (17), and Swann (18) had investigated how to optimize the performance of existing polarized light microscopes. They also showed that the mitotic spindle and astral rays in cleaving sea urchin eggs and cultured chick embryonic cells indeed displayed a longitudinal positive birefringence consonant with the presumed presence of molecules oriented parallel to the fiber axes. Each of the workers also noted the striking emergence, rise, fall, and disappearance of spindle birefringence (retardation) as a single cell progressed through prometaphase, metaphase, anaphase, and telophase. Each interpreted the observations in molecular terms, variously biased by the paradigm adopted.

By 1953, I was able to demonstrate clearly with the polarizing microscope (19) that "there is fibrous structure in living cells which in conformation is very close to what the cytologists have long observed in well-fixed preparations. There are continuous fibers, chromosomal fibers, and astral rays" (6). Coupled with Cleveland et al. (20) and Cooper's (21) earlier observations of fibrous structures in the spindles of certain other living cells, the issue of the "reality" of spindle fibers seemed to be settled.

Whereas these earlier studies vividly displayed some dynamic changes of spindle birefringence in living cells, changes which should reflect events taking place at the molecular level during cell division, a clearer outlook depended on better optical resolution and broader experience gained through observations and experimental manipulations of cells in division. With progressive improvements in the resolution and sensitivity of the polarizing microscope, culminating in the introduction of "rectified" optics (22), clearer images of individual spindle fibers were obtained, and the birefringence distribution within each fiber in a living cell could be clearly ascertained (Fig. 2). We could now seriously examine the nature of the spindle fibers and their roles in mitotic chromosome movement.

By the late 1950s to early 1960s, several dynamic attributes of the spindle fibers were discovered or confirmed.[5] *(a)* The birefringence of the spindle fiber could be reduced *reversibly* to an equilibrium value, or be abolished totally, by low temper-

[5] For other important approaches complementing the polarized light analysis, see later sections on spindle-associated movements and micromanipulation.

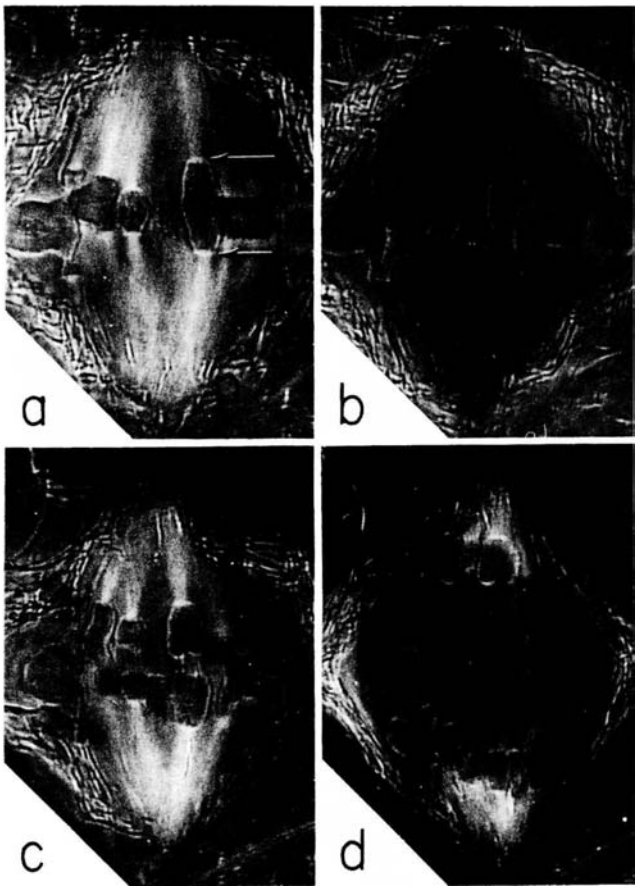

FIGURE 2 A *living* primary spermatocyte of *Pardalophora apiculata* (a grasshopper) as viewed with a rectified polarizing microscope (22). (*a*) Metaphase. Kinetochores of one bivalent are indicated by arrows (k) and polar regions by (p). Birefringent chromosomal spindle fibers run from each kinetochore toward a pole; the diffuse background birefringence of interpolar fibers is identified only with difficulty in the prints, but is readily measured. (*b*) Metaphase. The opposite compensator setting. (*c* and *d*) Anaphase. 22 and 36 min respectively, after *a*. From Nicklas (23). × 1,500.

ature (24) or with an antimitotic alkaloid, colchicine (25, 26). The morphological changes of the birefringent fibers (Fig. 3 *left*) suggested that the submicroscopic fibrils, from which the fibers resolvable with the light microscope were made up (18), were not simply coiling or becoming randomized but were, in fact, depolymerizing as the cells were cooled or treated with colchicine. (Also see important contributions by Beams and Evans [27], Östergren [28, 29], Pease [30], Wada [31], Gaulden and Carlson [32].) The fibrils repolymerized as the cells were rewarmed or the colchicine washed out. In other words, the fibers were not static structures, but rather existed in a dynamic state of flux. This strange capacity of reversible molecular assembly and disassembly, which was inducible by slight physiological perturbations, brought into line the seemingly paradoxical attributes of the achromatic spindle material. As emphasized by Östergren (29) and Wada (31) and puzzled over by Lewis (33), Wilson (2), Bělař (34), and others, there were indeed fibers made up of submicroscopic fibrils, yet the fibrils were made up of molecular subunits held together by labile bonds (Inoué [35, 36]). *(b)* The fibers were organized by "centers" (Boveri [37], Wilson [2], Wasserman [38]) such as centrioles (or equivalent structures), kinetochores, and, in typical plant cells, the cell plate material. As could be deduced from the higher birefringence adjacent to these centers and the temporal sequence of birefringent fiber growth in natural mitoses, as well as from the breakdown and regrowth behavior of the fiber which was microirradiated with a moderate dose UV-microbeam, the centers were capable of assembling or nucleating the fibrils from a preformed pool of unassembled subunits (36). *(c)* Depending on the activity of such centers and the physiological state of the cell, the spindle fibers could readily be built up, broken down, or reorganized. It appeared that the same molecules could enter one kind of fiber or another, depending on which center or polymerizing factor was active at that time (35, 36). *(d)* Chromosome movement ceased, and the chromosomes recoiled toward the metaphase plate when the chromosomal fiber birefringence was abolished in anaphase by cold. As shown in Fig. 3 *right*, poleward chromosome movement resumed in rewarmed cells after birefringent fibers had reappeared and reorganized into an anaphase configuration (36). (This paper also illustrates with many photographs, including excerpts from time-lapse motion pictures, changes in spindle-fiber birefringence that occur naturally in dividing plant and animal cells, as well as in experimentally modified cells.)

MICROTUBULES: In contemporary terms the birefringent submicroscopic fibrils of the spindle fibers and astral rays would be microtubules. By the early 1960s, electron microscopists had begun to describe mitotic microtubules, or "paired fibrils" (39–42); in 1963 "microtubule" still appeared in quotation marks (43). The equivalence of spindle fibrils and microtubules was therefore yet to be made.

By the mid-1960s, especially after the introduction of glutaraldehyde as a fixative for electron microscopy (44), mitotic and other microtubules were widely described and accepted as a basic cytoplasmic element as summarized by Porter (45). The lability and the reversible disassembling ability of the mitotic, and some cytoplasmic, microtubules (46–48) were shown to parallel the behavior of spindle fibrils deduced from their birefringence (49). Thus the ideas evolved that microtubules were the major structural element (fibrils) of the spindle fibers and astral rays,[6] and that the lability of the microtubules, in an equilibrium with a pool of their subunits, was responsible for the lability of spindle fibers (Inoué and Sato [49]).

The assembly of subunits into microtubules was seen to be mediated by hydrophobic bonds and to be entropy driven, as the assembly of tobacco mosaic virus A-protein (57), in the globular to fibrous transformation of actin (58), etc. The greater hydration predicted by this model for the subunits, as compared with the assembled microtubules, was consistent with the ability of D_2O, glycols etc., reversibly to increase the degree of spindle-fiber polymerization (49, 59). Low temperature and colchicine would both favor the disassembly state.

In the meantime, Taylor (60) succeeded in labeling colchicine with radioactive tritium and, in 1965 showed in an elegant study that the antimitotic action of colchicine was based on a tight but reversible, noncovalent binding of colchicine to a

[6] The quantitative correlation between the spindle fiber birefringence and microtubules has been questioned by some authors (e.g., 50–52), but has been affirmed after careful analysis by Sato et al. (53, also see 54 and 55). Marek '56) reports that the amount of microtubules found by electron microscopy is only half of that expected from the birefringence in living grasshopper spermatocytes. However, even in careful studies such as Marek's, it is not unlikely that a significant fraction of the more sensitive microtubules have been lost by fixation (cf. 49 and 53).

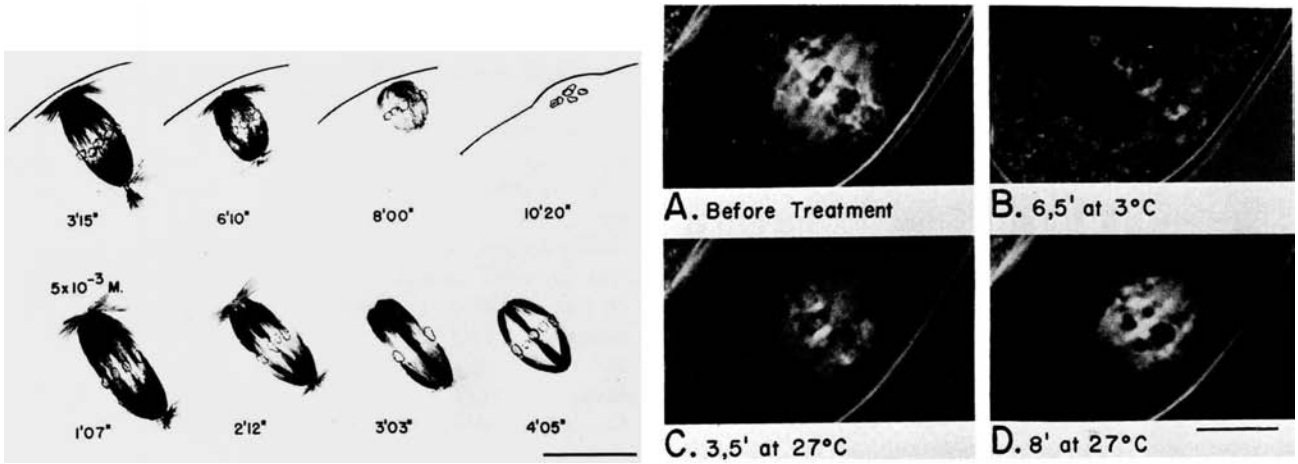

FIGURE 3 (*Left*) Effect of colchicine on the metaphase-arrested spindle in the oocyte of *Chaetopterus pergamentaceous* (a marine annelid worm). The changes in the position of the chromosomes and the morphology and birefringence of the spindle fibers were followed with a sensitive polarizing microscope. In 0.5 mM colchicine (*top*) the spindle fiber birefringence is lost in about 10 minutes. As the spindle decays, the chromosomal fibers shorten without thickening. The loss of birefringence indicates depolymerization of the microtubules making up the fibers. As the fibers shorten, the chromosomes and inner spindle pole are transported to the cell surface. The outer spindle pole is anchored to the cortical layer of the cell. At a higher concentration of colchicine (5 mM, *bottom*), the "continuous" (central) spindle fibers depolymerize and lose their birefringence first. Then the chromosomal fiber birefringence disappears, as the microtubules fall apart, before the fibers have shortened appreciably. The chromosomes are left "stranded." When colchicine is removed, the microtubules reassemble, and the elongating fibers transport the chromosomes and inner spindle pole away from the cell surface, eventually back to the metaphase configuration. Time in minutes (') and seconds (") after application of colchicine. From Inoué (25). Bar, 20 μm. (*Right*) Effect of chilling on pollen mother cell of *Lilium longiflorum* (an Easter lily) in early anaphase. The birefringence of the spindle fibers disappears in a few minutes as the microtubules depolymerize at 3°C. As the mitotic microtubules reassemble at 27°C, the birefringence returns rapidly. The chromosomes recommence anaphase movement in 8–10 min once the spindle organization has recovered. From Inoué (36). Bar, 20 μm.

critically small fraction (3–5%) of sites within the cell. Borisy and Taylor (61, 62) shortly thereafter isolated a colchicine-binding protein from extracts of sea urchin eggs and from isolated mitotic apparatuses. The binding-site protein showed a sedimentation constant of 6S and was identified by them to be the subunit protein of microtubules. An amusing sidelight of this study was that Borisy and Taylor found brain, which they chose as a control tissue expected to be free of dividing cells and hence of the (colchicine-binding, microtubular) spindle protein, to be a particularly rich source of the colchicine-binding protein.

Whereas the behavior of mitotic microtubules as seen in electron micrographs appeared to parallel the behavior of spindle-fiber birefringence as observed in living cells, the behavior of the in vitro, isolated mitotic apparatuses did not. Nor was the in vitro behavior of microtubules and their colchicine-binding 6S subunit, which was by that time isolated, characterized (63–67), and named tubulin (68) as similar as one would have liked to the behavior of microtubules in vivo. Isolated spindles were more stable in the cold than at room temperature, and they were insensitive to colchicine. Tubulin isolated from brain could be assembled into sheets and at times into microtubules, but only irreversibly so (66, 69).

LABILE MICROTUBULES: 1972 was a major turning point. Weisenberg (70) reported the in vitro reconstitution of *labile* microtubules from extracts of rat brain that contain a high concentration of tubulin. Unlike the earlier isolated spindles and reassembled microtubules, the new microtubules disassembled in the cold, and their assembly was inhibited by colchicine! In order to assemble such labile microtubules, it was important that the calcium ion concentration be kept low and that some magnesium ion and guanosine triphosphate (GTP) be present in the neutral (organic) buffer. These findings of Weisenberg's were rapidly confirmed (71, 72) and a new era in microtubule research had begun.

The labile microtubules which could now be assembled in vitro were reversibly disassembled by cold. Indeed, following Olmsted and Borisy (73), purification of tubulin and associated proteins have since been routinely accomplished by cold-warm recycling. Likewise, isolated and reconstituted microtubules were reversibly disassembled by hydrostatic pressure just as were the mitotic microtubules in intact dividing cells (74, 75). In vitro polymerized microtubules were in equilibrium with a pool of assembly-competent tubulin (76), and assembly was promoted by D_2O (77, 78). These properties of labile microtubules indeed seemed to parallel the behavior of mitotic microtubules in vivo.[7]

In detail, however, the assembly properties of the isolated tubulin, with or without accessory proteins,[8] still appeared to

[7] For a summary of the chemical and physicochemical properties of isolated microtubules and associated protein, see Haimo and Rosenbaum (this volume), the monograph by Dustin (79), records of two conferences (80, 81) and the following reviews (77, 82–86).

[8] Accessory proteins include high molecular-weight components "MAPS," presumably including dynein, lower molecular weight "tau's," and some with molecular weight not too different from tubulin (reviews, 86–88, and Haimo and Rosenbaum, this volume). Although they seem to affect the assembly and stability of microtubules, their role in mitosis does not seem very clear at this point. I will have little further to say about these components nor about the role of cyclic nucleotides and phosphorylation of tubulin although this is a field receiving much attention lately. (See especially [84], Haimo and Rosenbaum, this volume, and the references given at the end of the last footnote.)

be not quite the same as in living cells. In vivo, colchicine and Colcemid depolymerized labile microtubules[9]; in vitro, they acted primarily to prevent assembly and did not seem to take apart preformed microtubules (63, 73, 83). In vivo, D_2O shifted the equilibrium toward more microtubules (49, 94), whereas in vitro, it primarily raised the rate of microtubule assembly but not the amount of microtubules in equilibrium with tubulin (77; but also see 95). In vivo, the net assembly reaction appeared to fit a simple equilibrium model TUBULIN $\rightleftharpoons$ MICROTUBULES (assuming spindle-fiber birefringence to measure the concentration of its component, parallel aligned, microtubules) (35, 74, 94). In vitro, the tubulin (dimers) would be expected to enter and to leave at free ends of the microtubules and, as anticipated, the reaction was observed to take the form: MICROTUBULE + TUBULIN $\rightleftharpoons$ LONGER MICROTUBULES (76). These differences may in part be accounted for by the fact that the microtubules in vivo appear to be constantly, and rather rapidly, turning over; they are in a dynamic equilibrium.

DYNAMIC EQUILIBRIUM: The concept of a steady-state, or dynamic, equilibrium had been postulated earlier for spindle fibers in living cells.[10] Also, a UV microbeam of appropriate dose[11] could induce an area of reduced birefringence ("arb") on a spindle fiber, and it was found that this marker traveled poleward in a metaphase crane-fly spermatocyte at ¼–½ μm/min, a velocity approximately equal to the anaphase poleward velocity of chromosomes at similar temperatures (Fig. 4) (50, 97). Likewise, in a (Nomarski) differential interference contrast microscope, "particles or states" barely resolvable with the light microscope were seen traversing poleward along chromosomal spindle fibers in *Haemanthus* endosperm cells and tissue culture cells (98, 99). These transport phenomena could be interpreted as reflecting a dynamic assembly-disassembly of kinetochore microtubules, the assembly occurring at the kinetochore and the disassembly at or near the spindle pole. The component tubulin molecule would then travel along the microtubule in a fashion similar to a link in a chain that is constantly being assembled at one end and disassembled at the other end. (To date, however, the mechanical properties of the arb are unknown, and there exists no tagging experiment that unequivocally shows a poleward flow of tubulin along mitotic microtubules in living cells.)

For a while, it appeared that the steady-state equilibrium of labile microtubules in vivo might explain the difference of their response to colchicine treatment, their thermodynamic properties, etc., as compared with the in vitro system. But even in vitro, the story has become more complicated. Microtubules are now known to be polarized and show a preferred end of growth (e.g., 100, 101, and Haimo and Rosenbaum, this volume). Additionally, Margolis and Wilson (102) have shown recently that "equilibrium" microtubules in vitro are also in a dynamic, steady state. Under equilibrium conditions, the net assembly of GTP-bound tubulin at one end of the microtubule is balanced by the net disassembly of guanosine diphosphate (GDP)-bound tubulin at the other end. Therefore, even at steady state in vitro, there is a net flow or "treadmilling" of tubulin along the microtubules; that rate can approach 1/10 μm/min in the presence of 10 mM adenosine triphosphate (ATP) (103; but also see 104 and the section on models).

CENTERS: In living cells, spindle fibrils, or mitotic microtubules, are assembled sequentially around centrioles or satellites, kinetochores, and in plant cell phragmoplasts, the cell plate (reviews in 87, 105–107). Pickett-Heaps (108) has called these structures collectively microtubule organizing centers (MTOCs). Indeed, when such centers are isolated from living cells they have the capacity, as shown in Fig. 5, to initiate the assembly of microtubules onto or around themselves in vitro (reviews 86, 88, Haimo and Rosenbaum, this volume).

In mitosis, microtubules would have to be properly assembled and dynamically anchored onto appropriate organizing centers in order to: form a functional bipolar spindle; proceed successfully through metakinesis and anaphase separation of chromosomes; and coordinate mitosis with cytokinesis. Thus the MTOC are somehow activated at the right time, location, and orientation (36). There are many studies on the structure and composition of centrioles and kinetochores (e.g., 88, 113, 114), but little is yet known of how the activity of these centers, and how the assembly capability of tubulin, are regulated. (See [110, 115–119] for suggestive results. For experimental dissection of centriole replication and cell division, see Mazia et al. [120] and Sluder [121]. The problem of *de novo* formation of centers is reviewed in [113; also see 122–124 and Haimo and Rosenbaum, this volume].) The concentration of assembly-competent tubulin can be altered by application of colcemid long before the cell enters mitosis (125), as might be expected if the activities of the centers primarily govern when and where assembly is to take place (36). On the other hand, the disassembly of microtubules in anaphase may be governed in part by removal of assembly-competent tubulin from the tubulin pool (126).

SPINDLE-ASSOCIATED MOVEMENTS: In parallel with the characterization of microtubules, the birefringent major linear elements of the spindle fibers, several other lines of approach were used to characterize the mitotic spindle and to explore the mechanisms of mitotic chromosome movements. The movements of chromosomes and particles in and around the mitotic figures were analyzed in living cells by bright-field and phase-contrast microscopy. As early as 1929, Bělař (34, 127) observed anisotropic "Brownian" motion in the anaphase spindle mid-region, the motion being decidedly greater parallel to the spindle axis than it was transverse to this direction. In the 1950s, through extensive frame-by-frame analysis of phase-contrast, time-lapse motion pictures, Bajer and Molè-Bajer (e.g., 128) followed the predominantly poleward expulsion of particles lying in the region between the chromosomes and the spindle poles. Östergren et al. (129) found that long arms of chromosomes were likewise transported poleward in prometaphase. The poleward flow of "particles or states" at this stage

[9] Behnke and Forer (89) summarize evidence for the presence of microtubules with varying degrees of stability in living cells. Others have pointed out that kinetochore microtubules are often more resistant to colchicine, cold, hydrostatic pressure etc., then the astral and interpolar or nonkinetochore microtubules (e.g., 25, 90–93).

[10] The term "dynamic equilibrium" used in some of my earlier papers (e.g., 49) referred to a labile, equilibrium assembly of subunit protein coupled with the dynamic nature of the fibers. The latter point is stressed in Wilson (2), Östergren (29), Wada (31), and Inoué (36). The interpretation that their fibrils were also in a steady state-flux with a dynamic through-flow of subunits was gradually developed over the years. The distinction between the two types of "dynamic" properties are clearly defined (in 96, page 6).

[11] The dose of UV used for microbeam irradiation is highly critical because the irradiation also produces a diffusible toxic product that abolishes spindle birefringence. Failure in critical adjustment of the dose (best accomplished by observing spindle birefringence change) probably accounts for the diversity of results reported in the literature.

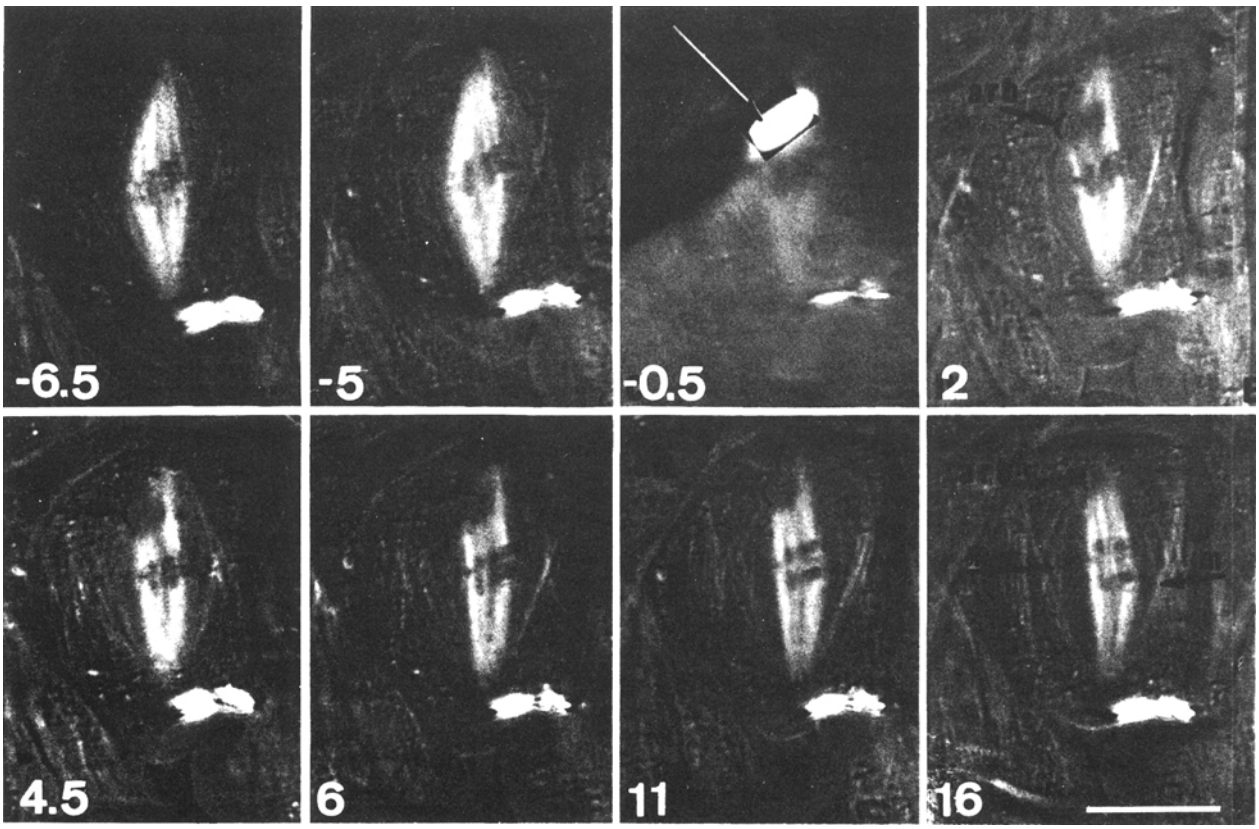

FIGURE 4 Ultraviolet (UV) microbeam irradiation of crane fly spermatocyte in late metaphase observed with a polarizing microscope. The bright patch (arrow) in the third frame shows the area to be irradiated by the heterochromatic UV microbeam. An area of reduced birefringence (arb) is induced in the spindle fibers by the microbeam irradiation. Note the gradual migration of the arb towards the upper spindle pole. Anaphase started 6 min after the irradiation. Mitochondrial sheath (m) surrounds the spindle. Time in minutes after irradiation. From Forer (97). Bar, 20 μm.

was mentioned earlier. In addition to poleward transport, time-lapse motion pictures at times displayed a striking lateral transport (130, 131) and compacting of nonkinetochore and phragmoplast microtubules (see [132] for grasshopper spermatocytes, [133] for *Haemanthus* endosperm cells). Bajer and Molè-Bajer (134) and Lambert and Bajer (135) found, by electron microscopy, that long stretches of microtubules are often "zipped" together in those cells (see later).

Mitochondria, yolk granules, and vesicles migrate radially toward and away from the spindle pole in a jerky, saltatory motion along astral rays (13, 136, 137). During prometaphase and metaphase, the centrospheres at the spindle poles can grow considerably in size, which perhaps reflects the transport and accumulation of vesicles into that region (but see 107). Similarly in telophase, small vesicles that are seemingly undergoing Brownian motion accumulate at the mid-region of a plant phragmoplast. There they fuse laterally to form the cell plate (138) by a process possibly reversing the pinching-off of vesicles from the Golgi body (Fig. 6). Whereas these movements have in common the transport of particles in a direction parallel to the lengths of microtubules, the nature of the transport mechanisms still remains to be solved (134, 137).

The whole spindle, as well as the nucleus, sometimes rock back and forth or spin around slowly. Such behavior is especially prominent in time-lapse motion pictures (e.g., 36, 139, and especially 140). Spindle-rocking is often accompanied by the "northern-lights" flickering of birefringence seen in the fibers, in and around the spindle. Some of these movements give the impression of being mediated by (microtubule) assembly-disassembly (36). Other aspects, such as the nuclear rotation, may be related to the revolution of the (actin-based?) polygonal cytoplasmic filaments studied in *Nitella* cytoplasm by Jarosch (141) and Kamiya (142; also see the variety of cytoplasmic movements described in 143, 144, and in Allen and Pollard in this volume).

In centrifuged living cells, particles accumulate along the centripetal side of the spindle, and spindle fibers and chromosomes are distorted. The pattern suggests a considerable mechanical integrity of the metaphase, but not anaphase, central spindle and of the chromosomal fibers which anchor the chromosomes to the poles (145, 146; summary in 6). Similarly, the premetaphase stretch of chromosomes, studied in detail by Hughes-Schrader (147, 148) in mantids and other insect spermatocytes, indicates, even before metaphase, a poleward force acting on the kinetochores of the unseparated sister chromatids.

These earlier analyses, and Östergren's classical studies on the paradoxical chromosome behavior during mitosis in *Luzula*,[12] reflect the dynamic mechanical behavior of spindle fibers (Östergren [29]). These intriguing mechanical properties

[12] In early metaphase of *Luzula purpurea* cells, the holokinetic chromosomes are interlocked in such a way that they could not undergo anaphase separation without breakage either of the chromosomes themsleves or of their kinetochore fibers. While the process of unlocking has not been seen in living cells, Östergren deduced from the abundance of normal anaphase figures in his fixed specimens that the kinetochore fibers must have been labile enough to be broken and reformed at the beginning of anaphase (29).

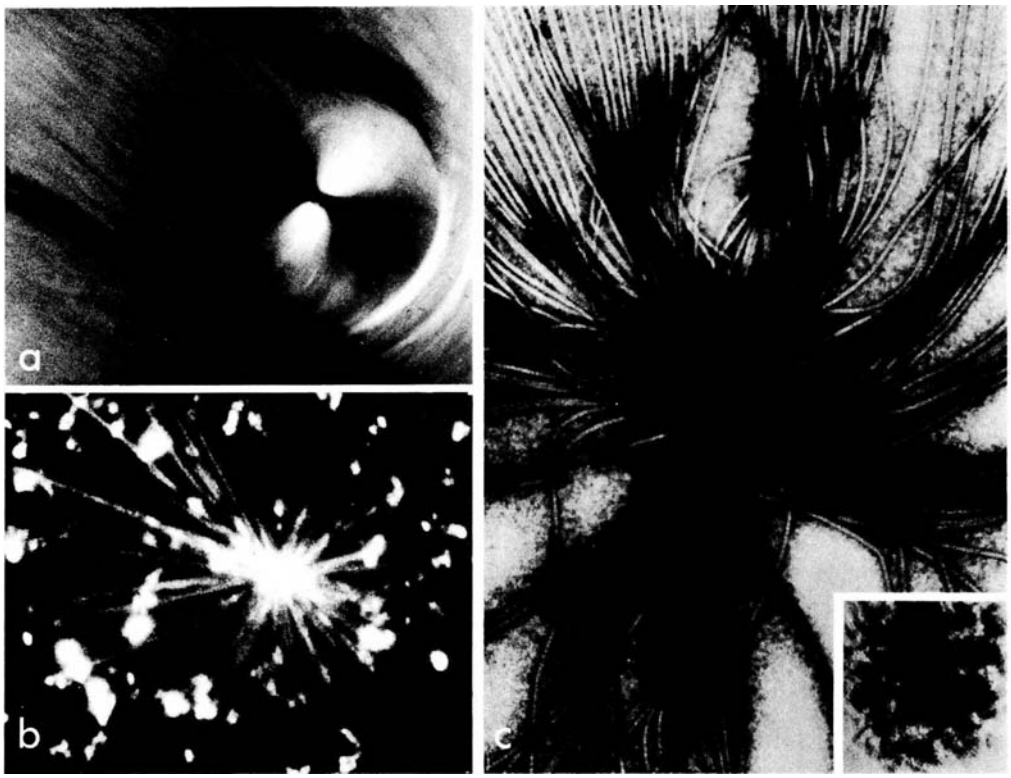

FIGURE 5 Artificial asters grown by addition of purified, heterologous tubulin onto isolated centrosomes. (*a*) Low power (~× 550) view in the polarizing microscope. Tubulin extracted from pig brain was polymerized onto a centrosome pressed out from a *Chaetopterus* oocyte in metaphase. The microtubules have grown to over 100 μm in length and show as long birefringent streamers. From Inoué and Kiehart (109). (*b*) Medium power (~× 2,200) view in dark field microscopy. Tubulin from chick brain was polymerized onto a centriolar complex isolated from HeLa cells blocked in "M-phase" with Colcemid. From Telzer and Rosenbaum (110). (*c*) Electron micrograph (~× 16,000) of a negatively stained preparation. Tubulin from pig brain was polymerized onto centrosomes isolated from CHO cells blocked with Colcemid. (*Inset*) The central region of the same micrograph printed lighter at a higher magnification (~× 30,000) to show the pair of centrioles. From Gould and Borisy (111). On isolated chromosomes, the kinetochores have similarly been shown to serve as microtubule-organizing centers (86, 111, 112).

of spindles have been investigated further by probing the interior of living cells with micromanipulation.

MICROMANIPULATION: The earlier micromanipulation studies of Chambers (149) and others were extended by Wada (150) and by Carlson (11) to analyze spindle structure. More recently, extensive and intricate manipulations of the chromosomes and spindle parts have become possible by use of the piezoelectric micromanipulator developed by Ellis (151). Thus Nicklas and co-workers (152, 153; review and interpretation in 23; also see 154) and Begg and Ellis (155, 156) were able to demonstrate the following: when a fine glass needle is inserted into a chromosome and gently tugged away from the spindle pole, the chromosome extends but the kinetochore-to-pole distance is virtually unchanged. Individual chromosomes (or chromosome pairs) can be swung about the spindle pole without disturbing other chromosomes (152, 155).

If the cell is already in anaphase, a chromosome can easily be pushed toward the spindle pole. That chromosome then waits until the other chromosomes catch up before it recommences its poleward travel. The chromosomes behave as though they were all being reeled in to the pole by individual fishing lines each attached to the kinetochore, but all sharing a common reel (155).

With the piezoelectric micromanipulator, Nicklas and Koch (157) detached individual chromosomes from their spindle fibers by tweaking the fiber near the kinetochore. Detached metaphase chromosomes reestablish a connection to the spindle; a kinetochore is drawn toward the pole it now faces. That may or may not be the pole to which it was originally joined!

The mechanical strength of a chromosomal spindle fiber was found to increase in parallel with its birefringence (156). Fiber strength increases in prophase as the fiber birefringence grows. Likewise, the mechanical integrity of the fiber disappears as fiber birefringence is eliminated by colchicine, and recovers as the birefringence returns during recovery from colchine treatment. Strangely, the fiber is also more stable, and chromosomes spontaneously detach and reorient less frequently, when the fiber is under tension (153).

These micromanipulation experiments directly confirmed and, to some extent, clarified the twin paradoxical properties of the spindle fibers, mechanical integrity and lability. Ellis and Begg (158) have prepared a comprehensive, thoughtful summary of the mechanical properties of the fibers connecting the kinetochore and the spindle pole, as revealed by micromanipulation studies.

The earlier micromanipulation studies by Wada (150) are also interesting. Even though he acknowledged the labile attributes of the fibers, Wada (31) held firmly to his view that the nuclear membrane never breaks down during mitosis in higher eukaryotes. This view is contrary to the essentially universal observation that the nuclear envelope does break down during mitosis in such cells (see later for the many exceptions found in lower eukaryotic mitoses). Even so, the shape and volume of the spindle often do resemble those same attributes of the

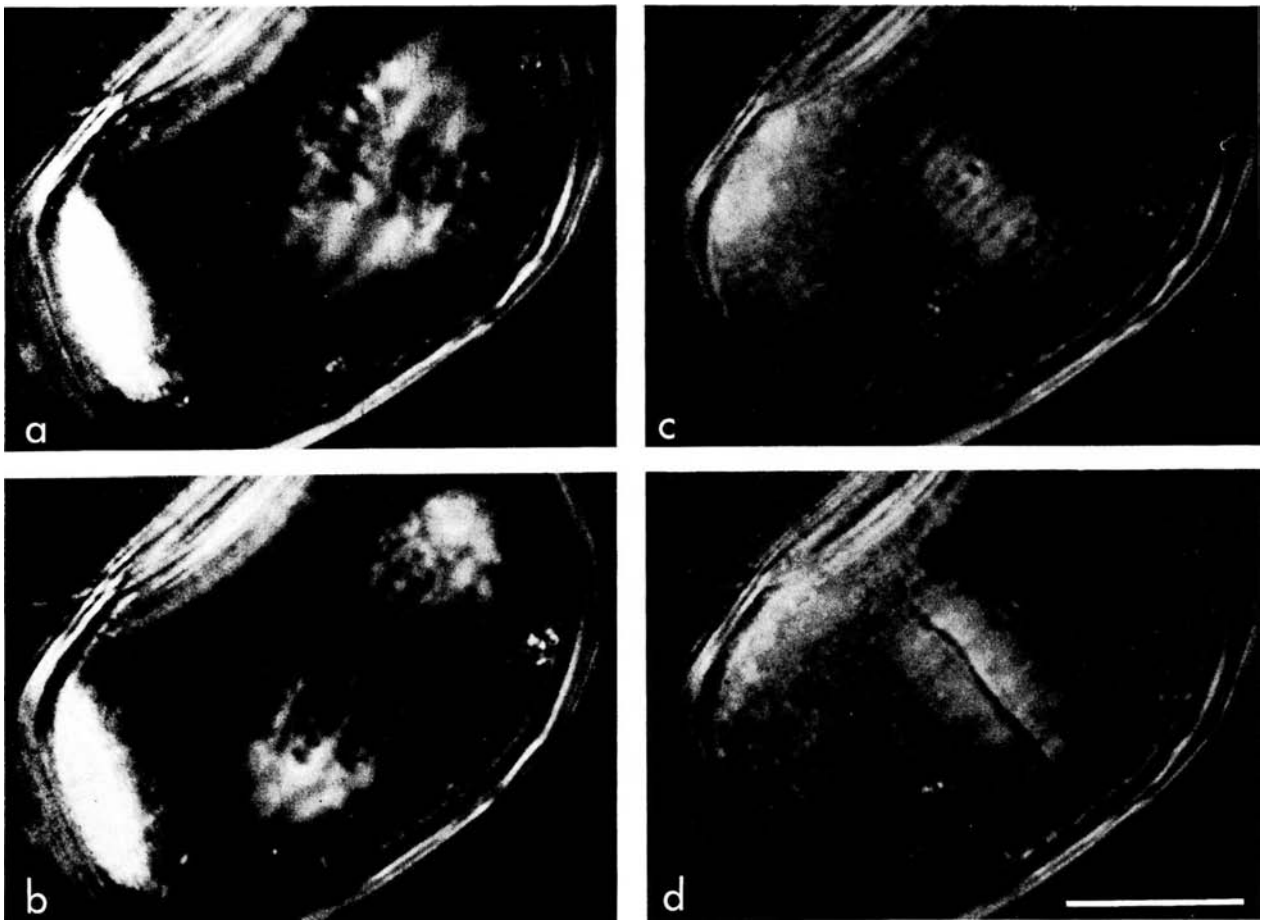

FIGURE 6 Pollen mother cell of *Lilium logiflorum*. Selected frames taken from a 16 mm time-lapse movie taken with a sensitive polarizing microscope. (*a*) Late metaphase. The chromosomes (dark gray) are still on the metaphase plate. They are not yet stretched but the strong birefringence (brightness) of the chromosomal fibers indicates that the cell is about to enter anaphase. (*b*) Mid-anaphase. The helical chromosomes, led by the birefringent chromosomal fibers, are just separating. The strong birefringence (signifying high concentration of microtubules) of the chromosomal fiber adjacent to the kinetochore persists from late metaphase to mid-anaphase. Notice weaker birefringence toward the poles and the absence of asters (cf., Figs. 1 and 11, and footnote 20). (*c*) Telophase. Chromosomes have formed the daughter nuclei. Between them the birefringence of the phragmoplast fibers is considerably stronger than was the mid-zone of the anaphase spindle (cf., *b*). Many new microtubules have been formed oriented parallel to the sparse microtubules that remained behind the separating chromosomes in late anaphase. Small vesicles are beginning to accumulate at the middle of the phragmoplast. (*d*) Cell plate formation. The vesicles have fused at the middle of the phragmoplast and have started to form the cell plate. The phragmoplast and the cell plate continue to grow laterally until the cell is completely divided. From Inoué (36). Bar, 20 μm.

nucleus before nuclear envelope breakdown. Nucleoplasm and the hyaline cytoplasm clearly must mix in establishing the spindle (e.g., see the extensive inclusion of ribosomes (?) amidst spindle microtubules in Fig. 7), but larger organelles[13] are excluded or expelled from the spindle region. In fact, the mitotic figure is frequently visible in living cells as a clear region from which most microscopically detectable granules are absent and which is outlined by mitochondria, yolk granules, etc. What accounts for this separation? This may be explained in part by the fact that the spindle is embedded in its own gel matrix. In addition, it may reflect another component that participates in mitotic cellular organization, the membranes.

[13] In some cells the nucleolus is not expelled from the spindle and is even regularly divided into two (e.g., 2, 6). Also see Cooper (161) for chromosome shaped "equatorial bodies" which retain the shape of chromosomes and remain on the metaphase plate as the chromosomes move poleward in anaphase.

MEMBRANES: Recently, increasing attention has been paid to the amounts of cytoplasmic membranes surrounding, although not completely enveloping, the spindle (e.g., Fig. 7, top right). As shown earlier by Porter and Machado (162) and more recently emphasized by Hepler (163), some lamellar or tubular cisternae also penetrate the spindle from the poles parallel to the chromosomal fibers. Harris has called attention to the many vesicles found in that region and especially the spindle-pole regions in sea urchin eggs (however, see 107). Are these, as Harris (164) postulates, calcium-sequestering or -releasing structures (in analogy with the sarcoplasmic reticulum in muscle cells)? Calcium seems to be accumulated within the vesicles or vesicular membranes (163, 165, 166). The distribution of vesicles and reticular membranes in and around the spindle, as well as the sensitivity of microtubules (70, 167), actin gels (168, 169), regulator-bound actomyosin (170, 171) etc. to micromolar concentrations of calcium ions, suggests some regulatory role for these membranes as discussed below.

CALCIUM: During the 1930s to 1950s, Heilbrunn sug-

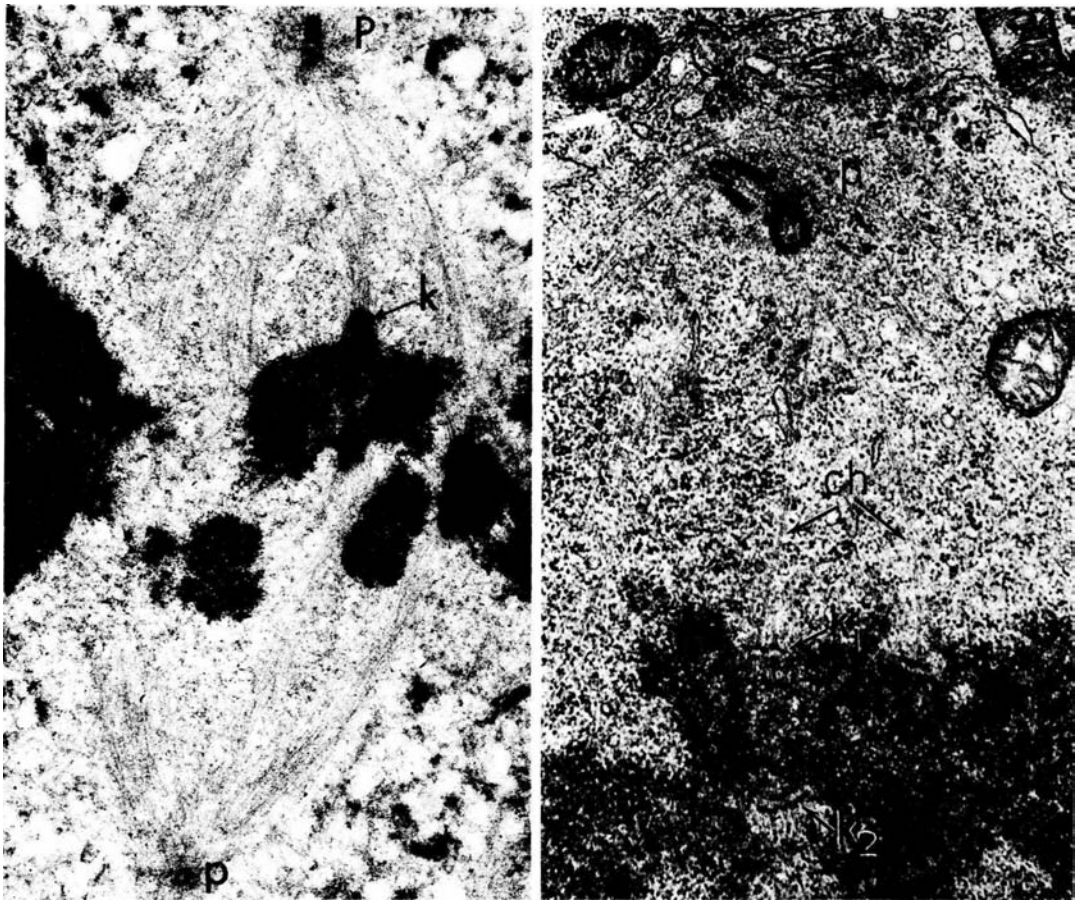

FIGURE 7 Electron micrographs. (*Left*) Thick (0.25 μm) section of a PtK_1 cell in early metaphase, observed at low power (~× 7,000) with a high voltage electron microscope. Both poles (p) of the spindle are clearly visible. Bundles of microtubules making up the chromosomal fibers run from the kinetochore (k) towards the spindle pole (cf., Fig. 2). The ribonucleoprotein stain employed in this preparation darkened the inner plate of the trilaminar kinetochore. From Rieder (159). (*Right*) Thin section of a rat kidney tubule cell in metaphase. The two kinetochores (k_1, k_2) of one chromosome clearly show the trilaminar structures. Chromosomal microtubles (ch) appear to terminate on the outer layer of the kinetochore. One pole (p) of the spindle is marked by a pair of centrioles. From Jokelainen (160). ~× 31,000.

gested a multifaceted physiological role for calcium ions (e.g., 172). While he was often scoffed at by his contemporaries, his now proven postulate regarding the sequestering and release of calcium and its role in the regulation of muscle contraction was prophetic (173). Heilbrunn further attributed to calcium ions the capacity to induce "mitotic gelation" in analogy with blood clotting. A calcium-activated ATPase was later found to be associated with the isolated mitotic apparatus (174). On the other hand, recent findings suggest that calcium ions solate, rather than gel, some of the components relevant to mitosis. In the presence of millimolar calcium, isolated microtubules depolymerize rapidly (70, 175). Purified microtubules reassociated with calmodulin, a calcium-binding protein similar to the muscle protein troponin C, rapidly depolymerize in the presence of calcium ions at even micromolar concentrations in vitro (167).

When microinjected into sea urchin eggs, millimolar calcium chloride or EGTA-buffered micromolar concentrations of calcium ions depolymerize spindle microtubules locally and instantaneously. The process is so rapid that the progression of birefringence loss could not be followed under continuous observation (Kiehart [165]). The portion of the spindle whose microtubules are depolymerized is so limited that it shows as a discrete, sharply delineated patch from which the birefringence has disappeared (Fig. 8). This observation complements

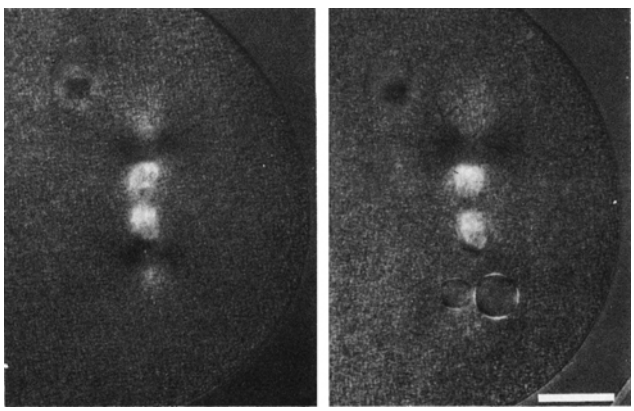

FIGURE 8 Microinjection of 1 mM $CaCl_2$ into *Asterias forbesi* egg at metaphase observed in polarized light. (*Left*) First injection at the upper left away from the spindle pole produced no effect on the spindle birefringence. (*Right*) Injection at the lower pole eliminated the birefringence of the aster and the tip of the spindle. Note the very sharp contour of the remaining spindle. When calcium-buffer solutions are injected, 5–10 μM equivalents of free Ca^{++} ions locally eliminate the spindle birefringence. Each pair of oil drops that had been used to cap the test solutions in the micropipette before the injection, indicates the volume of the test solution and the approximate site of injection. From Inoué and Kiehart (109). Bar, 30 μm.

Rose's and Loewenstein's findings (176) that when calcium ions are microinjected into *Chironomus* salivary gland cells previously loaded with aequorin (a calcium-dependent, light-emitting protein), light is emitted only in that portion of the cell into which the calcium solution is directly applied. In other words, although it is a small, diffusible ion, calcium is sequestered so rapidly in the cytoplasm that the relatively high injected concentration is limited to the region of the cell that receives the microinjection directly.

The cytoplasmic membranes and mitochondria are likely candidates as calcium sequestrants. The calcium-dependent aequorin glow is no longer limited to the site of injection, but is spread out in the presence of respiratory poisons (176). Similarly, Sawada and Rebhun (177) have found that the birefringence of the spindle in some cells is abolished when the cell is exposed to respiratory poisons or uncouplers of oxidative phosphorylation. These agents, as well as caffeine, probably poison the calcium-pumping ATPase, making the cell membranes leaky to calcium ions and inducing the mitochondria and endoplasmic reticulum to dump their accumulated calcium ions. In caffeine-microinjected cells, there is a drop in spindle birefringence that is not sharply delineated, but diffuse, presumably because in contrast with calcium ions, caffeine is not sequestered by the cytoplasm and therefore diffuses normally (165).

Petzelt's calcium-dependent ATPase appears to be membrane bound (178). Membrane-delimited vesicles and cisternae are seen by electron microscopy to be concentrated at the spindle pole and in a sheath surrounding the spindle (163). Thus, as postulated by Harris (164), these membranes may well play an important role in calcium regulation of the cytoplasm, and they may do so in highly localized cell regions. Welsh et al. (179) observed, by fluorescent antibody staining, a higher concentration of calmodulin in the half-spindle. The anaphase location of calmodulin near the spindle poles, and the changes observed in their distribution during late anaphase, suggested to Marcum et al. (167) that calcium is an endogenous regulator of microtubule assembly through the activity of calmodulin (also, 180).

Models for Mitosis

We shall now consider some current models which have been proposed to account for the (anaphase) movement of chromosomes. The models have been reviewed (23, 134, 181) and extensively discussed (e.g., 79–81, 144, 182), except for the recent model that incorporates the treadmilling of tubulin along microtubules (183).

ACTIN AND MYOSIN: One of the oldest models for poleward movement of chromosomes invoked the contraction of a muscle-like fiber which linked the chromosome to the spindle pole (pages 178–184 in [2], pages 70–75 in [6]). From the earliest days, however, this model has been repeatedly questioned. Shortening chromosomal fibers generally do not get thicker; anaphase velocity is so much lower than the contraction velocity of skeletal muscle (of the order of 10 nm/s, in contrast with 100 μm/s for muscle); and the lability of the spindle fibers does not fit the properties of muscle.

For many years there was no reason to resurrect this model, although the counter arguments were not airtight. But recently the model has again gained some support. Actin and myosin, the two major proteins responsible for force production by muscle fibrils (review; e.g., 184, 185), were detected in the half-spindle regions of glycerinated cells. Fluorescent antibodies made against these proteins stained the spindle (186, 187), as did fluorescein-conjugated heavy meromyosin or subfragment-1, which carry the active ATPase sites of the myosin molecule (188–191). (However, the levels of immunofluorescent staining for actin and myosin are not greater in the spindle, according to the latest report from Aubin et al. [192].) In electron micrographs of glycerinated cells, some actin filaments were seen to terminate at or about the kinetochore and to run approximately parallel to microtubules in the half-spindle (e.g., 190, 193).

Whereas these observations on glycerinated cells are suggestive and have attracted considerable attention, the data in themselves do not imply a functional role for actomyosin or an actin system in the poleward movement of chromosomes. Like tubulin, actin is one of the major protein constituents of most cells (each at times amounting to several percent or more of the total cell protein). The spindle region in dividing cells excludes granular organelles such as mitochondria and yolk, so that, on this basis alone, one might expect to find a somewhat higher concentration of nonparticulate cytoplasmic constituents, including actin, in the spindle region of some cells (165, 194; also see Fig. 2 in 195 for a model demonstrating this point).

Two types of tests for the functional role of actomyosin and actin in mitosis have yielded negative results. The microinjection of an antibody against starfish-egg myosin (previously shown to suppress hydrolysis of ATP by egg myosin) prevented many successive cleavage divisions but did not interfere with mitosis in the same starfish eggs (Mabuchi and Okuno [196]). This is consistent with the strong evidence that cleavage is brought about by an actomyosin contractile ring (review, 197). In the eggs injected with anti-egg myosin (Fig. 9), birefringent spindles formed at regular intervals, chromosomes moved toward the spindle poles and spindles elongated normally in anaphase, and nuclear envelopes were reconstituted on schedule, despite the absence of eight or more cleavages (Kiehart [165]).

Likewise, in Cande's detergent-permeabilized tissue culture cells, cleavage was suppressed by the application of heavy meromyosin or subfragment-1, which had been treated with *N*-ethyl maleamide (198). These treated fractions of myosin bind to actin competitively and prevent the interaction of actin with normal myosin (199). In the permeabilized cells, cleavage was arrested, but anaphase movement was not affected by application of the modified myosin fragments. Chromosome movement, especially the part dependent on spindle pole-to-pole elongation was, however, reversibly inhibited by vanadate, a potent inhibitor of ciliary dynein ATPase (200). This ion showed little effect on cleavage in lysed cells, reinforcing the idea that different molecular mechanisms are operating in chromosome movement and cleavage.

In living cells, cleavage is suppressed, or regresses, when cells are bathed in solutions that affect actin gelation. Cytochalasin B and D are reported to weaken actin gels (201, 202), but not to affect mitosis when applied in concentrations adequate to suppress cleavage (e.g., 197). In contrast, colchicine and podophylotoxin, which prevent mitosis and even disassemble mitotic microtubules, do not affect cleavage once the cleavage message has been delivered from the spindle to the potential furrow (26, 203). Actin and myosin have been extracted from the cell cortex, and electron micrographs show a clear band of actin filaments in the cell cortex oriented circumferentially in the cleavage furrow (review, 196). Taken together, these data strongly support the role of an actomyosin system in cytoplas-

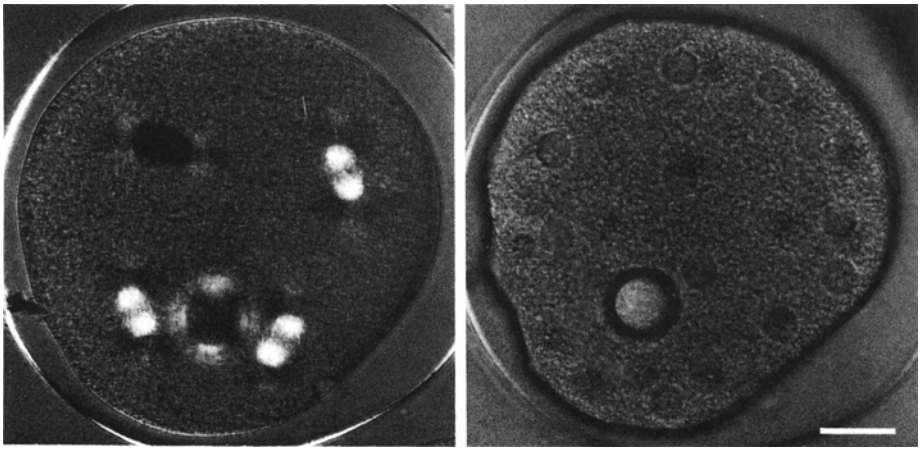

FIGURE 9 Egg of a starfish, *Asterias forbesi*, microinjected with an antibody made by Mabuchi and Okuno (196) against myosin of another species of starfish, *A. amurensis*. Cleavage is suppressed for up to nine divisions, but mitosis is unaffected. One nanogram of IgG, containing the antimyosin, was injected before first cleavage. (*Left*) Third division spindles seen in polarized light. (*Right*) Over 30 nuclei are visible 2.4 hours later. From Inoué and Kiehart (109). Bar, 30 μm.

mic cleavage, but do not favor the involvement of actin or an actomyosin system in mitotic chromosome movement.

ASSEMBLY-DISASSEMBLY OF MICROTUBULES: This model postulates that assembling microtubules, by their extension, push organelles apart, and slowly disassembling microtubules, by their shortening, pull organelles together. I conceived the model through the observation of *Chaetopterus* oocytes exposed to colchicine or cold. As the spindle-fiber material slowly depolymerized in these metaphase-arrested cells, the chromosomes and the inner spindle pole were transported toward the outer spindle pole, which is anchored to the cell cortex (Fig. 3). As the fibers reassembled upon removal of the depolymerizing agent, the chromosomes and inner pole were transported away from the outer spindle pole. Too high a dose of colchicine or overrapid chilling simply caused the spindle fibers to fall apart without appreciable displacement of chromosomes or pole (24, 25). This model at once seemed to explain the labile, yet cohesive, nature of the forces that held together the ephemeral fibrils of the spindle (36), as well as the slowness of chromosome movements.

The subsequent discovery of labile microtubules added credibility to the assembly-disassembly (or dynamic equilibrium) hypothesis (35, 49, 204), but its validity has been repeatedly questioned, presumably in part because the proposal is not intuitively compatible with macroscopic mechanics.[14] Nevertheless, Salmon (74) and Fuseler (126) performed experiments utilizing hydrostatic pressure and cold as microtubule depolymerizing agents, and confirmed that spindle shortening and chromosome movements are induced in living metaphase cells by the depolymerizing agents.[15] They also demonstrated a strict proportionality between the velocity of induced spindle-fiber shortening (and of natural anaphase movement) with the rate of microtubule depolymerization. Although slow depolymerization of microtubules induced a shortening of chromosomal fibers, the induced movement ceased altogether when the rate of microtubule depolymerization became too great, whether the depolymerization was induced by pressure, temperature, or colchicine (review, 96, 205). In Salmon's words (74), "Polymerization of microtubules does produce pushing force and, if controlled microtubule depolymerization does not actually produce pulling forces, at least it governs the velocity of chromosome-to-pole movement."

Whether or not it turns out that shortening microtubules can exert pulling forces in addition to the pushing forces generated by their growth, the dynamic anchorage of microtubules is essential for force transmission through the assembling and disassembling microtubules. In this context, dynein, the ATPase associated with ciliary and flagellar microtubules (206, 207) has lately received much attention. Cytoplasmic dynein has just been isolated and characterized (208), and its role in anaphase movement, at least in pole-to-pole elongation, finds much experimental and observational support, as discussed below.

THE SLIDING MODELS: In 1969 McIntosh et al. (209) proposed that mitotic chromosome movement was brought about by a combination of microtubule sliding (in analogy with muscle contraction and ciliary and flagellar beat) and microtubule assembly and disassembly. Though the details of this model soon needed to be revised (23, 210), it nevertheless struck a favorable chord with many investigators. The labile and dynamic attributes of the spindle fibers were ascribed to demonstrated properties of microtubules, and force production could be attributed to (dynein) cross bridges, whose ability to induce relative sliding of ciliary microtubules was soon to be established (211; review, 212).

While seeming to provide a rational model, some predictions of which were quite readily testable, the model failed to account for certain properties of some spindles. In studying mitosis in yeast and other lower eukaryotes,[16] Roos (213), Peterson and Ris (214), Heath (215) and others came upon connections between chromosome and pole (plaque) that consist of single microtubules! Some of the nonkinetochore microtubules that make up the central spindle appeared to span the whole distance between the spindle-pole structures, even as the spin-

[14] Contractile force production by a disassembling microtubule can be explained by viewing the labile microtubule as a cylindrical micelle, as explained in Inoué and Ritter (96).

[15] Salmon has now induced spindle-shortening and chromosome-to-pole movement in isolated metaphase spindles (of the type shown in Fig. 1), by depolymerizing the labile microtubules with micromolecular concentration of calcium ions.

[16] For mitosis in lower eukaryotes, which show many interesting and potentially instructive variations on mitosis and mitotic organelles, see (4, 20, 55, 215–218) and the excellent review by Kubai (219).

dle elongated. Further, the central spindle microtubules appeared not to be in locations where they could interact with the "kinetochore" microtubules.[17] The single "kinetochore" microtubules appeared to shorten and to bring the "chromosomes" to the spindle pole independent of spindle pole-to-pole elongation.

In serial sections of cultured cells, Brinkley and Cartwright (221) did not find the number distribution of microtubule cross sections predicted by the McIntosh et al. model. On the other hand McIntosh et al. (222, 223) do report finding the distribution of microtubules appropriate for their model. Manton et al. (224) in an elegant study on mitosis and meiosis in a centric diatom, counted microtubule numbers in serial sections that are compatible with the overlapping of central spindle microtubules. In contrast, central spindles in some protozoa are known to elongate much more than twice their initial length, so sliding alone cannot account for spindle elongation; at least some spindle fiber growth is required (e.g., 55).

Despite these reservations, Tippit et al. (225) and McDonald et al. (226, 227) provide a most striking illustration of the overlapping microtubules in a configuration highly suggestive of interactions between oppositely polarized microtubules. In the mid-region of a diatom (*Melosira*) spindle, the cross sections of microtubules are arranged in a regular orthogonal array. And every other microtubule appeared to be connected to opposite spindle poles! In later anaphase, the overlap of the central spindle microtubules progressively decreased, although concomitantly there was growth of some microtubules (also see 228).

Although dynein has not yet been unambiguously demonstrated between opposing microtubules (summary regarding intertubule arms [229]), extensive periodic cross bridges have been seen in the metaphase (extranuclear) central spindle in a hypermastigote protozoan (Fig. 10). These observations, which might suggest the involvement of dynein-mediated sliding, are in fact fortified by two functional tests.

In isolated mitotic apparatuses, Sakai et al. (230) observed chromosome movement, which, while considerably slower than in living cells (but see improved movement reported in [231]), appeared to exhibit general features of in vivo anaphase movements. The movement which required a labile mitotic apparatus was prevented by excess tubulin in the medium and especially by vanadate ions and antibodies formed against dynein (review, 232).

These conclusions were complemented by observations in another type of cell model by Cande and Wolniak (200). In detergent-extracted rat-kangaroo cells in tissue culture, chromosome movement could be stopped by vanadate ions in the +5 oxidation state. After the chromosomes had stopped, they could be restarted by converting the vanadate to the inactive +4 state via the addition of norepinephrine. As shown by Gibbons et al. (233), the +5 vanadate is a potent inhibitor of ciliary and flagellar ATPase (however, see [234] for a lower vanadate sensitivity of cytoplasmic dynein).

Whereas these ongoing experiments require further confirmation,[18] the experiments on isolated spindles and extracted cell models strongly suggest the involvement of dynein in anaphase chromosome movement. It would seem quite likely that a dynein-mediated sliding mechanism is at least in part responsible for the pole-to-pole extension of the anaphase spindle. It is not as clear whether a dynein-mediated sliding is involved in the chromosome-to-pole movement. Perhaps dynein is a dynamic anchor for the kinetochore microtubules, but then we still must ask, how does microtubule disassembly govern the velocity of poleward chromosome movement?

FIGURE 10 Electron micrograph of central spindle in a hypermastigote protozoan *Barbulanympha* sp. Extensive cross bridges are seen in this micrograph, reinforced by superimposing two transparencies of the same image translated once along the microtubule axes. From Inoué and Ritter (96); also see Ritter et al. (55).

TREADMILLING: Recently, an alternative to the McIntosh et al. model, placing a greater emphasis on the role of microtubule assembly-disassembly was introduced by Margolis et al. (183). These authors found in vitro a "treadmilling" of tubulin through microtubules that were in an assembly steady state with soluble tubulin dimers in the presence of an adequate and continuous supply of GTP.[19] They postulate for dividing cells that all mitotic microtubules add microtubule subunits at the equatorial region of the spindle and at the kinetochores, and that the microtubules lose subunits at the spindle poles. They also propose that nonkinetochore microtubules, which overlap at the equator, slide by each other at a rate needed to keep the spindle poles separated. Kinetochore microtubules are thought to form a parallel linkage to the treadmilling interpolar microtubules (183). Depending on the relative rates of tubulin incorporation into the kinetochore and nonkinetochore microtubules and their rates of disassembly, one could increase or decrease spindle length as well as the distance between kinetochores and the spindle poles. Margolis and Wilson (103) report that the in vitro rate of microtubule treadmilling can come close to anaphase chromosome velocity. Because dynein

[17] In yeast, mitosis takes place within the nuclear envelope. The poles of the intranuclear spindle lack centrioles but are organized around spindle pole plaques on the nuclear envelope. The trilaminar kinetochore structure generally seen on chromosomes of higher eukaryotes has not been observed in yeast and chromatin appears to associate directly with microtubules (213). Ris and Witt believe that even in organisms with a trilaminar kinetochore, the kinetochore microtubules are directly linked with the centromeric chromatin (220).

[18] Both the Sakai et al. isolates (231), and the Cande cell models (198) have yet to be refined before the inference of these results is fully accepted. Both models run "downhill" rapidly, and to the best of my knowledge, vanadate and dynein antibody inhibition of mitosis has not yet been observed in living cells.

[19] Weisenberg (personal communication), Bergen and Borisy (235), and Karr and Purich (104) emphasize that treadmilling is not caused simply by assembly at one end of the microtubule and disassembly at the other. Rather, both assembly and disassembly take place at each end, but their rates differ in such a way that the *net* assembly at one end is greater than the *net* disassembly at the other end.

has now been used successfully to "decorate" microtubules and to indicate their polarity (Haimo and Rosenbaum [236]; also [101, 237, and Haimo and Rosenbaum, this volume]), we should soon be able to learn whether the polarities of mitotic microtubules conform to those stipulated in the Margolis and Wilson or McIntosh et al. model.

OTHER PROPOSALS: Before we leave the models for anaphase chromosome movement, we should take special note of the work by the Bajers and co-workers who emphasize the lateral transport seen in spindles and the lateral interaction believed to take place between mitotic microtubules (summary, 131, 134, 238). Since the late 1940s, the Bajers have extensively analyzed chromosome and particle movements and spindle behavior directly in healthy, dividing plant and animal cells and in cells treated with a variety of antimitotic agents. Their analyses on electron micrographs of cells that had been followed and recorded up to the time of fixation with time-lapse cinematography, suggested the importance of the changing lateral association of microtubules seen within individual kinetochore fibers, as well as between kinetochores and nonkinetochores microtubules (134). In general, the presence of intrakinetochore fiber association correlated with cessation of chromosome movement, whereas association between kinetochore and nonkinetochore microtubules was evident whenever chromosomes were moving poleward (but also see [93]).

Little is yet known of how these microtubular organizations are controlled, nor whether there is or is not sliding of microtubules associated with the lateral interactions, but the Bajers alert us to the possible role of microtubule interactions that could play an important role in anaphase chromosome movement (238). In contrast, Thornburg (239) proposed that viscous coupling associated with *intra*microtubular conformational change might propel the microtubules with their attached chromosomes.

Coordination of Cytokinesis with Mitosis

Once the chromosomes are partitioned into two equivalent (or, in meiosis, nonequivalent) groups by mitosis, how are the daughter nuclei placed in the proper cytoplasmic environment? This question is not only important for the successful completion of cell division, but also for determining the future role of the nucleus, because it is the cytoplasm surrounding the nucleus, rather than the unequal division of the nucleus itself, that generally determines how a particular cell is to differentiate (2, page 1059), (also see [5, 240–242]).

In astral mitosis,[20] a close correlation has long been noted between the metaphase spindle axis and the cleavage plane. When a cell was left undisturbed, the cleavage furrow almost always started from the cell surface nearest the spindle and in a plane bisecting the pole-to-pole axis of the spindle (Fig. 11). In centrifuged eggs, the cleavage furrow would appear in a new location dictated by the displaced spindle (e.g., 145). In fact, the correlation was so universal that most postulates for cleavage-furrow induction ascribed a major role to the mitotic spindle (2, 105, 203, 245, 246). Not only was there present a spatial correlation between spindle axis and cleavage plane; in the late 1030s to 1040s, Katsuma Dan and his co-workers showed a striking geometrical relationship between the extending anaphase spindle and the progression of cleavage. In sea urchin and jelly fish eggs, small particulate markers, which were applied directly to the cell surface near the impending cleavage furrow, moved along the exact path predicted from the separation of the astral centers as the spindle elongated. Dan assumed that the cell cortex was connected to the astral centers by interdigitating, inextensible astral rays by which the elongating anaphase spindle drew in the cell cortex, thus forming the cleavage furrow (summary in 247). Whereas this hypothesis could also account for the many unexpected cleavage patterns found in eggs deformed into toroids (248), two sets of experiments negated Dan's hypothesis.

In 1953, Swann and Mitchison (26) applied high doses of colchicine (3 mM in seawater) to metaphase sea urchin eggs and showed that cleavage nevertheless proceeded after destruction of the birefringent asters and spindle, providing the chromosomes had progressed to mid-anaphase (also see 27). Further, in 1956, Hiramoto (249) managed to suck out the entire spindle and asters from a dividing sea urchin egg and showed that a cleavage furrow appeared in the expected location so long as the cell had reached metaphase before spindle extraction (further, detailed analysis in [250]). He thus eliminated the possibility that cleavage was mechanically effected through a noncolchicine-sensitive element of the spindle and aster. These experiments clearly showed that the late metaphase-to-anaphase spindle and asters were unnecessary for cleavage, but it was equally clear that at an earlier stage the mitotic figure did determine the cleavage plane.

If the spindle before metaphase was artificially reoriented, the cleavage furrow appeared perpendicular to and bisecting the spindle in its new position (summary in 105). During a brief critical period, the spindle could even initiate up to ten cleavage furrows in succession, if the spindle were squeezed along the length of a sand-dollar egg previously deformed into a cylinder (251)! Clearly, then, a message[21] must be sent from the spindle to the cell surface where the cortical layer contracts and produces the cleavage furrow[22] (but see 254 and 255).

While the furrow was normally localized where the two asters (whose foci lay at the two poles of a spindle) overlapped, two asters not joined by a spindle could also induce cleavage (203, 246). The message for cleavage induction therefore comes not directly from the spindle itself, but from the spindle poles or astral centers. The speed of the message, and the duration required for the cortex to respond, were determined by displacing the spindle (251). Interestingly, the message travels along the astral rays at about 6 μm/min, approximately the rate at which microtubules grow.

In anastral mitoses,[20] especially in cells of vascular plants, a large number of microtubules appears between the separating chromosomes in late anaphase (138, 256). The ellipsoidal bundle of microtubules, the phragmoplast, has long been thought to arise from central spindle fibers (e.g., Strasburger, 1888, in

[20] Astral mitosis: with asters (Figs. 1 and 11). Typically, but not always nor exclusively, found in animal cells. Anastral mitosis: without asters (Fig. 6). Typically found in, but not limited to, higher plant cells. See Dietz (243) for experimental dissociation of asters from the spindle pole in a living cell, and Aronson (244) for analysis of attractive forces between (astral) centers and nuclei.

[21] The notion of a cleavage-inducing message (substance X which acted through polar relaxation) that traveled along the astral rays was proposed by Swann (18) and Mitchison (252).

[22] Sadly, I must leave out a series of intriguing accounts on the search for the mechanism of cleavage itself. Many interesting experiments were performed and ingenious hypotheses constructed (excellent summaries in [203, 246, 251, 253]). For our present purpose, we proceed by accepting the finding that the "contraction" of a cortical actomysin system is responsible for cell cleavage see (e.g., Schroeder. [197]).

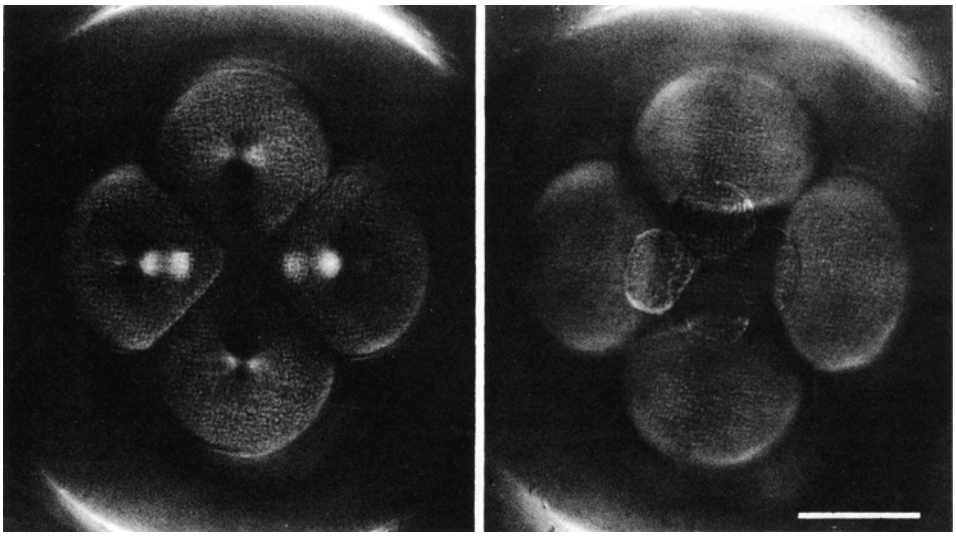

FIGURE 11 Fourth cleavage division in the egg of a sand dollar, *Echinarachnius parma*. The characteristically asymmetric cleavage of the eight-cell stage embryo is viewed from its vegetal pole in polarized light. (*Left*) Early anaphase. The positively birefringent spindles, and the asters at the center of each of the four cells in focus, stand out in bright or dark contrast. The spindles are tilted toward the observer at the vegetal pole (the middle of the picture). There, the astral birefringence is weak and the spindle fibers do not converge at the poles. (*Right*) Telophase. The four cells have cleaved perpendicular to the spindle axes and have given rise to four micromeres and four macromeres. Portions of the (birefringent) fertilization membrane show as bright crescents at the top and bottom of the pictures. From Inoué and Kiehart (109). Bar, 50 μm.

[2] page 160]). Time-lapse recording and direct observations with polarized light microscopy (Fig. 6) clearly showed the late anaphase waning of the central spindle fibrils (microtubules) and the dynamic waxing of the phragmoplast fibers (microtubular bundles), as well as the alignment of small "granules" (already observed by Becker [257] to be vesicular) at the midzone of the phragmoplast to form the cell plate (19, 133, 258; also see [259]). As summarized by Bajer and Molè-Bajer (134) and Hepler and Palevitz (260), these vesicles, which were postulated to be Golgi products (261, accumulate and fuse laterally in the midzone of the phragmoplast microtubule bundles to transform into the cell plate that divides the cell body into two. Cytokinesis thus takes place in the middle of the telophase spindle and insures the partition of the daughter nuclei into two cell bodies by a mechanism alternate to cleavage.[23]

In both astral and anastral mitoses, cytokinesis is coordinated with mitosis by an organized arrangement of mitotic microtubules and is not controlled directly by the chromatin or nuclei. In this respect also, the ephemeral achromatic fibers of the spindle and asters express the multifunctional, dynamic organization of the hyaline cytoplasm common to all cells.

Concluding Remarks

In this brief historical sketch, I have highlighted some of the research on the dynamic aspects and functions of the mitotic spindle that took place principally over the last quarter-century. This has been an exciting period in which the happy convergence of the morphological, physiological, and biochemical approaches, and the development and application of new methodologies, have led to major progress.

Although we are still searching for the exact molecules that move chromosomes in anaphase, we have learned much about the dynamic physiological behavior of microtubules, the ephemeral fibrils of the mitotic apparatus, and the hyaline kinoplasm of all cells.

The spin-off from these studies has improved our understanding of cell behavior in many unexpected directions: nerve and muscle growth, organogenesis, gametogenesis, secretory functions, phagocytosis, drug action, etc., (e.g., 79, 80). Thus, the major investments made by the investigators and the sponsors are bearing fruit, both for a better grasp of cell division and its regulation, and in the basic physiology of cytoplasmic organization applicable to an unexpectedly wide range of biomedical fields.

In so short a sketch, much interesting and important work could not be included, and my presentation is by no means balanced. Some important aspects of mitosis have not even been mentioned in this selective narrative. Fortunately, there are several excellent monographs and reviews that can remedy this situation. Several have been cited in the text, and the articles in the following references should provide a good introduction: for mitotic mechanisms and diversity (23, 134); for cell motility including mitosis and cytokinesis (81, 144); for mitotic microtubule assembly and its control (79, 86, 178); for the cell cycle and its regulation (265, 266); for mutants affecting mitosis (267, 268); and for an overview of mitosis and cell division (105, 269). Additionally, some earlier references, especially Wilson (2), Bělař (4, 34, 127), Wassermann (38), Gray (270), Hughes (139), and Schrader (6) contain much information and many ideas which sould be of contemporary and lasting value.

[23] Cytokinesis by cleavage and by phragmoplast formation are probably expressions of two extremes. It is quite possible that both contribute to cytokinesis in many cell types. Also note that the spindle is not always a single bipolar body, but may be made up of two or more smaller spindles arranged in parallel (e.g., 262, 263); also see (e.g. 264) for multipolar origin of a bipolar spindle.

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Motility

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Definition and Scope

Motility is the ability of living systems to exhibit motion and to perform mechanical work at the expense of metabolic energy. Motility and mobility are often confused. The distinction is clear in the simplest motions observed in living cells with the light microscope: Brownian motion of particles demonstrates their *mobility* under the influence of thermal agitation. On the other hand, their saltatory motion, a form of *motility*, may transport the same particles much greater distances using metabolic energy.

The scope of motility, as it is presently understood, includes a variety of diverse phenomena: (*a*) bacterial (prokaryotic) flagellar movement; (*b*) gliding in unicells (bacteria, blue-green algae, diatoms, and desmids, etc.); (*c*) saltatory motion of particles in cytoplasm; (*d*) organelle movements (deformation or translocations of chloroplasts, mitochondria, the costa and axostyle, acrosomal filament extension, etc.); (*e*) cytoplasmic streaming (in protists, plant, animal, and fungal cells); (*f*) ameboid movement (cell movement by means of cytoplasmic streaming in lobopodia, filopodia, axopodia, retraelopodia, etc.); (*g*) movements of tissue cells (degree of relatedness to ameboid movement uncertain); (*h*) platelet motility (shape change, transformation, and clot retraction); (*i*) contractility (of muscles, spasmonemes etc.); (*j*) axoplasmic transport in nerve; (*k*) mitotic movements; (*l*) cytokinesis (plant and animal types differ); and (*m*) eukaryotic flagellar and ciliary movement.

The coverage of the whole field of motility obviously is impossible in the available space. It is possible, however, to give the reader a general impression of the activity and ferment in the field, as well as some key references to the literature.

The Literature of Motility

Researchers have known of the principal phenomena of motility for a long time. Descriptive accounts can be found as early as van Leeuwenhoek's letters to the Royal Society. It is clear that fascination with movement as an attribute and manifestation of life motivated the minds of early biologists. However, science must develop both a conceptual framework and an armamentarium of methods before it can study complex phenomena and, in the case of cell motility, these prerequisites became available about two decades ago.

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The conceptual framework arose in branches of science that seemed at the time to have little relationship to biology: fluid dynamics, rheology, colloid and polymer chemistry, and thermodynamics. Biophysics and biochemistry were the interdisciplinary sciences through which these concepts found their way into the minds of cell biologists. Before 1960, the literature of motility was scattered and of uneven quality. Much of it was descriptive, poorly documented, unquantitative, and highly speculative. Conferences and symposia had a strong and catalytic influence on this field. In 1961, under the leadership of P. J. Gaillard and J. F. Danielli, a conference was held on "Cell Movement and Cell Contact" at Noordwijk, the Netherlands, at which a fruitful discussion on mechanisms of cell movement took place. Several of the papers given at that meeting are still widely quoted (e.g., references 1–4).

In 1963, a "Symposium on the Mechanisms of Cytoplasmic Particles" was held in Princeton, New Jersey, with the deliberate intention of promoting interaction between scientists studying different kinds of nonmuscle motility and muscle contraction. The volume, *Primitive Motile Systems in Cell Biology*, which was a result of that conference (5), had a profound influence on the development of the field. It stimulated motility researchers to explore the molecular basis of motility, using muscle as a model. It also stimulated some muscle researchers to look at nonmuscle systems. The conference also introduced microtubules to scientific audiences (6). Finally, the value of film as a means of documenting and communicating the phenomenology of motility was demonstrated.

There have been many meetings on motility since 1960. Those that resulted in publications are cited in Table I. The largest meeting was that held at Cold Spring Harbor (7), at which 92 papers were given, and 250 people attended. The field has grown so rapidly that it is almost impossible to have a true meeting of minds unless the subject matter (or the attendance) is restricted.

The most recent meetings of which the proceedings have been published are the First John M. Marshall Symposium held at the University of Pennsylvania in 1977 (9) and the Yamada Conference I on "Cell Motility Controlled by Actin Myosin and Related Proteins," held at Nagoya, Japan, in 1978 (10).

The journal literature on motility has grown at a rate substantially larger than the literature of cell biology as a whole. Papers on the subject can be found in more than 50 periodicals, including two new journals: *Cell Motility* (United States) and *Journal of Muscle Research and Cell Motility* (United Kingdom).

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TABLE I

Motility Symposia with Published Proceedings Since 1960

Year	Title	Organizer(s)/editor	Reference
1961	Cell Movement and Cell Contact	P. J. Gaillard and J. F. Danielli	*Exp. Cell Res.* Volume 8: Suppl.
1963	Primitive Motile Systems in Cell Biology	R. D. Allen, E. Bovee, D. Marsland, and L. I. Rebhun	(references)
1963, 1964	Conferences on Cell Dynamics	M. Rosenburg	(reference 8)
1967	The Contractile Process	A. Stracher	*J. Gen. Physiol.* 50: Suppl. 6
1968	Aspects of Cell Motility	P. L. Miller	*Symp. Soc. Exp. Biol.* Volume 22
1973	Locomotion of Tissue Cells	M. Abercrombie	*CIBA Symp.*
1974	Molecules and Cell Movement	S. Inoué and R. E. Stephens	Society of Gen. Physiologists Volume 30, Raven Press, New York, 1975
1975	Contractile Systems in Non-Muscle Tissues	S. V. Perry, A. Margreth, and R. S. Adelstein	Elsevier North-Holland, Inc., New York, 1976
1976	Cell Motility	R. D. Goldman, T. D. Pollard, and J. Rosenbaum	*Cold Spring Harbor Conf. Cell Proliferation* (1976).
1977	Conference on Cell Shape	J. P. Revel	Proceedings on the Conference on Cell Shape. Alan R. Liss, Inc., New York, 1977.
1977	Motility in Cell Function	F. A. Pepe, J. W. Singer, and V. T. Nachmias	Academic Press, Inc., New York, 1979
1978	Cell Motility: Molecules and Organization	S. Hatano, H. Ishikawa, and H. Sato	University of Tokyo Press, Tokyo, 1979
1979	Contractile Proteins in Plants	D. S. Fensom	*Can. J. Bot.* 58(7), 1980.

Steps to Understanding Motility

PHENOMENOLOGY: There is a strong tendency to oversimplify the descriptions of motile phenomena. In some cases hypotheses have served as "filters" preventing observers from recording details that did not fit with theory. To avoid this problem, investigators learned to utilize objective recording methods, such as film or videotapes, and to make them freely available to others. A pioneer in this effort was Lewis (11), who made splendid films of the movements of amebae, tissue cells in culture, and embryonic cells *in situ*. Scientific films have progressed a long way from *ad hoc* productions intended for a scientific meeting to documentary films that include dimensional, temporal, and experimental data. In Göttingen, the German Federal Republic supports a nonprofit "Institut für den Wissenschaftlichen Film," (IWF) the purposes of which are to make and to disseminate films of this type in collaboration with scientists from all over the world. The distributor of IWF films is the Audiovisual Services at Pennsylvania State University, University Park, Pa.

The latest wave in phenomenological documentation is television. Parpart (12, 13) was a pioneer, a generation ahead of most other biologists. As early as the 1960s, he used a videcon camera and monitor to permit more than one observer to study saltatory movements in *Arbacia* eggs (13). Most recently video cameras (some compatible with computers), recorders, projectors, and accessory instrumentation have been widely adopted; they are so convenient and inexpensive to use that they may well replace cinerecording for all applications except high-speed filming and presentation to large audiences.

BIOPHYSICS: Motility research depends heavily upon methods of observation and recording, especially with modifications of the light microscope. Improvements in optical microscopy, such as phase-contrast (14), interference, and differential interference contrast (15), dark-field (16), and fluorescence (17), microscopes, have all seen service in motility studies. Rectified polarizing microscopes (18) have had many uses in the detection of birefringence owing to microtubules in mitotic spindles (19), and phase-modulation methods (20), have been used to detect strain birefringence in ameba cytoplasm (21).

Some aspects of motility have required special devices for recording images at low light levels. Depending on the degree of sensitivity required, equipment has included silicon-intensified tube (SIT) video cameras or image-intensifier videcons (22). In general, the detection of calcium ions in cytoplasm requires devices of the highest sensitivity.

ULTRASTRUCTURE: Since 1960, the most important and rewarding, yet perhaps the least reliable, approach to the study of motile systems has been electron microscopy. The fault lies not with the investigators or instruments, but with the unsatisfactory state of the art of specimen preparation. The history of electron microscopy applied to motile systems can be divided into chapters according to the preparation method used, such as osmic acid fixation, potassium permanganate fixation, glutaraldehyde fixation, thin section-CTEM, critical point-dried whole mount-HVEM, and freeze-fracture, deep-etch. Presumably the list of useful preparation techniques and, hence, the list of new structures to be found, is not complete.

Perhaps the main contribution of electron microscopy to the study of motility has been the categorization of ultrastructural entities found in different systems. The concepts of "microtubule-dependent motility" and "microfilament-dependent motility" were derived ultrastructurally and, in some cases, led to a more precise molecular characterization of certain motile systems.

Often the results of ultrastructural analysis correlate well with and extend those of optical microscopy. In the mitotic spindles, the positions and orientations of microtubules corresponded well with the predictions of the Wiener theory based on form birefringence data (23).

In other cases, fixation alters or destroys ultrastructural details. For example, the most birefringent region of a moving ameba is its endoplasm (24), and the birefringence of this region can be modulated in vivo by an applied force (21). When the cell is broken, "flare medium"-packed fibrils consisting of actin filaments emerge and engage in extracellular

motility (25). Despite this evidence of functionally important endoplasmic ultrastructure, fixation by any presently known procedure causes the birefringence to disappear, and virtually no F-actin filaments remain when the fixed cell is sectioned and observed in the electron microscope.

Newer specimen preservation techniques show considerable promise of circumventing *some* of the fixation damage by rapid freezing, freeze-fracture, and deep-etch of fixed (26), or living material without cryoprotection (27, 28). However, these techniques may also produce artifacts of a different kind, and the results should be interpreted with caution.

MOLECULAR APPROACHES: The point of departure for the molecular basis of motility was muscle biochemistry, where the major proteins responsible for contractility were isolated and partially characterized before 1960. The pioneering effort to extend muscle biochemistry to a nonmuscle motile system was that of Loewy (29), who demonstrated an actomyosin-like, adenosine triphosphate (ATP)-induced solution of *Physarum* extracts.

The best review of the contribution of muscle biochemistry to motility research is that of Pollard and Weihing (30). A particularly important contribution was that of Huxley, whose sliding-filament theory (31) and method of decorating F-actin filaments with heavy meromyosin to determine their polarity (32) were easily adapted to other motile systems (33).

Independent of muscle biochemistry was the early work on tubulin. The discovery of microtubules (34) and the colchicine-binding assay for tubulin (35) laid the ground work for the development of research on microtubule-based motility (see Haimo and Rosenbaum, this volume).

The study of both actin- and tubulin-related motility have profited by the availability of antibodies to purified tubulin, actin, myosin, tropomyosin, and other proteins (36–38). The fluorescent-antibody technique of labeling contractile and cytoskeletal proteins has ushered in a new era of what might be called "biochemical morphology," especially of cells grown in culture.

Antibodies (fluorescent or not) can also be injected into living cells to inactivate certain proteins and, in this way, demonstrate their function. The pioneering effort in this regard was the injection of myosin antibody into sea urchin eggs, where it inhibited cleavages without preventing mitosis (39). Taylor and Wang (40) have injected fluorescently labeled G-actin into cells, where some of it has polymerized into F-actin. A different labeling procedure makes it possible to label tubulin and microtubule-associated proteins (41).

Mainstreams, Eddies, and Backwaters

Looking back over the past two decades, one notices that the principal gains in knowledge have been in those areas of motility research in which the problems were evident and the techniques for their investigation were at hand or could be developed. Examples include rotational and shuttle streaming, movements of ameboid and tissue cells, mitotic movements, muscle contraction, and ciliary and flagellar movement. The latter three are discussed elsewhere in this volume.

Some interesting forms of motility remain to be investigated with the same degree of thoroughness and, for that reason, may be considered as "backwaters" in the motility field. Examples are gliding in unicells, intracellular organelle movements, and modes of cytoplasmic streaming other than shuttle and rotational streaming. These subjects await new discoveries, concepts, and methods before they can join the mainstream of motility research.

Intermediate between these extremes are some "eddies" in which intense excitement has been generated as the result of new insights or findings. The discovery that bacterial flagella rotate rather than undulate (42, 43) is an example; this finding led to a flurry of activity aimed at understanding the molecular biology and genetics of the rotatory motor of bacteria (44). Other examples of such interesting eddies are saltatory movement, reticulopodial and axopodial movements in foraminifers and heliozoans respectively, and movement along the slime ways of *Labyrinthula*, a marine slime mold (45; N. Nakatsuji, S. Sher, D. Solomon, T. Nakatsuji, and E. Bell. Manuscript submitted for publication.).

Progress in the Mainstreams

SHUTTLE STREAMING IN *Physarum*: In motility research, nothing could be more in the mainstream than shuttle streaming in the acellular slime mold *Physarum*, where the most rapid flow of cytoplasm has been documented: 1,300 $\mu m \cdot s.^{-1}$

The classical work of Seifriz (see references 46 and 47) on *Physarum* streaming laid the groundwork for the important studies of his student, Kamiya, who early in his career invented the important double-chamber method (48) of measuring the motive force for streaming. By using this and other quantitative methods of equal elegance and ingenuity, Kamiya and his students systematically investigated the effects on streaming of environmental factors, physiologically active substances, such as ATP, and drugs. Kamiya's reviews (48–51) should be consulted for details.

The most basic biophysical question to be asked about shuttle streaming in the early 1960s was the site of the motive force. Seifriz (46) had suggested that regions in the ectoplasmic gel (channel walls) contracted rhythmically, like beating hearts, and forced the streaming cytoplasm back and forth. Kamiya and Kuroda (52) showed that the velocity profiles for normal streaming and pressure-induced flow were identical. Therefore, it was assumed that a hydrostatic pressure gradient was the motive force. This assumption derived support from direct observations in polarized light; diffusely birefringent regions of fibrils periodically changed their length and birefringence (53). Wohlfarth-Bottermann (54) showed that cytoplasmic fibrils formed in response to the gravitationally-induced resistance to flow. In an effort to determine the site of the motive force in a double-chamber preparation, Allen et al. (55) constructed a differential thermometer into the agar floor of a Kamiya double chamber. With a sensitivity sufficient to display thermal noise of 2×10^{-5}°C, it was a simple matter to measure periodic temperature increases of ca. 10^{-3}° at each end inasmuch as it served as the source of the cytoplasmic stream. Therefore, it was concluded that the excess heat must be produced by the "tail" of the slime mold as a by-product of contraction.

In 1952, Ariel Lowey (29) demonstrated that *Physarum* extracts are sensitive to solation if ATP is added indicating the probable presence of an actin-myosin contractile system in slime molds. However, there were difficulties in isolating and purifying these proteins because of contaminants that altered their physical properties. In addition to Lowey, other pioneers in this effort were Nakajima (56) and Hatano and Oosawa (57) in Japan and Adelman and Taylor (58, 59) in the United States.

Ultrastructural studies carried out by many capable electron microscopists during nearly two decades failed to define the organization of the contractile material in *Physarum* until Nagai et al. (60) showed that during the transition from resting to contraction and relaxation, the parallel F-actin filaments that apparently are cross-linked by myosin dimers transform into a "felt-work" without straight F-actin filaments. A likely key to the explanation for this kind of ultrastructural transformation, which is very different from that in muscle, is found in the work of Matsumura and Hatano (1978), who showed that synthetic *Physarum* actomyosin undergoes reversible superprecipitation when ATP is added.

There is evidence of calcium control of the contractility and rheological changes in *Physarum* cytoplasm (62). Hatano and Oosawa (63) found that caffeine treatment causes slime molds to break down into "droplets" (cytoplasts) in which streaming proceeds in a narrow range of calcium concentration. Recently, Kuroda (64) reduced the droplets to models one step simpler in organization by removing their membranes in a modified "flare medium."

Physarum has turned out to be the only living nonmuscle material in which it has been possible to control and measure cytoplasmic contractility under physiological conditions. Kamiya et al. (65) described an apparatus in which isotonic and isometric contractions could be recorded alternatively. These studies have been continued in the laboratories of Kamiya (66) and Wohlfarth-Botterman (67) and have defined the way in which a "simple" nonmuscle contractile system responds to stretch and tension.

AMEBOID MOVEMENT: Until 1960 there had been four decades of virtual unanimity about the mechanism of ameboid movement (see reference 68 for an excellent review of the long history of this subject). Mast's (69) description of the phenomena of ameboid movement was in terms of the tail-contraction, sol-gel theory, to which he subscribed. Little research was done because methods were not available to study the molecular basis of tail contraction or sol $\rightleftharpoons$ gel transformations.

A serendipitous experiment, in which washed amebae were broken in glass or quartz capillaries, showed that cytoplasm could stream bidirectionally when released from the cell, in some cases for an hour or more (70). The responses to this report were mixed. Although the experiment was easy to repeat, many people did not believe the results. Others found reason to doubt the obvious interpretation that pressure cannot cause bidirectional flow, and sought ways to rationalize the result in terms of Mast's appealingly simple and long accepted theory.

In the early 1960s, as a result of the observed streaming in isolated cytoplasm, there was a period in which the number of hypotheses to explain ameboid movement exceeded the amount of solid information upon which any viable theory could be built. However, the development of these ideas was essential, and it was later possible to test some of them.

One, the frontal-contraction hypothesis, was that the motive force for pseudopod extension was a contraction localized at pseudopodial tips (71). This idea was based on the geometric details of streaming in isolated cytoplasm and was compatible with what was known about the details of streaming and pseudopod extension and retraction in intact cells.

An advantage of the frontal contraction hypothesis was that its predictions could be tested by biophysical methods. It was reasoned that endoplasm could be drawn forward by a tensile force from frontal contraction only if it exhibited viscoelastic behavior. Polarization microscopy showed not only that the endoplasm was birefringent (72), but also that endoplasmic birefringence could be modulated by tension applied to the tips by suction (21). The dynamics of change in birefringence established that the birefringence was a result of strain and not flow, showing that the endoplasm is, in fact, viscoelastic.

For more than a decade, the frontal-contraction hypothesis was the subject of considerable controversy (73–75; see also the discussion throughout *Primitive Motile Systems in Cell Biology* [5]).

In the meantime, other hypotheses were under consideration. For example, it was proposed that "active shearing" of concentric layers of cytoplasm could be responsible for streaming (76), and Bingley and Thompson (77) showed some evidence supporting the possibility of an electrophoretic mechanism. The most ingenious idea was the "domestic closet-bowl theory" (78). These hypotheses were not tested sufficiently to receive serious consideration in the literature.

The tail-contraction theory remained a subject of discussion until after 1970, when it was put to a direct test by a capillary suction experiment, in which it was shown that even high negative pressure gradients applied to the tip of one pseudopodium could not prevent others from extending (79). We can say that this result and others directly supporting the frontal-contraction hypothesis discredited the tail-contraction theory. Even the possibility that the tail might contribute to the motive force seems remote in the light of recent results which show that destruction of the tail by a microsecond laser beam does not instantaneously alter the rate of streaming (80). The shift in views regarding the mechanism of ameboid movement can be seen in the reviews of Jahn and Bovee (81), Seravin (82), Allen (71, 72), Allen and Allen (83), and Taylor and Condeelis (84).

One of the most important lines of investigation on ameboid movement was initiated by Thompson and Wolpert (85), who demonstrated streaming in extracts from pooled ameba cytoplasm. Pollard and Ito (86) continued this work and showed that streaming in extracts required both thick and thin filaments. Since that time, workers have learned a great deal about the molecular basis of motility. Most of the story is in an article by T. D. Pollard in this volume.

Perhaps the most dramatic and revealing experiment on ameboid movement was the demonstration that contractility, rheological behavior, and the streaming of isolated ameba cytoplasm could be brought under direct chemical control. Taylor et al. (25) showed that by controlling the concentrations of calcium ions, ATP, and magnesium ions, cytoplasm could be switched back and forth from states comparable to rigor, relaxation, and contraction in muscle. Furthermore, in a solution containing the correct balance of Ca^{++}, Mg^{++}, and ATP, streaming could occur in fountain or loop patterns similar to those in both the intact cell and broken cells in capillaries (70; see also reference 87).

Although contractility provides the motive force for ameboid movement, it has long been clear that the rheological (sol $\rightleftharpoons$ gel) cycle in cytoplasm is also central to the process. For a time, it appeared that changes in the degree of cross-linking between actin and myosin might account for the rheological cycle. A second possibility was that either or both types of filaments might disassemble, especially if the intracellular free calcium concentration were much below the micromolar level (88). A third possibility, suggested by extract experiments on *Dictyostelium* and proposed as the "solation-contraction coupling hypothesis" is that contraction can occur only when gel for-

mation, which is under the control of actin-binding proteins ("gelation factors"), is prevented by micromolar concentrations of free calcium ions or a pH above 6.8–7.0 (89). This hypothesis differs only in detail from that of Goldacre and Lorch (90), who also proposed solation-contraction coupling. The main problem with this hypothesis is that it fails to consider how the coupling could occur if the motive force is delivered at pseudopodial tips and the solation occurs in the tail.

Much of the recent work on the molecular basis of ameboid movement has used extracts of the small amebae *Dictyostelium* or *Acanthamoeba*. Unfortunately studies on extracts do not tell us what is going on in living cells, but instead indicate what could be happening and, therefore, what should be looked for. Whereas actin is presumably similar among the giant amebae and the smaller ones that are used for biochemical studies, the myosins are different in their molecular weights, solubilities, and enzymatic properties, and the actin-binding proteins hardly correspond at all (see review by Hitchcock [91; 84, 92]). Consequently, extraction experiments on one species can provide only clues as to what might be the case in another species.

With regard to the mechanisms of movement in the *Chaos-A proteus* group, it seems wiser to rely on biophysical evidence itself rather than on theoretical constructs that conflict with observation. Taylor (93) has made some very important observations on the effect of excess calcium ions injected into the cell. One of these effects appears to be a temporary loss of gel/sol differentiation. Cooling, which presumably allows calcium to enter the cell, has a similar effect (R. D. Allen. Unpublished observations.). In this case, the gelation that follows frontal contraction does not occur, with the result that the frontal contraction continues as the cytoplasm turns over the rim of the ectoplasmic tube. Thus, the tube continues to shorten anteriorly, and the contraction can continue when gelation fails. This situation seems to agree with the concept that contraction and gelation are separate, rather than coupled, processes.

Tissue Cell Movement

Tissue cell movement in animals bears some resemblance to ameboid movement. When tissue cells settle on a suitable substrate, they attach and spread (for a review, see references 94 and 95). In doing so, they form elongated processes of various shapes comparable with pseudopodia that are found on various ameboid cells—filopodia (thin, filamentous pseudopodia) and flat lamellipodia, some of which lift off the substratum where they are described as "ruffled membranes" (96, 97).

In fibroblasts, there is usually a ruffled membrane that extends in the direction of locomotion, and a tail that drags behind, attached to the substratum until it tears loose and recoils elastically toward the cell body. Harris and Dunn (98) found centripetal transport of particles on both surfaces of moving fibroblasts, including the ruffled membrane.

Movement in tissue cells is not restricted to surface movements and cell locomotion, for there is saltatory as well as Brownian motion of cytoplasmic particles. Often saltation is polarized in the long axis of the cell, parallel to the direction of locomotion.

Studies on tissue cell movement have, for the most part, lacked the kind of biophysical data that was generated in studies on amebae from 1960 to 1975. Consequently, the site of the motive force for movement in tissue cells remains obscure. Abercrombie (1) considered that the ruffled membrane on the advancing edge of fibroblasts was the "locomotor organelle" of the cell. If this turns out to be universally correct, then there may be a parallel to be drawn with present views of the mechanism of ameboid cell movement.

An important difference between ameboid cells and tissue cells of animals is in their rates of movement. With the exception of white blood cells, tissue cells in general move so slowly that time-lapse photography is necessary to record and study their movement. Tissue cells lay down points of attachment to the substrate; during locomotion some of these are broken and others are established. The surface-reflection interference microscope and its improvements have made it possible to study adhesion to the substratum (99–102). Revel et al. (103) have used scanning electron microscopy to study adhesion to the substratum.

The cytoplasm of fibroblasts is known to have viscoelastic properties from the behavior of iron particles moved inside the cell by a magnet. Crick and Hughes (104) characterized the cytoplasm as rather like "mother's work basket—a jumble of beads and buttons of all shapes and sizes, with pins and threads for good measure, all jostling about and held together by 'colloidal forces'."

An ultrastructural basis for viscoelastic behavior in cytoplasm became evident even in the earliest electron microscope studies of thin sections of moving tissue cells, for example secondary mesenchyme cells of sea urchins (105). By the mid-1960s it was evident that cytoplasm contained more than a single "linear element." The microtubule was the first to be identified on morphological grounds (34), and the identification of tubulin as the protein of which microtubules were made soon followed (35). By the mid-1960s, it was suspected that some of the smaller filaments were about the correct size to the F-actin, but not until the heavy meromyosin-labeling technique was available (33) could microfilaments be positively identified as F-actin and their polarity determined. The introduction of cytochalasin as a drug to disrupt microfilaments and to inhibit microfilament-based motility provided a second important tool with which to study the role of F-actin in nonmuscle motility (106).

A third type of filament intermediate in size between microtubules and microfilaments was the ~10-nm "intermediate" filament found in many tissue cells and neurons (107). So far it appears that these are not directly involved in cell motility. Excellent examples of cell ultrastructure showing all three types of filaments are found in the papers of Goldman and Knipe (108).

The central role of F-actin microfilaments in the ultrastructure attachment and motility of tissue cells was best shown in the work of Goldman et al. (109), who combined a number of techniques in demonstrating the different roles of actin.

The localizations of several cytoskeletal and contractile proteins have been determined on a number of cell types after fixation using fluorescent antibody techniques for actin (36), myosin (37), and α-actinin (111). The elegant images of symmetrical geometric localizations of these various proteins so far have not shed much light on the mechanisms of motility, other than to reassure us that the pieces of the puzzle are being put together. The "big picture" has not emerged.

The details of how the various cytoskeletal and contractile proteins are assembled in the cytoplasm has been investigated by high-voltage electron microscopy and stereo electron micrographs, which permit the observer to see the relationships of

cytoskeletal elements stereoscopically in thick sections or whole-mount, critical-point dried cells. With these techniques, preparations up to 2 μm thick can be seen with enough clarity to identify nearly all ultrastructural details visible in thin section. Thus Wolosewick and Porter (112) have depicted and described the microtrabecular lattice that envelops the microfilaments, microtubules and intermediate filaments, polysomes, etc. It is now clear that this lattice alters during motility, drug treatments, etc., but it is not yet certain what the microtrabecular lattice is biochemically, or what role it plays in cell movement.

Rotational Cytoplasmic Streaming in Characean Cells

The very rapid rotational streaming in characean cells was first observed by Corti in 1794. The large size of these cells has made them the ideal material in which to study streaming in plants (for reviews, see references 49, 50, 92).

In the frequently studied *Nitella* internodal cells, the cytoplasm streams in a spiral path beneath the spiral rows of chloroplasts embedded in the cortex. Two oppositely directed streams each occupy a little less than 180° of the cell circumference and are separated by "indifferent zones."

In 1956, Kamiya and Kuroda (113) made the important observation that the endoplasm exhibits shear only in its outer micron or two adjacent to the cortex. It was therefore suggested that the site of application of the motive force might be found at the corticoendoplasmic interface. It was also suggested that the motive force must be "active shearing" at this location.

In the same year, Jarosch (114) made some remarkable observations and ciné records of the behavior of filaments in cytoplasts obtained by stripping the contents from cut cells. He discovered that the cytoplasts contained chloroplasts and nuclei that could rotate on their own axes or "swim" in the cytoplasm. Many filaments very near or below the resolving power of the microscope could be seen to undulate, make serpentine movements, or form circles or polygons, which either rotated or served as substrates for the unidirectional motion of particles. These fascinating observations were discussed in terms of a theoretical model involving screw mechanics at a time when helical biopolymers and "treadmilling" in F-actin and microtubules had not yet been remotely considered (115). These unorthodox ideas provoked some amusement at the time but, in the light of newer findings, some of Jarosch's views should be reconsidered and tested by nanosecond fluorimetry techniques. However, as will be seen, some alternative theoretical schemes are easier to test.

The observations of Jarosch (114, 115) suggested that some kind of filaments might be found at the corticoendoplasmic interface, and this prediction was indeed confirmed. Kamitsubo (116) discovered the subcortical fibrils with the light microscope, and Nagai and Rebhun (117) observed ultrastructurally that each subcortical fibril was a bundle of from 50 to 100 microfilaments with diameters of 6–7 nm—about the size of F-actin—attached to the inner surface of the chloroplast rows.

Kamitsubo (118) continued his study of subcortical fibrils by showing that in centrifuged cells these fibrils could fold over to form polygons similar to those seen by Jarosch (114, 119).

Kamitsubo (120) also devised the "*Nitella* window technique" for banishing chloroplasts from an area ca. 100 μm in diameter, through which the subcortical fibrils and endoplasm could be seen clearly. He showed that cytoplasmic particles in the vicinity of the subcortical fibrils could suddenly "hitch on" and be transported at streaming velocity, whereas nearby particles engaged only in Brownian motion.

Similar *Nitella* window preparations were used by N. S. Allen (121) under improved viewing conditions to observe and record the undulations of a population of endoplasmic filaments considerably more numerous than the subcortical fibrils. The endoplasmic filaments could be counted, and their aggregate length was computed to be about 50 m for a cell 2 cm in length. Endoplasmic filaments are branches of subcortical fibrils; therefore it is not surprising that they can also cause particles to be transported along them.

The chemical nature of subcortical fibrils was revealed as F-actin by the experiments of Palevitz and Hepler (122), who successfully labeled them with heavy meromyosin and found that the polarities of microfilaments in a bundle were identical. The polarity with respect to the direction of streaming was later determined by Kersey et al. (123) to be counter to the direction of streaming. This finding was consistent with the hypothesis that a myosinlike molecule attached to particles might move along F-actin bundles by a sliding interaction similar to that in muscle.

Although the chemical nature of the subcortical fibrils is established and myosin has been isolated from *Nitella* (124), there is less information about endoplasmic filaments because of their destruction by fixatives. Even their existence has been called into question, because until recently the sole evidence for their existence has been films made with a sensitive differential-interference contrast microscope (121).

Recently, Allen and Ruben (27) and Allen (28) have demonstrated the existence of extensive loose bundles of 6–7 nm of microfilaments throughout the endoplasm by a rapid freeze-fracture, deep-etch technique carried out on unfixed cells without cryoprotection. In addition to the microfilaments, some of which exhibit the 37 nm helical repeat expected of F-actin, there are reticular "webs" of thinner filaments surrounding cytoplasmic particles in the vicinity of F-actin bundles. In some cases, it is possible to see thinner (ca 4 nm) filaments, which interact tail-to-tail and have a bifurcation leading toward two globular heads, suggestive of a putative oligomeric myosin network.

By opening characean cells at the ends and perfusing with appropriate physiological solutions, Williamson (125) was able to create a surviving membrane-free model system sensitive to cytochalasin and responsive to ATP. He could observe the adhesion of particles of the subcortical fibrils and that they did not move until exogenous ATP was added. Nagai and Hayama (126) have observed the ultrastructure of particles adhering to the subcortical fibrils in such preparations and have detected periodic structures believed to be myosin.

The subcortical fibrils have received greatest attention from workers interested in rotational streaming. However, it is now clear that these structures are but a small portion of the motile machinery, which extends throughout the endoplasm. Active shearing, whatever its mechanism, clearly takes place at the surfaces of endoplasmic filaments as well as subcortical fibrils.

Some Eddies

Space permits only the briefest mention of some of the areas of research, related to those discussed earlier, that offer opportunities for the next decade of discovery.

SALTATORY MOTION: It is commonly believed that sal-

tatory motion may be the most basic form of motility. Although there have been a number of classical descriptive papers, little progress has been made in understanding the molecular basis of the process (for review, see Rebhun [127]).

AXOPODIAL MOTILITY IN HELIOZOANS: Heliozoans are protozoans of the class Sarcodina that move by bending their stiff axopodia at their bases and feed by means of more typical ameboid food-cup pseudopodia (128). Tilney and Porter (129) described the ultrastructure of axopodia, which have a central rod consisting of a paracrystalline array of microtubules. For a while—as a result of this and other studies at the time—microtubules were regarded as part of the motive-force production mechanism in cells. Edds (130) performed a simple experiment to test this idea. He inserted a glass needle, with a diameter about that of the microtubular axoneme, through the cell and out the other side in such a manner as to cause an "artificial axopodium" to be produced. Particles saltated within this artificial axoneme at an almost normal rate, and were unaffected by concentrations of the microtubule-inhibitor colchicine, which was sufficient to cause other axopodia to collapse. This result clearly showed that the axonemal and other microtubules played no role in particle motions. Edds (131) demonstrated the presence of two kinds of filaments: thin filaments, which could be labeled with heavy meromyosin and are therefore F-actin, and unidentified thick filaments with an unusual morphology. Much more remains to be learned about the motility of the heliozoans, for they can be raised in mass culture for biochemical work.

RETICULOPODIAL NETWORKS OF FORAMINIFERS: The foraminifera are sarcodines that spread extensive reticulopodial networks for the purpose of feeding and locomotion. The filopodial strands within the networks exhibit bidirectional streaming marked by the transport of cytoplasm and particles at two or more velocities. The literature on the phenomenology is cited in Jahn and Rinaldi (132) and Allen (133).

The presence of microtubules in the reticulopodial network has been shown by several investigators (e.g., reference 134), and recently it has been found that there are close physical proximity, and therefore, likely interactions between microtubules and unidentified microfilaments, which do not appear to be actin (135).

At the light microscope level, it has been possible to observe the interaction of cytoplasmic particles with from one to a few microtubules and the "sliding" and "zipping" activities of microtubules in *Allogromia* as a result of the new AVEC methods of videomicroscopy (136, 137). The same method has revealed new details of microtubule-associated movements in neurons (axonal transport) and other vertebrate tissue cells (138). The same method has recently made it possible to record the transport of massive numbers of synaptic vesicles in intact axons (139) and in isolated axoplasm, where transport persists and can be studied for hours (140).

Foraminiferan reticulopodia may offer a unique opportunity to study a form of motility dependent upon an interaction between two types of linear elements.

MOTILITY OF AND ON *Labyrinthula* SLIMEWAYS: The marine slime mold, *Labyrinthula*, a parasite on eel grass, consists of spindle cells that move in a unique manner within a membrane-bounded "slime way" secreted by these cells. The slime ways themselves form lamellipodial extensions. Bell and co-workers have recently shown that the slime ways contain both actin and myosin and that the movement of spindle cells is regulated by calcium ions (see Nakatsuji et al. [Manuscript submitted for publication.] and Nakatsuji and Bell [45] for a review of most recent findings).

Conclusion

I have selected some of the systems in the mainstream of motility research for a brief progress report and have referred to reviews and symposium volumes where more references are available. I have also pointed out some of the conceptual and technical advances that have made motility research the vibrant field that it is today. It is possible to predict that, in the next decade or two, the eddies mentioned here will have grown into mainstreams and that few backwaters will remain.

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Cytoplasmic Contractile Proteins

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In reviewing work on cytoplasmic contractile proteins and the contributions made to this field by *The Journal of Cell Biology*, some perspective is gained by first pointing out that most cell biologists were not aware of the existence of these important cellular constituents more than 12 years ago. Times have changed, and today cytoplasmic contractile protein research is one of the busiest areas in cell biology. My purpose here is to highlight some of the important events in the growth of this field and to forecast some future trends. More exhaustive coverage of the field is found in recent books (1, 2) and review articles (3–7). Closely related historical reviews on cellular motility by R. D. Allen and muscle by Franzini-Armstrong and Peachy are included in this volume.

Without question, the most important landmark in this field was the independent purification of actin and myosin from the slime mold *Physarum* by Hatano and co-workers (8, 9) in Japan and by Adelman and Taylor (10) in the United States in the late 1960s (Fig. 1). To be sure, there were earlier reports by Loewy (11) and others (12) describing "actomyosin-like" proteins in nonmuscle cells, but all of these preparations were too crude to be characterized convincingly. However, once highly purified contractile proteins were available, it was straightforward to establish that they shared many important features with their muscle counterparts and to make a strong argument that they participate in cellular motile mechanisms.

A second major event was the publication in *The Journal of Cell Biology* in 1969 of a paper by Ishikawa et al. (13) that described a morphological technique for identifying actin filaments in cells by electron microscopy. Their technique was simply to treat glycerated cells with muscle heavy meromyosin that decorated cytoplasmic thin filaments with arrowhead-shaped complexes (Fig. 2), identical with those originally observed along heavy meromyosin-decorated pure actin filaments (Fig. 3) by Huxley (14). Armed with this technique, morphologists found actin virtually everywhere in nature (reviewed in reference 3). More recently a second generation of morphologists has used fluorescent antibodies to localize actin (15), myosin (16), and additional accessory proteins (17) in many cell types.

This work has led to a large number of studies characterizing the cytoplasmic contractile protein molecules and their distributions in cells. Other lines of investigation in this area have included efforts to demonstrate the involvement of the contractile proteins in specific cellular movements and in cytoplasmic structure.

Cytoplasmic Contractile Protein Characterization

ACTIN: Actin is the most thoroughly characterized cytoplasmic contractile protein. Initial reports were concerned with establishing the existence of actin in various nonmuscle cells and tended to include only a superficial characterization of several different properties. In the last five years, the sophistication of the analysis has increased considerably, so that knowledge about some cytoplasmic actins now approaches what we know about muscle actin.

The concentration of actin varies among different cell types, but it is always one of the most, if not *the* most, abundant cellular proteins (Table I). In highly motile cells it constitutes 10–15% of the total protein and is present in concentrations of 100–250 μM. Although it is clear that there is a vast excess of actin over myosin in nonmuscle cells, it is by no means clear

FIGURE 1 The founding fathers Doctors Fumio Oosawa, Sadashi Hatano, Mark Adelman, and Ed Taylor grind up a sample of *Physarum polycephalum* under the watchful gaze of Ariel Loewy.

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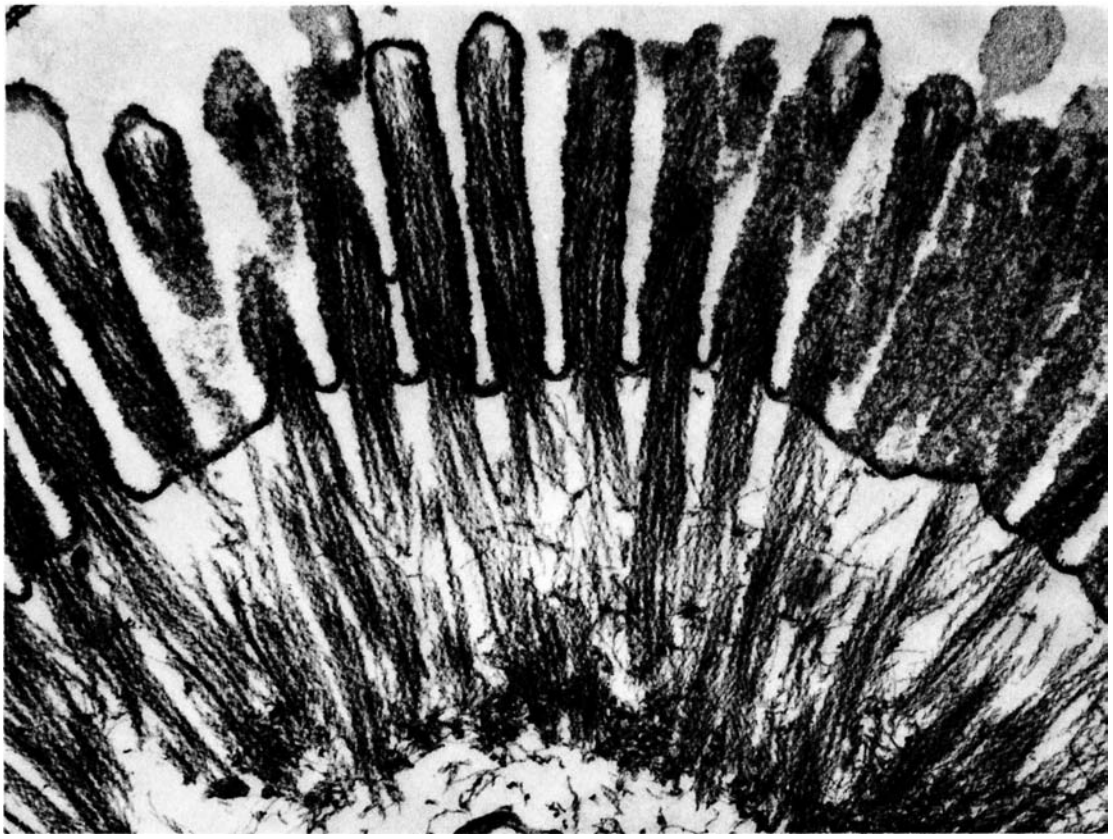

FIGURE 2 Electron micrograph of the actin filaments of the intestinal epithelial cell brush border decorated with myosin subfragment-1 arrowheads. This method was introduced by Dr. Hal Ishikawa in 1969 and improved by adding tannic acid to the fixative by Dr. David Begg, who contributed this micrograph.

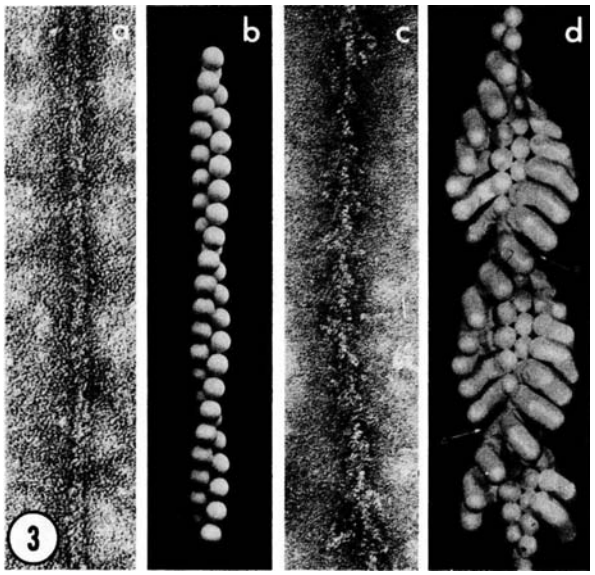

FIGURE 3 Electron micrographs by the author of (*a*) a negatively stained *Acanthamoeba* actin filament and (*c*) a negatively stained *Amoeba proteus* actin filament decorated with muscle heavy meromyosin. Models of (*b*) an actin filament and (*d*) an actin filament decorated with myosin heads from the work of Moore et al. (*J. Mol. Biol.* 50:279 [1979]).

what all this extra actin is doing in the cell. A leading speculation, discussed in detail below, is that the bulk of the actin is used as a structural protein.

Actins from all major branches of the phylogenetic tree have now been sequenced (18–20). About 95% of the residues are identical in muscle and cytoplasmic actins, but as a group all of the cytoplasmic actins are more similar to each other than they are to muscle actin. This suggests that there have been different evolutionary pressures on muscle and cytoplasmic actins. The *N*-terminal is the most variable region of the actin molecule, and minor sequence and compositional differences in the first few residues account for the distinctive isoelectric points of the three different isoactin classes found in vertebrates (19). The unusually basic isoelectric point of *Acanthamoeba* actin (21) is attributable to a histidine at position 228 where the other actins have a neutral residue.[1]

TABLE I
Contractile Protein Content

	Total protein	Concentration	Actin: myosin ratio
	%	(μmol/kg)	
Actin			
Rabbit muscle	19	900	6
Human platelet	10	240	110
Acanthamoeba	14	250	70
Myosin			
Rabbit muscle	35	144	
Human platelet	1	2.2	
Acanthamoeba-I	0.3	1.3	
-II	1.2	2.3	

[1] Elizinga, M., and T. D. Pollard. Unpublished observation.

The three-dimensional structure of the actin molecule is not yet known. However, both two- and three-dimensional actin crystals of muscle and nonmuscle actins are now being studied by electron microscopy and X-ray diffraction (22, 23, 23a) so this information should soon be available.

Given the highly conserved nature of the actin molecule, it is not surprising that all actin filaments are indistinguishable by electron microscopy (see reference 3) (Fig. 3). As established by X-ray diffraction of live muscle (24) and confirmed by electron microscopy of many different actin specimens, actin filaments consist of a double-helical array of more or less globular actin molecules (14). The polymer is 6 nm wide, the molecules are 5.5. nm long, and the helix repeats every 37 nm. The similarities among various actin filaments extend beyond the double-helical structure of the filaments to the myosin-binding sites. They must be nearly identical, because all known actins bind myosin in precisely the same way to form polarized arrowhead-shaped complexes which repeat with every turn of the underlying actin helix (see reference 3) (Fig. 3). As stressed by Huxley (14), the polarity of actin filaments revealed by myosin decoration is an essential feature for tension generation. In every case studied, the force generated by actin-myosin interaction *pulls* the actin filament in the direction pointed by the arrowheads.

In those cases where cytoplasmic actin polymerization has been studied in detail (21, 25, 26), the process has been shown to be generally similar to actin polymerization in muscle. The only substantial differences were found under nonphysiological conditions. Actin polymerization involves three major steps (27). First, several monomers bind together to form a short oligomer. This slow step provides a nucleus. Next, monomers rapidly add to the nucleus to elongate the filament. Elongating filaments grow in both directions with a strong bias toward the "barbed" end (the end with the arrowhead barbs if the filament were decorated with myosin) (28). The third step is the annealing of two filaments end-to-end to form a longer filament. Filaments will grow until the concentration of monomer is reduced to the so-called "critical concentration" of monomer that remains in apparent equilibrium with any amount of filament. Rather than a true equilibrium, this is more likely to be a steady state with net actin monomer addition at one end balanced by net loss from the other end (29). The regulation of the polymerization process is discussed below.

All actins are capable of binding myosin reversibly and in the presence of adenosine triphosphosphate (ATP) of activating the myosin Mg-ATPase activity (see reference 3). This cyclic interaction of myosin, actin, and ATP is thought to be the physiologically relevant, force-generating enzyme activity of the proteins. When cytoplasmic actins were compared with muscle actin for the ability to stimulate the muscle myosin ATPase, all of the actins gave the same V_{max}, but muscle actin had a higher affinity for the myosin than did the cytoplasmic actins (26).

As mentioned above, the classic method for identifying actin filaments in cells is decoration with myosin fragments (either heavy meromyosin or subfragment-1). Studies of many cells have shown that most 6-nm filaments in cells are composed of actin (13) (Fig. 4). Some cytoplasmic actin filaments are preserved in thin sections of fixed cells, but there is concern that the conventional fixation process, especially extended exposure to OsO_4, fragments actin filaments to form "microfilament networks (30)." Negative staining (31) and freeze drying (32), which preserve straight actin filaments, are two alternate methods for preparing cells for electron microscopy. They promise

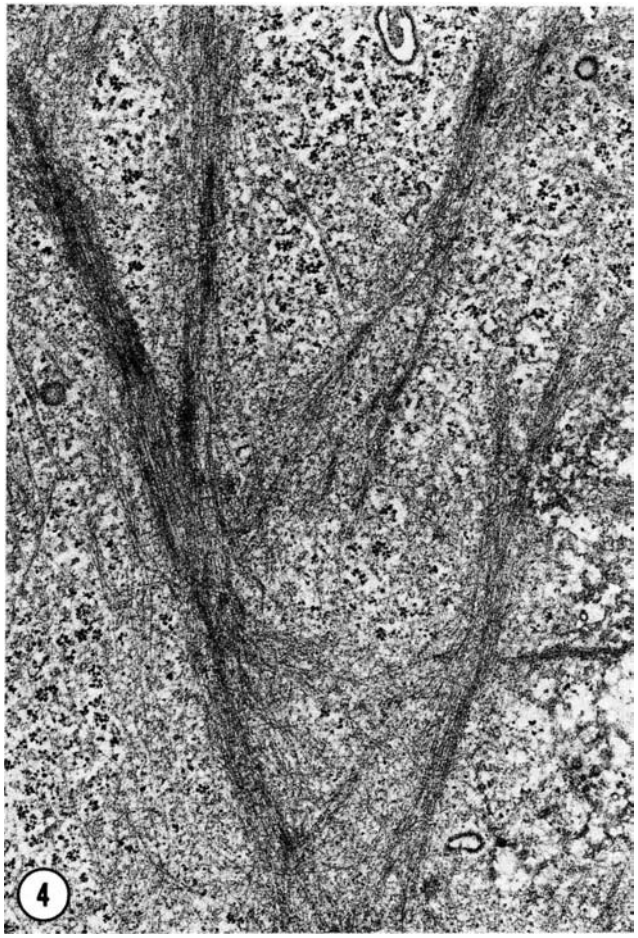

FIGURE 4 An electron micrograph of a HeLa cell showing bundles of actin filaments called "stress fibers," a few microtubules, and numerous free ribosomes. Micrograph by Dr. Ira Herman.

to be extremely useful in mapping out associations among the fibrous elements of the cytoplasm, although they are currently limited to extracted cell models.

Fluorescence microscopy with labeled antibody (15) or labeled heavy meromyosin (33) allows one to evaluate quickly the overall distribution of actin in the whole cell. As much for aesthetic reasons as anything else, considerable attention has focused on the so-called "stress fibers", which are prominent in some tissue culture cells (Fig. 5). These fibers are composed of actin filament bundles with associated myosin (16) and accessory proteins (17). They are potentially contractile (34) and may be involved with cytoplasmic retraction in some forms of cell movement. However, most cells with prominent stress fibers exhibit little locomotion (34a) (Fig. 5). On the other hand, rapidly motile cells have a diffuse distribution of actin and myosin.

A promising method for studying actin distribution in living cells is microinjection of actin labeled with a fluorescent dye (35–37). Image intensification of the fluorescence allows direct observation of actin dynamics. Alternatively, the injected cell can be fixed and the actin distribution observed by conventional means.

Actin filaments are highly concentrated in the peripheral cytoplasm (cortex) of many cells, which suggests that they may be attached to the plasma membrane. This was shown to be true by isolating membranes from cells such as *Acanthamoeba* (38) and the intestinal absorptive cell (39) and by showing biochemically and microscopically that actin filaments are

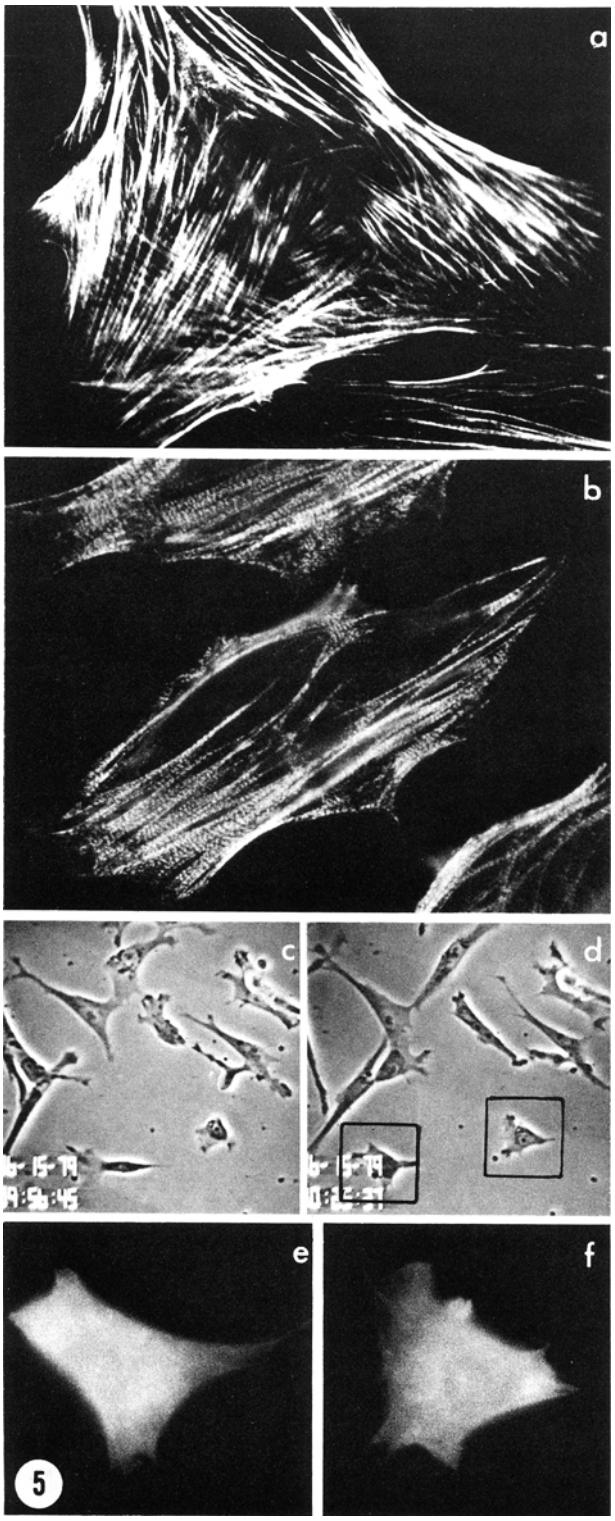

FIGURE 5 Tissue culture cells stained with fluorescent antibodies. (*a*) PtK-2 cells stained with purified anti-actin showing continuously labeled stress fibers. (*b*) HeLa cells stained with purified anti-platelet myosin showing punctate labeling of stress fibers. (*c,d*) Phase-contrast time lapse videotape records of the movements of living chick embryo cells taken 30 min and 1 min before fixation and staining with fluorescent anti-actin. (*e,f*) Fluorescence micrographs of the two cells indicated in (*d*) showing the diffuse staining of these migrating cells. Micrographs by Dr. Ira Herman.

attached. Most importantly, the barbed end of the actin filaments always seems to be attached to the cytoplasmic surface (Fig. 2) (34, 39, 40). Consequently, any tension generated by myosin interaction with these filaments will exert a "pull" on the plasma membrane, just as in the case of the Z line in striated muscle.

In spite of the profound functional significance of these membrane attachments, essentially nothing is known about their molecular basis. Early enthusiasm about the Z-line protein α-actinin being involved directly with attachment (39) has not been substantiated, although both α-actinin (17, 41, 42) and a 130,000-mol wt protein (43) are found near actin attachment sites in tissue culture cells and the zonula adherans of epithelial cells.

MYOSIN: The work on cytoplasmic myosins lagged behind studies of cytoplasmic actin for a number of years. Initial problems were the small amount of myosin in most cells and the lack of simple purification procedures. Moreover, no morphological work was possible until antibodies were developed, because myosin cannot be identified in nonmuscle cells by conventional light or electron microscopy. Now there are reliable purification procedures (for example, see reference 44) and methods for localization (16).

In contrast with muscle, where myosin is the major protein, myosin is a minor protein in nonmuscle cells (Table I). This is appropriate for myosin, the force-generating, energy-transducing enzyme in these contractile systems, because the forces required for cellular motility are orders of magnitude less than those developed by muscle.

Unlike the actins, which are all rather similar, the myosins are remarkably variable. Even though the myosins from various muscles are all the same size and shape, they differ in primary structure and enzyme activity (45). Nonmuscle cells even have myosins with different sizes and shapes (Table II) (38, 46–49), and at least one cell, *Acanthamoeba*, has multiple myosins (46–48). Given this diversity, one might ask what defines myosin? I feel that the essential features of myosin are the ability to bind reversibly to actin filaments and actin-activated ATPase activity. Other features, such as the capacity to form bipolar filaments, are common to most myosins, but probably are not essential.

All myosins consist of "heavy chains" and "light chains" (Table II). In most cases, one end of the two heavy chains forms an α-helical-coiled coil "tail"; the remaining part of the heavy chain, together with the light chains, form two globular

TABLE II

Cytoplasmic Myosin Classes

	Molecular weight	Polypeptides	Reference
Metazooan	~470,000	2 × 200,000 2 × 18,000 2 × 16,000	44, 52, 90
Slime mould	~500,000	2 × 210,000 18,000 16,000	132
Acanthamoeba myosin-I	~180,000	1 × 130,000 or 125,000 1 × 17,000 or 25,000 1 × 14,000	46, 133
Acanthamoeba myosin-II	~420,000	2 × 175,000 2 × 16,500 2 × 17,500	47, 48

"heads" (Fig. 6) (50, 51). In both muscle (50) and nonmuscle cells (52) the heads contain the catalytic site for ATP hydrolysis and the actin-combining site. The tails form the backbone of myosin filaments.

Except for the globular *Acanthamoeba* myosin-I, which has no tail (46), all muscle and cytoplasmic myosins form bipolar filaments under physiological conditions (Fig. 6) (48, 49, 53). Although they differ in size, all of these myosin filaments have the same geometrical features. In the center is a bare zone, the same length as the myosin tail, composed of an antiparallel overlapping array of myosin tails. This bare zone is flanked by terminal regions of variable length where the myosin heads protrude from the surface of the filament at 15-nm intervals (24). Thus myosin filaments have a plane of mirror symmetry in the center of the bare zone. The thickness of the filament depends on how many heads are present in each 15-nm interval (53). The filaments formed by most purified cytoplasmic myosins are small compared with the myosin-thick filaments in muscle, which are composed of 300–400 myosin molecules (54) and are 18 nm wide and about 1.5 μm long. Platelet myosin filaments, for example, are composed of about 30 myosin molecules and are 10–11 nm wide and 0.3 μm long (53). *Acanthamoeba* myosin-II filaments are even smaller (48).

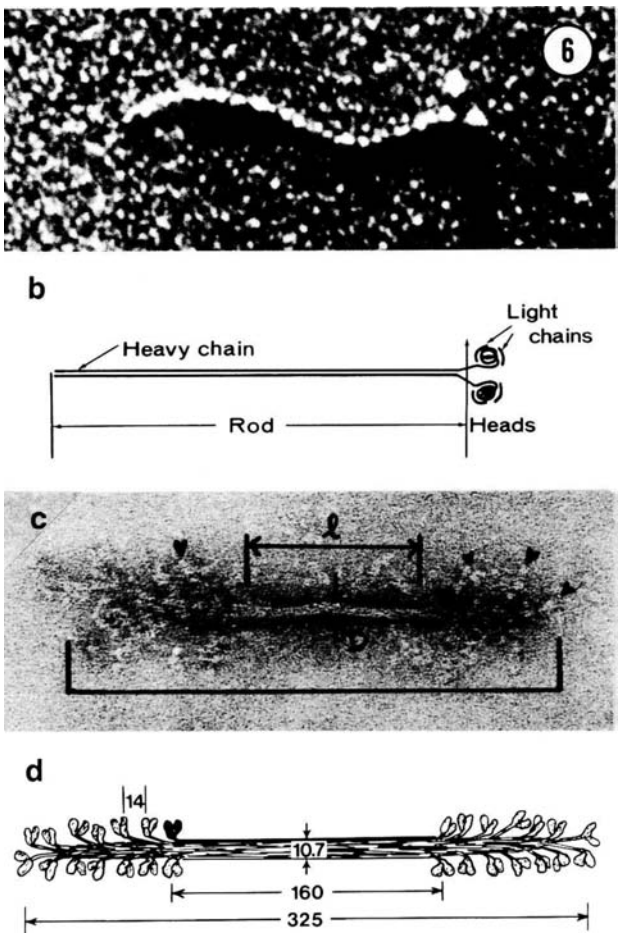

FIGURE 6 Platelet myosin. (*a*) An electron micrograph of a shadowed platelet myosin molecule contributed by Dr. K. Burridge. (*b*) A drawing of a platelet myosin molecule showing the constituent polypeptides. (*c*) An electron micrograph of a negatively stained bipolar filament formed from purified platelet myosin taken by the author. *l* is the length of the bare zone, *D* is the diameter of the bare zone, arrowheads mark some of the myosin heads. (*d*) A two-dimensional model of a platelet myosin filament showing the dimensions of the filament in nanometers from the work of Niederman and Pollard (53).

Such myosin filaments are not seen in electron micrographs of nonmuscle cells. It has been argued (53) that this is the consequence of the low concentration of myosin and the small size of the filaments, but it could equally well be that little of the myosin is usually assembled into filaments. Early results from electron-microscope localization of myosin with ferritin-labeled antibodies suggest that all of these factors contribute to the apparent absence of myosin filaments (54a).

Participation of Contractile Proteins in Cellular Movements

Establishing mechanisms for the generation of cellular motile force has been difficult compared with that for muscle, where direct physiological studies of contractile protein activity manifested as tension generation or shortening are straightforward. The biochemical properties of the cytoplasmic contractile proteins made it seem obvious almost from the beginning that they must be responsible for cellular movements, but even the obvious must be proven. In fact, very few cellular movements have been shown to be powered by the contractile proteins. The following discussion is divided into two sections: first, the arguments that the cytoplasmic contractile proteins can generate tension and motion, and second, the evidence that they are responsible for a specific movement—cytokinesis.

THE ARGUMENTS: One suspects from the parsimony of nature and the similarity of the structures of actin and myosin filaments in all cells, that the well-studied mechanism generating force in muscle is universal. Because the myosin heads at each end of the filaments are oppositely polarized, one filament can cross-link two or more oppositely polarized actin filaments. In muscle, a structure of this kind is repeated with crystalline precision many times in parallel and in series to make sarcomeres and myofibrils (24). No actomyosin contractile structures with such clearly defined geometry have been identified in nonmuscle cells, but filaments of cytoplasmic actin and myosin can form loosely organized networks with the same essential geometrical features (52, 53). This shows on mechanical grounds, at least, that the contractile proteins of nonmuscle cells can generate tension and motion by the same sliding filament mechanism used in muscle.

The most highly ordered cellular actomyosin structures appear to be the brush border of some epithelial cells and the stress fibers of tissue culture cells. In the brush border, a large number of microvilli protrude from the surface. Each microvillus contains a bundle of actin filaments attached at their barbed ends to the tip of the microvillus (39). The pointed ends of the filaments are in the terminal web, where they are associated with myosin (55). Geometrically, the structure is similar to a sarcomere folded in the middle of the A band. The natural motion of microvilli is not known, but a sliding-filament mechanism can account for the movement of the microvillar bundles into the terminal web in demembranated models treated with ATP (56). Stress fibers are less ordered. They consist of a bundle of parallel actin filaments (57) with intermittent concentrations of myosin (16), α-actinin (17), tropomyosin (17), and a new actin-binding protein called filamin by some (58). However, the detailed arrangement of these proteins in stress fibers has not been established, so that the mechanism of their contraction (34) is unknown.

In addition to these structural considerations, the following facts strengthen the argument that the cytoplasmic contractile proteins generate forces for cellular movements by a mechanism similar to that of muscle. It is possible to construct functional actin-actin (59), myosin-myosin (60), and actin-myosin hybrids (review in reference 3) from quite diverse muscle and nonmuscle cells. For example, any myosin will bind to any actin, and any actin will activate myosin ATPase (26), which show that the essential features of the active sites are conserved. Furthermore, actomyosin threads (61) and gels (62) have been formed from a number of purified myosins and actins and shown to contract upon addition of ATP.

THE EVIDENCE: Cytokinesis is the most thoroughly studied example of a cellular movement believed to be powered by actin and myosin. Elegant micromanipulation studies (63) established that the cleavage furrow itself develops enough tension to bring about the deformation of the cell during cytokinesis. In the base of the furrow is a narrow band of parallel actin filaments, called the contractile ring, which encircles the equator of the cell (64). The volume of a contractile ring decreases during cytokinesis, which suggests that it disassembles (65). Both myosin (16) and α-actinin (66) are present in the cleavage furrow and, in many cells, are more highly concentrated there than elsewhere in the cytoplasm (Fig. 7). Surprisingly, actin does not seem to be concentrated in the furrow, compared with other regions of the cell cortex (Fig. 7) (67, 68), which suggests that the unique feature of the contractile ring is the parallel alignment of the filaments. It has been suggested that tension on preexisting random actin filaments brought about by contraction confined to the equatorial region might align the filaments and form the contractile ring (66).

Microinjection studies have provided direct evidence for the participation of myosin and actin in cytokinesis. Injection of myosin antibodies into living echinoderm eggs inhibited cytokinesis (Fig. 8) (69), presumably by inactivating myosin. The injected cells survived and continued nuclear division, but cytokinesis was permanently blocked. Microinjection of frog eggs with heavy meromyosin inactivated with a sulfhydryl reagent also blocked cytokinesis (70), presumably by binding permanently to actin filaments and interfering with their interactions with cellular myosin.

Although these experiments provide evidence for an actomyosin purse-string mechanism of cytokinesis, the details of the mechanism are unclear. No other cellular movement is understood in even this much detail.

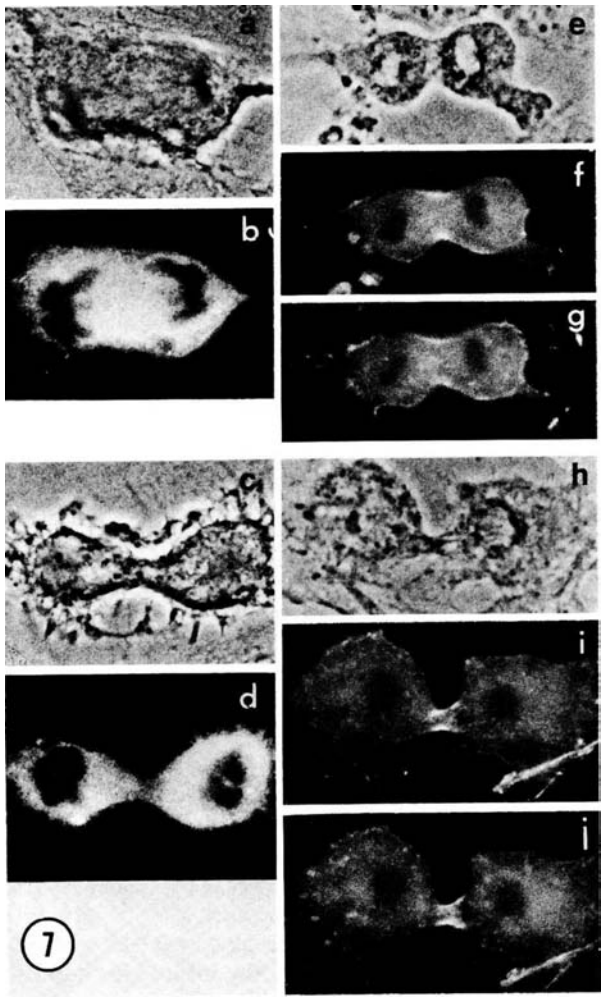

FIGURE 7 Localization of actin, myosin, and α-actinin during cytokinesis. (*a,c*) Phase-contrast and (*b,d*) fluorescence micrographs of PtK-2 cells stained with purified anti-actin showing diffuse distribution of the fluorescent antibody and no apparent concentration in the cleavage furrow. From the work of Herman and Pollard (67). (*e,h*) Phase-contrast and fluorescence (*f,g,i,j*) micrographs of chick embryo cells double stained with fluorescein-labeled anti-α-actinin (*f,i*) and rhodamine-labeled anti-myosin (*g,j*) showing the concentration of these antibodies in the cleavage furrow which is seen in many dividing cells. From the work of Fujiwara et al., (66).

Regulation of Cellular Contractile Protein Function

The regulation of the contractile apparatus must be a challenging task for the cell. Like muscle, nonmuscle cells must turn the contractile machinery off and on, but on top of that nonmuscle cells must also specify when and where actin and myosin filaments assemble and disassemble. I will cover filament assembly mechanisms before considering the regulation of motility.

There are a number of reasons to believe that cells must have mechanisms that specify the number, the sites of assembly, and lengths of their actin filaments, in addition to having some way to regulate the interaction of the filaments with each other, with membranes, and with other cellular structures, such as microtubules. For example, the ephemeral contractile ring in cytokinesis organizes, exerts tension on the plasma membrane, contracts, and disappears in a matter of minutes (65). Other filamentous structures, such as the brush border of intestinal or renal tubular epithelial cells, are much more stable, but some mechanism is responsible for the precisely ordered arrays of membrane-associated actin filaments, which are all the same length (39).

The total amount of actin filament in a cell is probably determined by the concentration of available actin, but not all of the actin is available for polymerization. Some of it seems to be sequestered. For example, some sperm have a cup of actin complexed with other proteins stored for eventual polymerization during the acrosomal reaction (71). In other cells, a substantial fraction of the actin is bound to a 16,000-mol wt protein, profilin, which inhibits the nucleation of actin polymerization (72, 72a). Together with a limited number of polymer initiation sites, profilin could maintain the total number and overall lengths of the filaments.

A more specific way to determine the length would be to

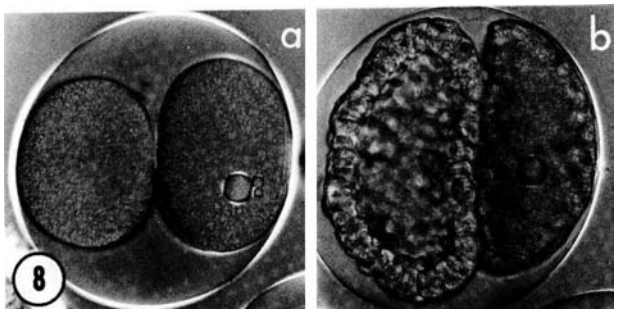

FIGURE 8 Microinjection of antimyosin blocks cytokinesis in *Asterias forbesi* embryo cells. (*a*) At the two-cell stage, the cell on the right was injected with 0.8 ng of antimyosin immunoglobulin together with a small droplet of vegetable oil to mark the injection site. (*b*) 12 h later the control cell on the left had divided normally many times, whereas only nuclear division occurred in the injected cell, showing that cytokinesis was completely inhibited. Micrographs by Dr. Daniel Kiehart from work by Kiehart, Mabuchi, and Inoué (unpublished).

cap one or both ends of the filaments, to prevent subunit addition or loss. Such a capping protein has been purified from *Acanthamoeba* (72b). It consists of 28,000- and 31,000-mol wt subunits and blocks monomer addition at the "barbed" end of actin filaments. A protein named β-actinin may cap the "pointed" end of muscle actin filaments (73), but its mechanism and the identification of its subunits are not settled. *Physarum* has a 43,000-mol wt protein called plasmodium actin (74), which seems to limit the length of actin filaments and may also act on one or both ends. Macrophages have still another length-regulating protein of 90,000 mol wt called gelsolin (75). It has the interesting feature that it requires Ca^{++} for its action.

It is clear from electron microscopy that at least some actin filaments grow from morphologically identifiable nucleating sites. For example, the filaments in intestinal epithelial cells seem to grow in the pointed direction from dense membrane plaques, which eventually become the tips of the microvilli (76). In some sperm, the actin filament bundle in the acrosomal process grows in the barbed direction from a filamentous structure named the actomere (77). The molecular components of these and other nucleating structures are not known. The only nucleating molecule known is the *Acanthamoeba*-capping protein (72b). At very low concentrations (1/1,000 actin molecules), it initiates actin polymerization and, because it also blocks growth in the barbed direction, determines both the site and direction of polymerization.[2]

Before anyone investigated the regulation of cytoplasmic actin-myosin interaction, it was shown by experiments with glycerinated cells (78), caffeine-treated slime-mold fragments (79), and demembranated cytoplasmic models (80) that the free Ca^{++} concentration controlled cytoplasmic contraction and streaming. The threshold Ca^{++} concentration for movement was in the micromolar range.

The source of Ca^{++}-stimulating movements in living cells has not been established. Because Ca^{++} also stimulates contraction in muscle, the first thoughts about mechanisms regulating cellular motility turned to the vertebrate skeletal-muscle paradigm of tropomyosin and troponin (81). This is a negative control mechanism, because it turns off the spontaneous interaction of actin and muscle myosin, which occurs regardless of the Ca^{++} concentration. Tropomyosin-troponin regulate contraction by blocking the actin-myosin interaction when the Ca^{++} concentration is low. When the Ca^{++} concentration is in the micromolar range, the inhibition is removed and the actin, myosin, and ATP continue their cyclic interaction until the Ca^{++} concentration falls and the block is restored.

When Cohen and Cohen (82) purified tropomyosin from platelets, it seemed as though the earlier history of similarities to muscle would be repeated in nonmuscle cells. Next, brain tropomyosin (83) was shown to form a functional, Ca^{++}-regulated hybrid with muscle troponin, actin, and myosin. When a troponin-C-like molecule was found in brain (84) and adrenal gland (85), only troponin-T and troponin-I were missing to reconstitute the whole regulatory system. But cytoplasmic troponin-T and troponin-I have not been found and may not exist. Instead, something new and much more interesting emerged: positive regulation of cytoplasmic contractile systems.

The first example of a positive regulatory mechanism was found in *Acanthamoeba* (86). When myosin-I was first purified, it was found to lose its actin-activated ATPase during the final steps of the purification. This lost activity was restored by adding back a partially purified 95,000-mol wt "cofactor" protein. From the stoichiometry of the reactants, it was speculated that the cofactor protein acted on the myosin rather than upon actin. A crude cofactor protein was also found in macrophages (87).

Independently, Adelstein and his colleagues found that platelet myosin light chains were phosphorylated and that the phosphorylated form of myosin had much higher actin-activated ATPase activity than the dephosphorylated form (88). In the initial experiments, Ca^{++} and cAMP had no effect on the kinase or the actomyosin ATPase, so it was not clear that myosin phosphorylation had anything to do with control of cellular movements. However, through subsequent experiments with both smooth muscle and nonmuscle cells, it was shown that Ca^{++} regulates the light-chain kinase through the calcium-binding protein calmodulin, the troponin-C-like protein purified previously (89, 90). Actually, calmodulin seems to have been discovered several times. Calmodulin binds Ca^{++} and then binds to and activates the light-chain kinase, which, in turn, activates the myosin. Activation of contraction by light-chain phosphorylation has been shown directly in synthetic platelet actomyosin threads (61), so this mechanism, coupled with appropriate phosphatases, should be capable of turning cellular contractions on and off.

It has now been shown that the macrophage cofactor protein is a light-chain kinase (91), but the *Acanthamoeba* cofactor turned out to be a heavy-chain kinase (92). Dephosphorylated myosin-I has little actin-activated ATPase activity, whereas the phosphorylated form is highly active.

It is conceivable that myosin phosphorylation alone can account for the regulation of cytoplasmic contractility. However, the function of cytoplasmic tropomyosin has not been established, and it is possible that there are additional unrecognized mechanisms controlling cellular contraction.

Actin as a Structural Protein

Since the 1830s, when Dujardin proposed the existence of a

[2] Since this review was written, it has been shown by the laboratories of S. Craig (Johns Hopkins University), M. Mooseker (Yale University), and K. Weber (University of Göttingen) that villin from the intestinal epithlial cell is also a barbed-end capping protein which can nucleate polymerization. See the *Cold Spring Harbor Symposium on Quantitative Biology*, Volume 47 (1981), for recent summaries of this and other recent work on contractile proteins.

gelatinous contractile material in the cytoplasm, there have been repeated suggestions that the cellular contractile machinery also plays an essential structural role (see reference 93 for a review). By the 1930s, it was generally accepted that living cytoplasm is a gel-like material which varies in consistency with time, depending on the activity of the cell. Some believed that the gel was essential for motility. The direct demonstration of this connection at the molecular level came from experiments on amoeba cytoplasmic extracts. Thompson and Wolpert (94) found that cold, cell-free extracts of *Amoeba proteus* streamed when supplied with ATP and warmed to room temperature. Pollard and Ito (95) showed that the extracts increased in viscosity at the time they were warmed and that this consistency change is correlated with the formation of innumerable 6-nm filaments from a soluble precursor. These 6-nm filaments were later identified as actin filaments. It was argued that the polymerization and depolymerization of the actin might control cytoplasmic consistency.

In subsequent experiments on demembranated amoeba cytoplasm, Taylor et al. (80) found that conditions which influence actin-myosin interaction also affected the viscoelastic properties of the cytoplasm. They suggested modulation of actin-myosin interaction as an addition factor in the determination of cytoplasmic consistency.

A series of papers from several laboratories (62, 96–101), have established that the components of many cells solubilized by homogenization in the cold can form a solid gel when warmed to room temperature or above (Fig. 9). Initially, these experiments were carried out qualitatively by tipping test tubes, but now the kinetics of the gelation reaction can be followed with a sensitive low-shear, falling-ball viscometer (101). Generally, it has been found that gelation requires Mg-ATP and is inhibited by micromolar free Ca^{++}. Actin filaments are the major component of these gels, and under appropriate conditions myosin associates with the gel to cause contraction.

Because the gels have a much higher viscosity than actin alone, efforts have been made to isolate cross-linking molecules from these extracts (Table III). The first was actin-binding protein from macrophages (102). This large protein cross-links actin filaments to form a homogeneous gel. Independently, a similar protein was purified from smooth muscle and named filamin (58). Small- and medium-size gelation factors have been isolated from *Acanthamoeba* (101, 103).

Sea urchin eggs are particularly interesting, because the gels differ in structure from the others mentioned above. The sea urchin extract forms actin filament bundles, rather than isotropic actin filament networks. The bundles are held together by a 58,000-mol wt protein named fascin, which is spaced at 11-nm intervals along the bundles (102). Similar bundles with 10-nm periodicity are found in sea urchin egg microvilli (104).

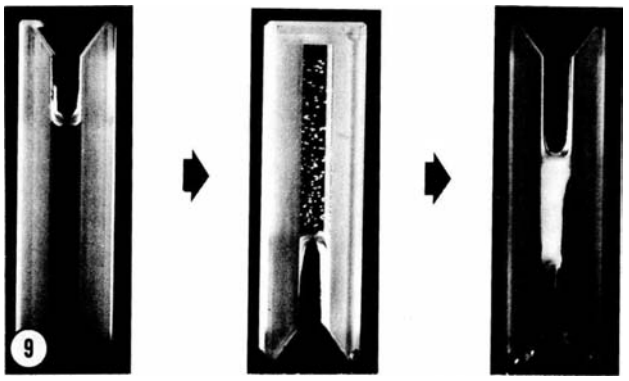

FIGURE 9 Gelation and contraction of a 140,000-g supernate of an *Acanthamoeba* homogenate induced by warming the cold extract to room temperature. From the work of the author (44).

TABLE III

Examples of Actin Cross-linking Gelation Factors

Source	Name	Subunit molecular weight	Calcium sensitivity	Reference
Acanthamoeba	Gelation factor-23	23,000	No	101, 103
	Gelation factor-29	29,000	No	
	Gelation factor-33	33,000	No	
	Gelation factor-38	38,000	No	
	Gelation factor-85	85,000	Yes	103a
Ascites tumor cells	Actinogelin	110,000	Yes	105
Sea urchin egg	Fascin	58,000 220,000	No	96, 102
Smooth muscle	Filamin	250,000	No*	58, 135, 136
Vertebrate nonmuscle cells	Actin-binding protein	250,000	No*	62, 134

* Unless an additional protein "gelsolin" is present (75).

There is general agreement that the Ca^{++} concentration controls, at least in part, the gelation process, but there is little agreement about the mechanism. The simplest case is that cross-linking by one of the gelation factors is inhibited by Ca^{++}. This is true for a 110,000-mol wt gelation factor from ascites tumor cells (105). Another possibility is that additional proteins participate, as suggested for macrophages (75). There the Ca^{++}-requiring protein gelsolin inhibits gelation by reducing the length of the filaments and by thus increasing the concentration of actin-binding protein required for gelation. The actin filament-capping protein (72b) may be an additional factor in the gelation process because it strongly inhibits the self-association of actin filaments, which normally contribute to the stabilization of the gels (106).

These cross-linked actin networks are thought to be responsible for the gelatinous nature of the cytoplasmic matrix and are likely to be a component of the latticework of cytoplasmic matrix fibers, which Porter, Buckley, and others (107, 108) have referred to as microtrabeculae. With improved methods involving extraction, quick-freezing, drying, and metal coating (32), the elements of this lattice have now been shown to include actin filaments and intermediate filaments. Almost certainly this actin filament network is responsible for limiting the Brownian movement of the organelles and probably contributes to the maintainence of cellular shape and the nonhomogeneous distribution of intracellular components. The gel may also be important to the cell as a scaffolding for certain enzymes (109) and may bind the "free" polyribosomes (110).

In addition to these associations of actin filaments with each other, they may also interact with microtubules (111). This association is weak and requires the microtubule-associated proteins found on the surface of the tubules. The functional significance, if any, of this interaction is not established. It could be purely structural, as suggested by electron microscopy

of critical point-dried specimens (107, 108) or the contractile proteins attached to microtubules may power microtubule-dependent movements (111).

Drugs That Act on Cytoplasmic Contractile Proteins

Since the pioneering work of Carter (112) and Schroeder (113), we have known that micromolar or submicromolar concentrations of cytochalasins inhibit some cellular movements. The cytochalasins are a group of closely related ~500-mol wt organic molecules produced by molds and distinguished by alphabetical designations. Cytochalasin B has been used the most widely. Cytochalasin D has the highest potency in inhibiting cellular movements (114).

There was early speculation (115) that actin ("microfilaments") is the target of the cytochalasins, but the first experiments with purified actin (116) failed to reveal a direct effect of micromolar cytochalasin B on polymerization. It was found that some cytochalasins inhibit glucose transport into cells (117), although this could not be correlated with the effects on motility (114).

More recently, it was shown that submicromolar concentrations of cytochalasin B, D, and E inhibit gelation of cytoplasmic extracts (93, 98, 101, 118) and reconstituted systems of purified actin with various cross-linking molecules (12, 101, 106). This has been traced to a direct substoichiometric effect on actin filament network formation, which occurs when a few molecules of cytochalasin bind to an actin filament consisting of hundreds of actin molecules (106, 119). Two mechanisms have been suggested: either cytochalasin reduces the length of the filaments (119) or it inhibits the self-association of the filaments (106). These same concentrations of cytochalasin also reduce the rate of actin filament growth (106, 120–122) by blocking monomer addition at the barbed end of the filament (106), so the cytochalasin-binding site(s) is most likely at the barbed end of the filament. Under physiological conditions, micromolar cytochalasin B strongly inhibits actin filament network formation and gelation (106, 118) with only minimal effects on polymerization rate (106), so it seems that the inhibition of cellular motility is caused by the structural change.

Phalloidin is a second alkaloid that reacts directly with actin. This molecule is a product of the poisonous mushroom, *Amanita phalloides*. It binds to actin molecules in actin filaments and stabilizes the filaments (123) under a variety of conditions where actin filaments usually depolymerize, including exposure to DNase I (124), 0.6 M KI (125), OsO_4 (126), ultrasonication (125), and high temperature (127). This stabilization probably accounts for the abundance of actin filaments in electron micrographs of phalloidin-treated cells (128). Although phalloidin does not readily enter cells, it can be microinjected (129). This treatment inhibits cellular locomotion in tissue-cultured cells (129) and amoebas (130). Presumably, this means that depolymerization of actin filaments is required in some way for normal locomotion, but other explanations are also conceivable. Phalloidin can be attached to fluorescein and has been used to stain actin filaments in tissue-culture cells (131).

Future Trends

The major question in motility is: How is tension generated during actin-myosin interaction? It seems to me that research on muscle is more likely to answer this fundamental question than is work on cellular motility. However, cellular systems continue to reveal fascinating new insights into contractile proteins, which have escaped attention in muscle, so that one cannot exclude the possibility that research on cellular motility will contribute to this area. For example, much of the actin crystallography is being done on cytoplasmic actins.

In nonmuscle cells, much of the future work on contractile proteins will focus in two areas: (*a*) investigation of the mechanisms which control assembly and tension generation; and (*b*) elucidation of the macromolecular anatomy of contractile protein systems. By macromolecular anatomy, I mean definition of the molecular associations. This would include, for example, a detailed structural analysis of the actin filament and how the various associated proteins bind to the filament. In particular, this analysis will require definition of the molecular interactions, which link the components of the system together and to other cellular constituents.

Both of these issues promise to be extremely complex. One can safely predict that a number of new proteins will have to be discovered and characterized before a detailed understanding of either regulation or molecular anatomy will be realized. I also predict that no single approach will provide the answers. It is, after all, the phenomena observed in living cells that have prompted the molecular analysis of cellular contractile systems during the last 15 years. It is tempting to accept the resulting biochemical purification and characterization of the components as the culmination of the work. Although this is (and will continue to be) essential for progress, one will always have to return to the intact, preferably living, cell to test the ideas generated in the biochemistry laboratory. This two-way street between the living cell and molecular analysis is what cell biology is all about.

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Striated Muscle—Contractile and Control Mechanisms

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A new era in muscle research had its beginning in the early and mid-1950s. The new structural and biochemical techniques that became available to muscle-cell biologists were combined with clever physiological experiments to provide a vigorous and fresh new approach, the hallmark of which has been the understanding of close relationships between function and underlying structure. As a result, striated muscle has become more than a black box containing springs and viscosities and delimited by a membrane with unusual electrical properties. Within a muscle fiber, specific patches of membrane have specific functions in the control of muscle activity, enzymes are suitably located for their functional contribution, and the contractile material is disposed in a highly organized array of interacting filaments and bridges. Springs and viscosities have been moved to the description of muscle contractility at the molecular level. Beyond these successes, what has been learned from muscle is applied to many other biological phenomena, e.g., excitation-secretion coupling in secretory cells and motility in many kinds of nonmuscle cells.

In this chapter, we attempt to review that progress. Of necessity, we have limited our coverage to certain topics and our references to a few key papers and reviews. In general we have attempted to summarize and to simplify, and in doing so we may be guilty of obscuring controversies and omitting details. The following three sections follow the historical development of modern research on muscle. In the second part of the paper, current knowledge on selected topics is considered. Personal biases, as well as the purpose of this volume, encourage us to emphasize structural findings in this review.

Background

Some of the structural information underlying modern theories of muscle function was available by the early part of the century. However, the light microscope was being used at the limits of its resolution, and relatively crude fixation techniques were known to produce potentially severe artifacts. As a result, there seemed to be little hope of discovering which of several views of muscle structure was correct. Considerable imagination came into play, and resulting theories were often contradictory (cf. 1 and 2). Perhaps because of this, in the words of A. F. Huxley, "microscopy really went out of fashion from 1900 until after World War II."

From the vantage point of today's knowledge, we can select some authors whose views we now recognize to be remarkably correct. We will discuss here some that are relevant to the rest of this chapter.

FILAMENTS: Cross-striations of myofibrils have been known since Bowman (1840). Constancy of A-band length with shortening of the sarcomere was demonstrated subsequently (3). Birefringence of the A band had been noted and correctly interpreted as caused by the presence of longitudinally oriented protein rodlets. High-angle X-ray diffraction failed to produce evidence for changes in helical structure of muscle proteins during rigor, as expected from several contemporary theories of contraction, in which the emphasis had been on shortening of individual filaments rather than on interaction between filaments. It seemed clear to some workers, notably to H. E. Huxley, that the interesting things were happening at a level larger than the protein intramolecular structure (1 nm or less). Early, low-angle X-ray diffraction detected the regular arrangement of filaments and striking differences in equatorial reflections of active and rigor muscles, at the beginning of the modern era (4).

PROTEINS AND CONTRACTION: The two major contractile proteins, actin and myosin, had been separated from each other. Their association in the absence of adenosine triphosphate (ATP), to form "myosin B" or actomyosin, and their dissociation followed by superprecipitation in the presence of magnesium and ATP, had been demonstrated (5). Myosin was known to be an ATPase. A fortuitous biochemical observation later became an important technique in the study of the myosin molecule. Myosin incubated in trypsin shows a dramatic fall in viscosity and forms fragments, among which are a light fragment (light meromyosin [LMM]) and a heavy fragment (heavy meromyosin [HMM]) (6). HMM shows ATPase and actin-binding activity. LMM shows neither of these activities, and it is insoluble at normal ionic strength.

Initial evidence for a role of calcium in the activation of the contractile material was given (7).

Fenn and Marsh (8) described the relationship between two important mechanical parameters: load applied to the muscle and the velocity of shortening. Maximum velocity of shortening is a useful parameter for classification of fibers.

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ELECTRICAL PROPERTIES: The signal initiating the series of events that leads from excitation of the surface membrane to contraction of the fibrils was demonstrated to be a reduction of the resting membrane potential (9). An early study using intracellular electrodes found that a muscle fiber's electrical capacitance, measured at low frequencies, is far greater than that of a nerve fiber, both referred to outer surface area of fiber (10).

MEMBRANES: Reticular networks were a favorite subject of 19th century light microscopists, and often were rejected as artifacts (1). Some, however, were real. The black reaction of Golgi provided an Italian biologist of the turn of the century with "images of noteworthy subtlety and elegance" (11). Veratti's description of a fine reticular network in muscle cells, later to be identified with the network of tranverse tubules, suffered almost complete oblivion until it was rediscovered and republished in a special issue of *The Journal of Biophysical and Biochemical Cytology* in 1961 (11).

The other internal membrane system in muscle fibers is the sarcoplasmic reticulum (SR). Virtually unknown in the classical literature, it has been described in detail for a variety of species and fiber types during the period we will review. Marsh (reviewed in reference 12) discovered that the supernate of muscle homogenate, later recognized to contain the microsomal fraction, induces relaxation of a suspension of myofibrils. This effect of the "Marsh" factor was to occupy numerous investigators and to result in the definition of the role of the sarcoplasmic reticulum in the relaxation of muscle.

In The Beginning

SLIDING-FILAMENT MODEL OF CONTRACTION: In 1954 A. F. Huxley and Niedergerke (13), and H. E. Huxley and Hanson (14) simultaneously and independently proposed a theory of contraction that has become known as the sliding-filament model. It proposes that changes in the length of the sarcomere are caused by longitudinal sliding of two sets of filaments relative to each other, without changes in the length of the filaments themselves (Fig. 1).

COMPOSITION OF THE SARCOMERE: A rigorous comparison of the protein mass contained within the A band (measured by interference microscopy) with the amount of myosin extracted from the fibers, showed that the major component of the A band is myosin (15). The second set of filaments, which remained after extraction of myosin, was shown to contain actin.

Fibrils contain a large concentration of a few proteins for which good purification techniques were soon developed. Thus, muscle fibers were among the first cells to which the technique of immunocytochemistry was successfully applied. By this method, the location of actin and myosin in thin and thick filaments, respectively, was directly confirmed (16; see also review in reference 17).

The immediate source of energy for contraction was proven to be ATP in the careful biochemical study by Davies in 1964 (18). Thus splitting of ATP by myosin was confirmed to be the primary event in the interaction of actin and myosin.

FILAMENTS: In 1957, H. E. Huxley (19) published a complete description of the double hexagonal array of filaments forming the fibrils. The major structural features of the filaments and their disposition were beautifully illustrated (Figs. 2 and 3). Thick, myosin-containing filaments occupy the length of the A band, in the center of the sarcomere. Thin,

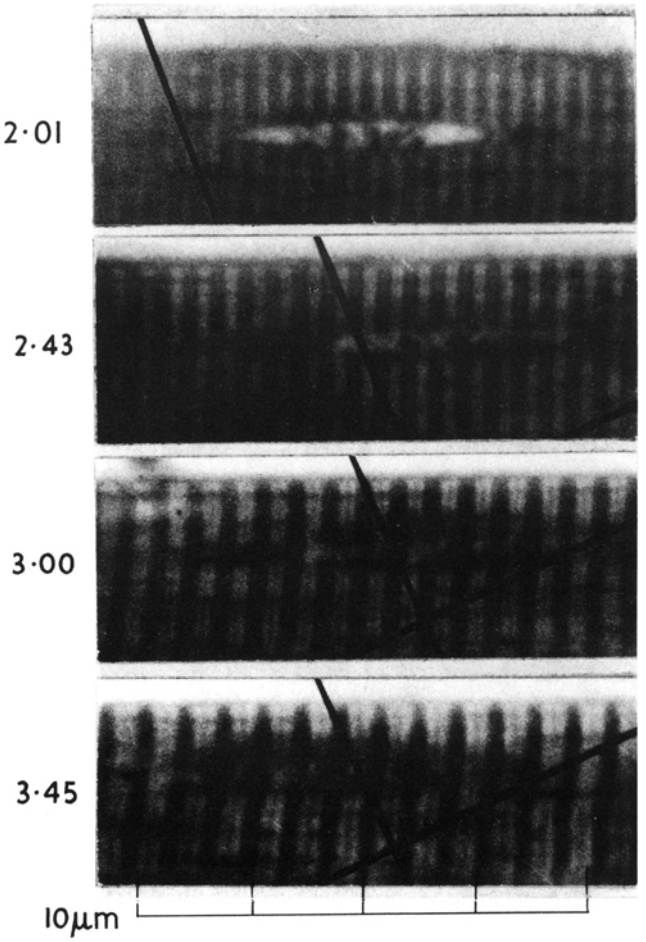


FIGURE 1 Interference-microscope views of a muscle fiber stretched passively to different sarcomere lengths, from 2.01 to 3.45 μm. The A band is dark. Constancy of A-band length in these micrographs first suggested the sliding-filament model of muscle contraction to A. F. Huxley and Niedergerke. Reprinted from reference 13.

actin-containing filaments attach at the Z line and interdigitate with the thick filaments at the borders of the A band. Both thick and thin filaments are well aligned and have a uniform length. The central region of the A band, which contains only thick filaments, is the H zone, observed earlier by light microscopy. The filaments form a highly ordered, double-hexagonal array (Fig. 3). Thus the stage is set for an interaction of a myosin filament with six adjacent thin filaments, and of each thin filament with three thick filaments (1:2 ratio). Bridges project out of the thick filament shaft all the way to its tapered ends, and seemingly attach to the thin filament. The central region of the thick filament has a bridge-free region (about 1,200 Å wide).

A MECHANICAL MODEL OF BRIDGE ACTION: A. F. Huxley (20) published a theoretical treatment of the sliding-filament model in which myosin cross-bridges, capable of moving around an equilibrium site, attach and detach from appropriate sites on the actin filaments according to a simple kinetic scheme. Cross-bridge action is a repetitive cycle of attachment, relative motion of the two filaments, and detachment. This early model could account quite well for such mechanical parameters as the force-velocity relationship and also for the relationship between load and energy utilization.

MEMBRANE STRUCTURE: Cell biologists were attracted

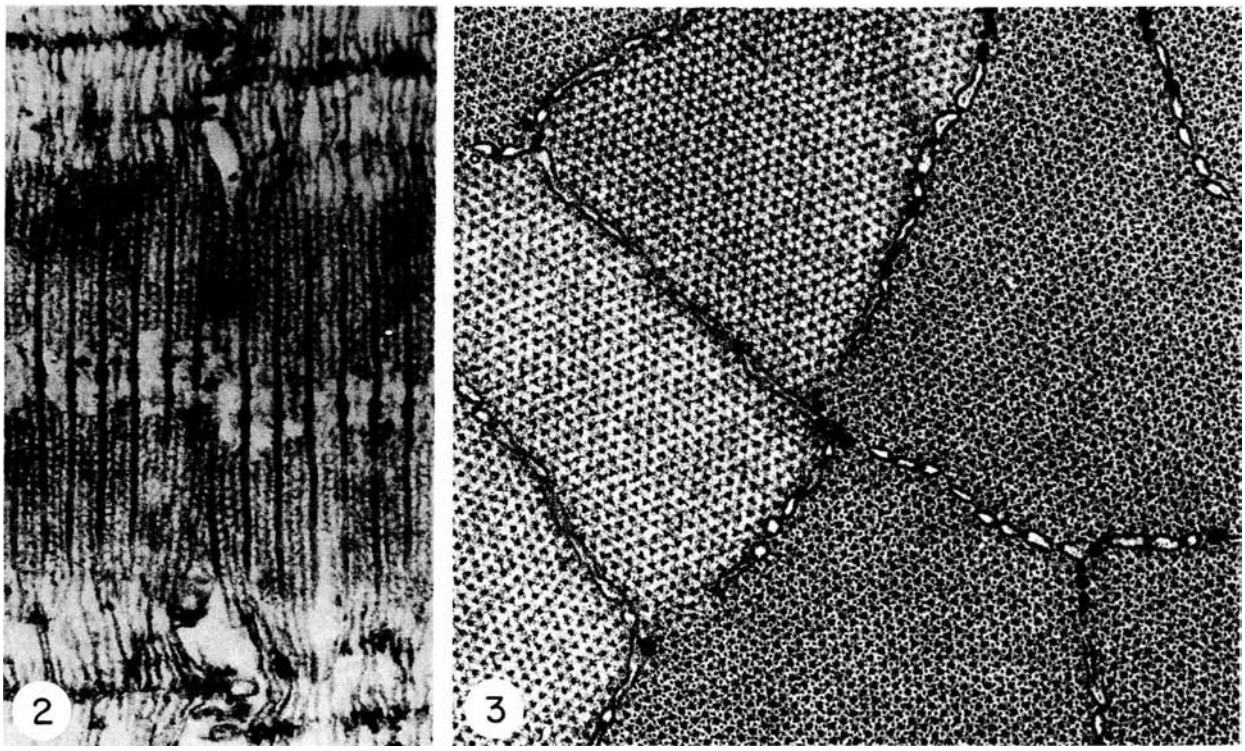


FIGURE 2 Disposition of thin and thick filaments is demonstrated in this longitudinal section of a single sarcomere from a rabbit muscle, fixed in rigor. Bridges, joining thick (myosin) to thin (actin) filaments are visible. The center of the thick filament is bridge-free. Images like these were of great importance in confirming the sliding filament model. Reprinted from reference 19.

FIGURE 3 Cross-section through several myofibrils, at the level of the A band, illustrating the relative disposition of thin and thick filaments in the sarcomere. At right, thin filaments occupy a trigonal position in the hexagonal lattice of thick filaments. At left, in the H zone, only thick filaments are present. Cross-links join thick filaments to each other at the M line (top, left), located in the center of the sarcomere. × 33,000.

by the elegant disposition of membranes in the form of a sarcoplasmic reticulum (the SR) with a repeating pattern relative to that of the sarcomere (21). Fittingly, the first full description of the SR was part of a series describing the structure and disposition of a cytoplasmic vesicular system, the endoplasmic reticulum, in a variety of cells. Porter and Palade (22) described the SR as an intracellular membrane system, separating its internal compartment from the rest of the sarcoplasm. The SR network is continuous transversely, but is longitudinally segmented at periodic intervals. Different muscle fibers contain a somewhat different expression of the SR, but with comparable structural characteristics. The most remarkable structural feature of the SR is the triad, formed by the apposition of two identical-looking sacs of the SR and an intervening intermediate element. In most muscles, triads are precisely located relative to the bands of the sarcomere (Fig. 4).

Not enough was known at the time to allow precise speculation on the function of the SR. However, two important points were made. First, the SR membrane allows its content to have a composition different from the cytoplasm that bathes the fibrils. Second, because of its continuity, the SR could be involved in the transmission of impulses within the muscle fiber. A first suggestion that this role might be played by a component of the triad came from the elegant serial-sectioning study of mouse skeletal muscle by Andersson-Cedergren (23). She established that the central elements of the triads form networks in a plane transverse to the fiber-long axis. These networks are called the transverse tubular system (transverse or T tubules being the individual segments of the network), and they are not part of the SR. Andersson-Cedergren recognized the T tubules as the obvious candidates for carrying excitation to the fiber's interior, and calculated that they should be able to conduct the impulse to the center of the fiber fast enough to account for known excitation-contraction (e-c) coupling delays. Further, she suggested that, as a result of the stimulus propagated along the T tubules, an activator substance is liberated from the adjacent SR and diffuses to the fibrils. A set of questions for further research was thus proposed.

E-C COUPLING: Much of the work done since 1955 on muscle membrane systems has been an effort to solve the problem of e-c coupling proposed by Hill (24). The entire cross section of the fiber is activated during a single twitch. The delay between depolarization of the surface membrane of a muscle fiber and its peak twitch tension is very short (a few to a few hundred milliseconds). Calculations showed that an activator diffusing from the surface membrane to the fiber's interior would take longer than the time available to reach a significant concentration at the center of the fiber. Thus, simple diffusion cannot account for the rapid activation of the fiber's entire cross section.

A. F. Huxley and co-workers set themselves the task of defining the link between excitation of the surface and contraction, in relation to the underlying structure (25). This work is a triumph of the concept of structure-function correlation. In these experiments (Fig. 5), a small patch of surface membrane was depolarized by an external electrode closely applied to the fiber surface. The tip of the pipette and the fiber's

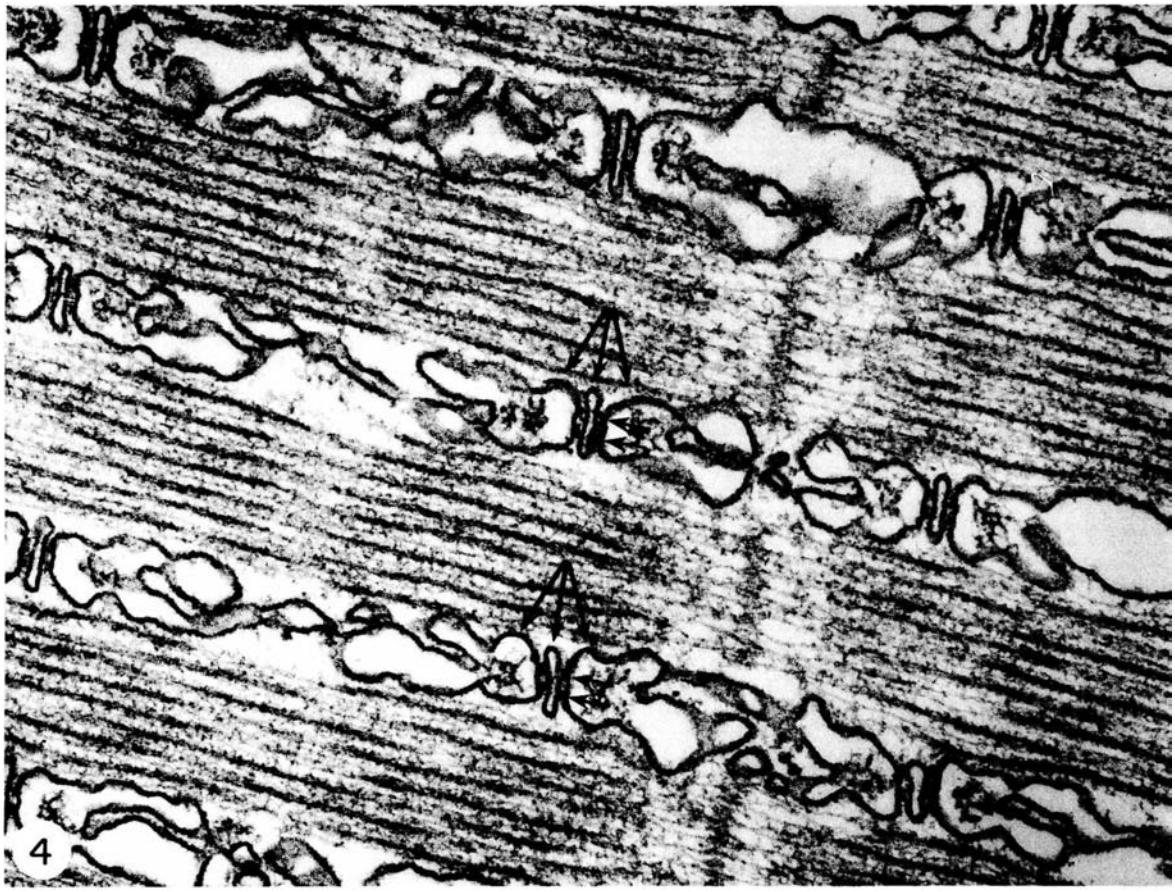


FIGURE 4 Longitudinal section of a fast-acting muscle from a fish. Numerous elements of the sarcoplasmic reticulum occupy the spaces between the fibrils. Triads (triple arrows) are located at regular intervals. Small arrows point to feet, joining SR to T tubules. The central element of the triad belongs to the transverse tubular (T) system. The precise disposition of membranes relative to the cross-striation suggested their role in the control of fiber activity to Porter and Palade (22). × 71,000.

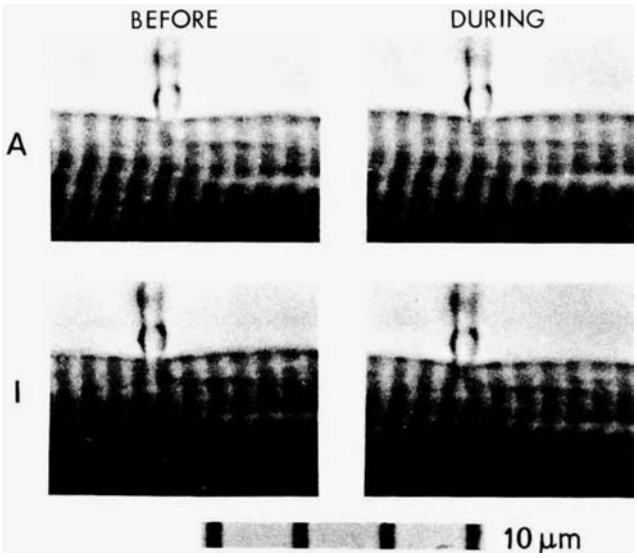


FIGURE 5 Local stimulation experiments in frog twitch fibers. The pipette is applied closely to the surface of the fiber (before, top and bottom) and current passed to it (during, top and bottom). When the pipette is opposite the A band, nothing happens (top). When it is centered over the I bands, a local, transversely spreading contraction of the two adjacent half-sarcomeres results from the depolarization (below, right). Inward spread of contraction coincides with the location of the transverse tubules. Reprinted from reference 26.

striations were visualized by interference or polarization light optics. In frog-twitch fibers, when the depolarized patch was at the level of the A band, no contraction was ever seen. On the other hand, when depolarization was at the level of the Z line, a small localized shortening of the two adjacent half-I bands was sometimes seen. Larger pulses produced a contraction that traveled further into the fiber in a transverse direction, i.e., in the plane of the Z lines. Muscle fibers from lizards and crabs produced a local, transversely spreading contraction when stimulated at the level of the A-I junction, and not at the Z line. Simultaneously with these experiments, electron microscopy showed that triads and T tubules are located at different levels of the sarcomere in different fibers: some fibers, as in the frog, have triads at the Z line (Fig. 6); some, as in the lizard, have triads at the A-I junction (Fig. 4), correlating with the location of sensitive spots. Crabs have dyads—structures that are similar to triads—near the A-I junction (see Fig. 21), again where sensitive spots are found (27). These results clearly suggest that some component of the triad and dyad must be involved in the inward spread of excitation.

Using single fibers, Hodgkin and Horowicz (28) established that, above a threshold value, a steep relationship exists between membrane potential and tension, and thus, presumably, between membrane potential and the release of an activator substance to the fibrils. These and the local stimulation experiments of A. F. Huxley and co-workers established the value of single fibers for observation of contraction by light-optical methods or measurement of mechanical performance not af-

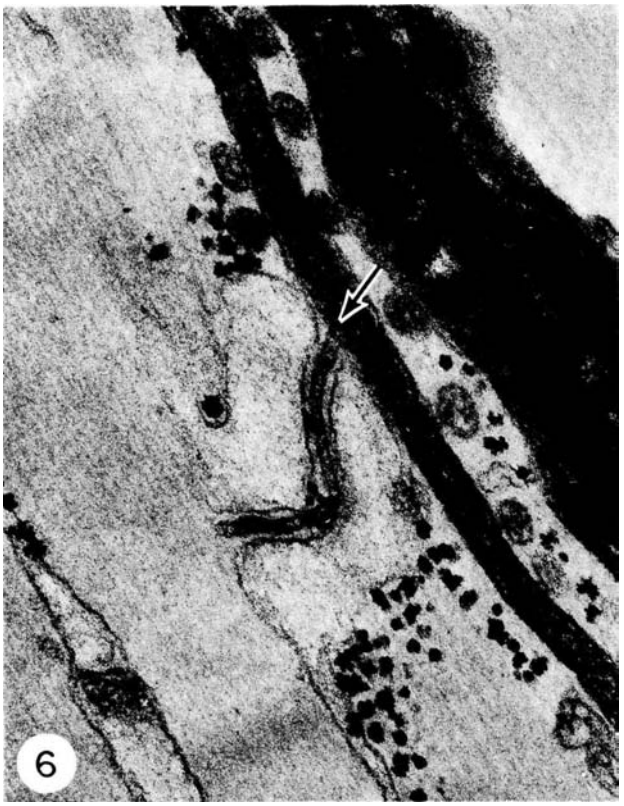


FIGURE 6 Longitudinal section at the periphery of a frog twitch fiber. Extracellular spaces and the transverse tubules are filled by an electron-dense tracer. By this means, continuity of extracellular spaces and lumen of T tubules is demonstrated. Opening of a T tubule (arrow), within the I-band region, coincides with the location of sensitive spots in local stimulation experiments. × 220,000.

fected by the geometry of the muscle and the connective tissue. They also established contraction as a sensitive indicator of the release of calcium to the fibrils and thus of the effect of a certain experimental procedure on e-c coupling parameters. Much of what is known about e-c coupling has been obtained in experiments of this type.

COMPARATIVE MORPHOLOGY: A fruitful area of morphological research was established in the early 1960s: this is the comparative study of muscle fibers from different sources and with different, but well-defined, functional properties. An initial collection of papers in this vein appeared in a special issue of *The Journal of Biophysical and Biochemical Cytology* (Volume 10, Supplement 4), which also contained at its beginning a translation of Veratti's work (11). Among the most interesting findings: Smith demonstrated the continuity between transverse tubules and the surface membrane in an insect muscle; Peachey established that some fibers of small size do not contain transverse tubules; Fawcett and Revel and Reger showed that fibers with a fast activity cycle contain an extraordinarily large amount of sarcoplasmic reticulum; Muscatello et al. isolated a microsomal fraction of SR origin and showed that it had ATPase activity.

From this initial comparative survey and from some early biochemical observations by Ebashi, involving calcium and the SR in the control of fiber activity, Porter (21) could conclude that the SR "plays an active role in the return of the fiber to the relaxed state." Many subsequent studies proved the truth of that statement.

Triumphant Confirmations

The next stage in the development of muscle research saw a number of significant contributions in morphology, biochemistry, and physiology. These provided confirmatory evidence for the theories of muscle contraction and its control, described in the previous section.

FILAMENTS: Native thin filaments and filaments formed by polymerization of actin in vitro were examined by Hanson and Lowy (29) (Fig. 7). The two types of filaments are apparently identical (but see section on Regulatory Proteins). Individual actin monomers are visible as small, approximately spherical structures, forming long chains with a subunit repeat of 5.5 nm. Two chains form a helical structure. The helical repeat varies somewhat with preparative procedures, showing that the two chains can unwind. In most preparations, the repeat is 36–37 nm (the helix is not integral).

Individual myosin molecules are easily damaged by negative staining, but they can be examined by shadow-casting techniques (31). They are very elongated and are made of two parts: a rod and a head region, with a total length of 150 nm (156 nm in the best recent estimate). The myosin head is globular and is approximately 5 × 10 nm (Fig. 8).

The best understanding of the structure of the thick filament and its relationship to the role of myosin in contraction came from examination of tryptic fragments (HMM and LMM) and of natural and reconstituted filaments and aggregates (31). HMM, which maintains the actin-binding and ATPase activity of the entire molecule, consists of the head and a short portion of the tail. LMM forms the rest of the tail and retains the self-aggregating characteristics of the entire molecule, forming paracrystals at low ionic strength. The length of LMM is estimated to be 96 nm.

The first model of a complete myosin filament was constructed bearing in mind LMM's self-assembly properties and the appearance of isolated myosin molecules (Fig. 9) (31). It was built by assuming that myosin molecules assemble in a tail-to-tail configuration in the center of the filament and then add on in a tail-to-head configuration in either side. The model fully accounts for structural and functional properties of the filament. (*a*) The central bare region, which is visible in thin sections of intact sarcomeres, does not bear bridges. (*b*) The two side regions bear bridges, and these are composed of the head portions of the myosin molecules that interact with actin. (*c*) More importantly, the model has a polarity that reverses in the center, because the myosin molecules in the halves of the filament point in two different directions. This satisfies a requirement of the sliding filament model: forces pulling the thin filaments of the two halves of a sarcomere toward the center must be in opposite directions on either side of the H zone.

Antibody-staining techniques add details to the basic model of the thick filament. For example, variations in the availability to staining of LMM sites with overlap of the filaments are interpreted as caused by changes in interfilament spacings at different sarcomere lengths. These produce a loosening of the LMM rod packing, as the bridges reach further from the thick filament shaft (33).

SLIDING-FILAMENT MODEL: The sliding-filament model requires the thin filaments to have a polarity that reverses at the Z line. The existence of this polarity was demonstrated by allowing actin and myosin to interact, in the absence of ATP, to form the so-called "decorated" thin filaments (31). When thin filaments are exposed to either intact

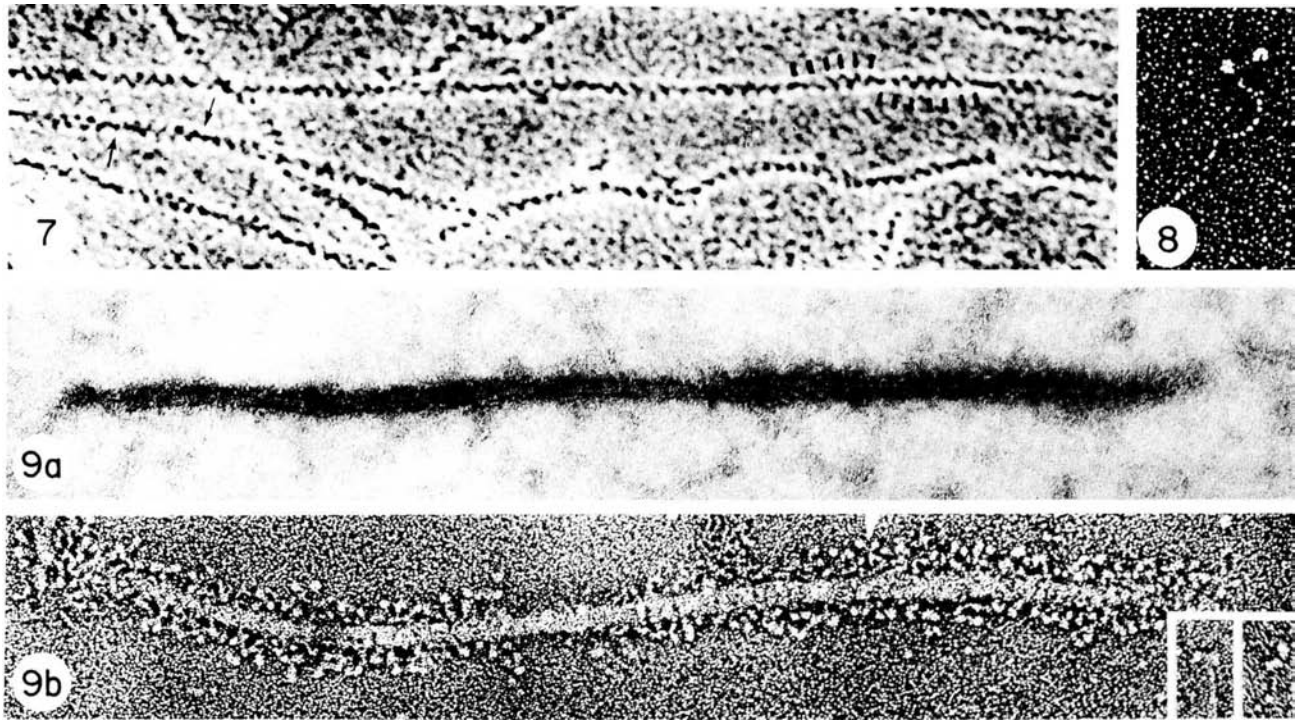


FIGURE 7 Negatively stained actin filaments (F-actin). The filaments are formed by two helically arranged strings of monomers. Individual G-actin molecules are visible (dashes). Reprinted from reference 30.

FIGURE 8 A single myosin molecule, rotary shadowed. The long tail and two heads are clearly visible. The first portion of the tail forms the LMM fragment, the rest of the molecule is the HMM, further divided into subfragments S1 (the heads) and S2, rods. Reprinted from reference 30.

FIGURE 9 (*a*) A thick myosin filament, negatively stained. The filament has a central, bare zone and two lateral regions, bearing bridges. H. E. Huxley (31) first suggested that bridges in the halves of the same filament point in opposite directions. Courtesy of F. Pepe. (*b*) In frozen-dried, rotary-shadowed filaments, individual myosin heads (S1 subfragments) that form the bridges are clearly visible. Single myosin molecules are shown in scale in the insert. Reprinted from reference 32.

myosin molecules or HMM, the entire actin filament is covered by myosin, forming an arrowhead configuration. When an entire I segment, consisting of a Z line and the two sets of thin filaments attached to it on either side, is decorated, the arrowhead tips point away from the Z line on both sides, thus revealing an inherent polarity in the thin filament array.

Since its first application in fibroblasts by Ishikawa, Bischoff, and Holtzer in 1968, the "decoration" technique has become widely used in all types of cells (34), and it is now one of the standard tools for identifying actin-containing filaments in nonmuscle cells.

The expectations of the sliding-filament model also were confirmed by physiological and X-ray diffraction experiments in living muscle fibers.

Isometric length-tension curve. If the force-generating elements are the individual bridges, then it is expected that the force of an isometric contraction would be proportional to the number of bridges capable of interacting with actin and, therefore, to the amount of overlap between thin filaments and the bridge-bearing regions of thick filaments. The isometric length-tension curve produced in 1940 by Ramsey and Street for single, isolated muscle fibers generally agreed with these predictions. One discrepancy in particular remained and was later clarified: A. F. Huxley and Peachey found that a small, residual, isometric tension produced by fibers stretched to the point where no overlap should exist arose from shorter sarcomeres, which still had some overlap near the myotendon junctions (2). The entire length-tension curve (Fig. 10) was obtained in a series of elegant experiments, in which the effects of the ends of the fibers were minimized by a complex apparatus that allowed only a central portion to be studied (35). The results were sufficiently precise that each segment and inflection point in the curve could be related to some feature of filament position in the sarcomere length (Figs. 10 and 11). The length-tension relationship established in these experiments was in astoundingly good agreement with expectations, and produced a strong argument in favor of the sliding filament model.

X-ray diffraction patterns of living muscle. The portion of the X-ray pattern containing meridional and offmeridonal reflections with an axial periodicity of 42.9 nm is dominated by the contribution of the cross-bridges (36). Early X-ray diffraction observations showed that these reflections are not altered during passive stretches. Improvements in X-ray diffraction cameras in the mid-1960s (36) allowed the first recording of diffraction patterns from stimulated muscles. It was found that axial periodicities of actin and myosin were virtually unchanged in isometric and isotonic contractions, i.e., no changes in the length of the filaments accompanies either the production of force or the shortening of the sarcomere (4, 37).

T TUBULES AND E-C COUPLING: T tubules are obvious candidates for the intracellular conduction of excitation, as demonstrated by the local activation experiments. However, this role would require the T tubules to have a functional, if not a direct, anatomical connection with the surface membrane

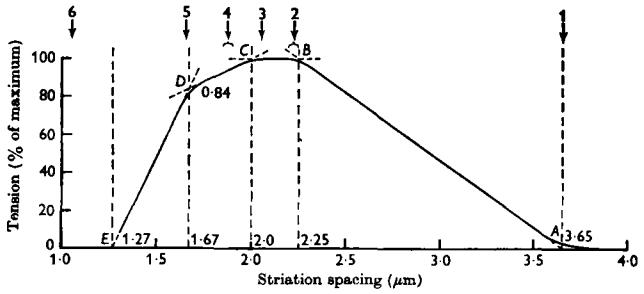


FIGURE 10 Summary of isometric length-tension results from single frog twitch fibers. The letters indicate critical relative positions between thin and thick filaments as the sarcomere length changes (see Fig. 11). Reprinted from reference 35.

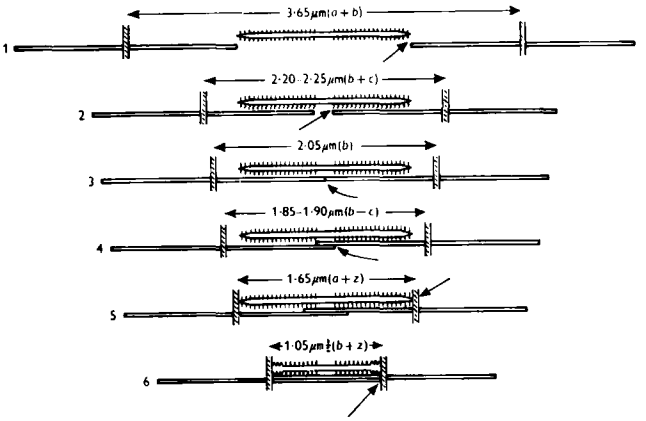


FIGURE 11 Schematic representation of critical stages in sarcomere length, at which infections in the length-tension curve are observed. Reprinted from reference 35.

of the fiber. A direct continuity could be demonstrated in insect muscles (38), but rarely in vertebrates. A new fixative, glutaraldehyde, finally allowed preservation of continuities between the T-tubule membrane and the plasma membrane in a fish muscle (39). In frog muscle, as a result of the tortuosity of the most peripheral segment of the tubules, openings are difficult to see. To date, even though numerous single openings have been seen (Fig. 6), the exact relationship between individual openings and the location of sensitive spots in local stimulation experiments is not clear.

Free exchange between extracellular fluids and the content of the T tubules was demonstrated directly in frog fibers by allowing the T tubules to be infiltrated by a fluorescent dye and by ferritin (40, 41). A number of different tracers have since been used in a variety of muscles (see Figs. 6 and 17–21), and the general conclusion is that molecules at least up to the size of ferritin diffuse into the T tubules. The SR is not directly infiltrated, and thus it is an intracellular compartment.

T tubules develop during muscle differentiation from initial small inpocketings of the plasmalemma (caveolae). These subsequently form long chains, penetrate into the fiber, and eventually form contacts with the SR (40).

T TUBULES AND ELECTRICAL PARAMETERS: Even before the real nature of the T tubules was established, specific models were proposed to explain the results of impedance measurements in muscle. Falk and Fatt (42) formulated the first equivalent circuit model for muscle that took into account the contribution of the transverse tubules. It was shown that a model in which T-tubule resistance and capacitance are in parallel to those of the plasmalemma could roughly account for the low frequency impedance data. The calculated surface area of transverse tubules in frog-twitch and slow fibers (43, 44) and in crustacean muscles (27) agrees with the capacitance data, assuming that the specific capacity of the muscle membrane is roughly the same as that of the nerve membrane.

SR AND RELAXATION: The function of SR in relaxation was defined in studies of isolated vesicles and in "skinned" fiber experiments. Once it was known (12) that the supernate of a muscle homogenate is composed of vesicles of SR origin (microsomal fraction, relaxing factor, muscle grana, vesicles), research started in earnest. Hasselbach and Makinose (reviewed in references 45 and 46) defined the properties of the calcium pump: the pump accumulates calcium into the SR against a concentration gradient, using ATP as a source of energy. The ATPase activity of the pump is stimulated by calcium and it requires magnesium. Weber and co-workers (47, 48) demonstrated that the relaxing effect of SR vesicles is uniquely attributable to their ability to reduce calcium concentration to below 10^{-7} M. At that calcium concentration fibrils are relaxed. One curious observation, which later acquired significance, was made in these studies: some actomyosin preparations could not be made to relax by lowering the calcium concentration. The explanation for this phenomenon is to be found in the Control Proteins section.

The presence of a calcium sink in the intact fiber was indirectly demonstrated by cleverly designed experiments using skinned fibers (49). More directly, when either skinned fibers or muscles fixed in glutaraldehyde were exposed to oxalate, calcium oxalate deposits were located within the SR (40, 50). Oxalate is currently used to locate calcium stores in other cell types.

COMPARATIVE MORPHOLOGY: A good correlation has been established between contractile activity of different muscle fibers and their content of SR and T tubules. The astounding variety of functional and morphological adaptations in the muscles of arthropods led G. Hoyle to suggest that these, rather than vertebrate muscles should be the primary target of modern research (51). Particularly beautiful is the disposition of SR and T tubules in fast-acting fibers of arthropods (reviewed in reference 40).

Myosin

In the words of Lowey (30), myosin is "an unusual protein; it cannot be classified as either a globular enzyme or a fibrous, structural protein. Rather it combines both classes of molecules in a functional, covalently linked unit."

A single myosin molecule has two heads connected to a common, double-stranded, α-helical, rodlike tail (Fig. 8). The two heads have equivalent ATPase activity (30).

Papain digestion separates the head regions, called subfragment 1, or S1, from the tail portion of HMM, called S2. S1 maintains the ATPase and actin-binding abilities of the entire molecule, and thus S1 must be located within the bridge, or, better, must form the bulk of the bridge. The tail portion of the HMM is thought to perform the function of allowing the S1 to reach actin filaments by swinging out from the thick filament shaft (52). The trypsin-sensitive region joining HMM to LMM is thought to act as a hinge. The ability of myosin to move out from the shaft of the filament is required by the constant volume behavior of the sarcomeres, which results in a variable separation between the peripheries of actin and myosins at different sarcomere lengths.

Under a variety of denaturing conditions, low molecular-weight subunits (light chains) separate from the myosin molecule. Myosin of "fast" muscle fibers contains three light chains. Two of the chains are related by a single identical thiol sequence and have been called alkali light chains (A1 and A2). Their removal affects myosin's ATPase activity. A third light chain has a different amino acid sequence; it can be removed without affecting ATPase, and has been called the dithionitrobenzene (DTNB) light chain (53). There are two DTNB light chains per mole of myosin and one per mole of S1. The molar ratio of A1 and A2 to myosin is not an integral number, but the ratio of the sum of the two is 2:1. Each S1 subfragment of myosin (i.e., each head) contains one DTNB light chain and either an A1 or an A2. A1 and A2 are the products of two related genes. Both isoenzymes are present in an individual muscle fiber, in its fibrils and filaments, and even, sometimes, within the same myosin molecule (51).

Myofibrils and Bridges

The basic model of thick filament structure proposed by H. E. Huxley in 1963 remains unchallenged in its main outline (reviewed in references 17, 33, and 54). Further details of the arrangement of the individual molecules to form the filaments are under close scrutiny. One question concerns the packing of the LMM subunits into the shaft of the thick filament. In one model (17, 33), LMMs are thought to lie parallel to the filament axis and to be staggered in a manner that brings HMM portions of the molecule out at appropriate intervals to form the bridges. The length of LMM (96 nm) determines the bridge period (14.3 nm). In an alternate model, LMMs are thought to be tilted around the long axis of the filament, rather than being parallel to it (54). Ultimately, a detailed model of the thick filaments should provide a useful description of the exact number and disposition of the force-generating portions of the molecule, i.e., the HMM bridges. As a first approximation (6), X-ray diffraction diagrams of frog muscles are interpreted as showing that the arrangement of myosin heads is a helix with a repeat of 43 nm and a translation of 14.3 nm between adjacent levels. Every 14.3 nm, two bridges arise on opposite sides of the filament: successive levels rotate by 60° around the filament axis. The structural regularity extends to a hexagonal superlattice, in which the nearest neighbors are not identical, but the next ones are.

Precise alignment of thick and thin filaments and accessory proteins in the sarcomere produce fine cross periodicities (Fig. 12). At the M line, in the center of the sarcomere, the thick filaments (in vertebrates) are bound to each other by cross-links, organized in three to five lines. The light (L) zone on either side of the M line is caused by the bridge-free regions of the thick filaments. In well-fixed material, the edges of the bridge-free regions, and those of the A bands are very sharp, indicating good alignement of bridges in adjacent filaments and precise uniformity in the length of the thick filaments. The disposition of bridges along the length of the filaments is not uniform: the absence of a single set of bridges near the ends forms a highly visible gap. The A band is crossed by lines with an approximate repeat of 43.0 nm. Eleven of these are most prominent and are probably results of the contribution of the bridges reinforced by the presence of accessory proteins, the C and H proteins (55). A 40-nm period in the I band is caused by the presence of control proteins in the thin filaments.

Excellent preservation of bridges and beautiful micrographs were obtained from asynchronous flight muscles of an insect.

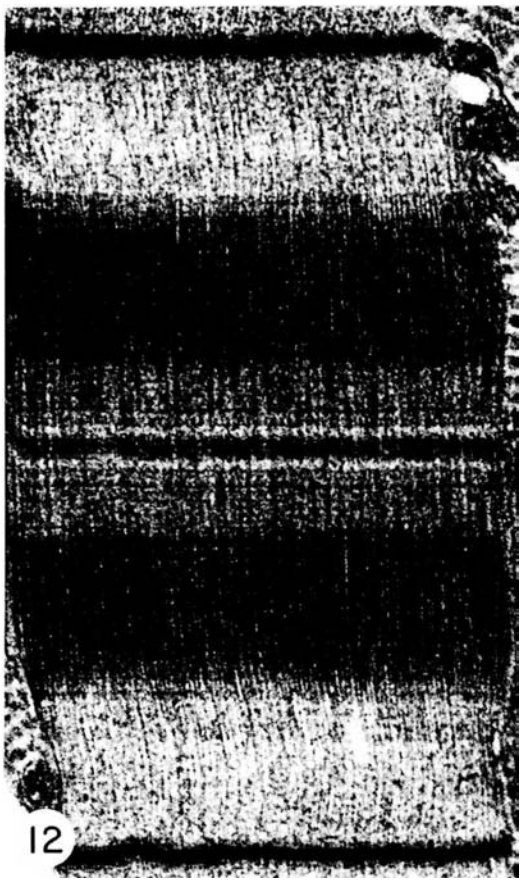


FIGURE 12 Longitudinal section of a sarcomere from the frog sartorius muscle, in which good alignment of fine periodicities is maintained. The I band's 40.0-nm period (caused by the presence of the tropomyosin-troponin system) is visible. The approximately 42.9-nm period in the A band is a result of the disposition of bridges and accessory proteins of the thick filaments. The bridge-free region of the thick filaments forms a lighter band on either side of the M line. The M line is composed, in this case, of three sublines.

In relaxed muscles, the pattern is dominated by the 14.3-nm repeat of the bridges arising from the thick filaments, and the helical arrangement can be determined quite accurately. The pattern from muscles put into rigor with low concentrations of ATP is dominated by the 2 × 38.5-nm repeat of the actin helix, which restrains the attachment sites of the bridges (56). Computer modeling gives the best fit with the data when the bridges are allowed considerable freedom, i.e., they have the flexibility of rotating around the thick filament by 30° and of moving up to 14.0 nm axially (57). In contracting muscles, the bridges may have a corresponding amount of freedom in the search for an actin attachment site. Such freedom of movement may be required by the known mismatch in the longitudinal periodicities of thick and thin filaments.

X-ray Diffraction

Striated muscle is admirably suited for studies by low-angle X-ray diffraction because most of the relevant periods are in the 10–40 nm range. Muscle is unique as a tissue, in that it can be examined usefully by X-ray diffraction in its living state both at rest and during activity.

X-ray diffraction studies have gone through several stages, encouraged by technical innovations and improvements toward

obtaining better results. Initially, the extremely long exposure times required reduced their application to the study of dead specimens. Improved cameras and sources allowed use of living muscle, but with the limitation that only steady-state situations, i.e., muscle in rigor or rest, could be used. Out of these observations came the first description of the hexagonal arrangement of thick filaments and of changes in intensity of equatorial reflections with the state of the muscle, later to be attributed to movement of the bridges relative to the thick filament. The constant volume behavior of sarcomeres with changes in length, i.e., the fact that interfilament distance is inversely proportional to the square root of sarcomere length is an important observation of x-ray diffraction, which must be kept in mind when considering bridge activity (36, 58).

In 1964–65, further improvements allowed the first experiments on contracting muscles. From work of this period came two very important confirmations of the sliding model: (*a*) the 14.3-nm bridge period does not change during contraction; and (*b*) arrangement of bridges changes in contracting muscles (4, 37).

Correlation with electron micrographs was used to assign meridional and off-meridional reflections to details of the architecture of thin and thick filaments. Direct evidence for the existence of bridges projecting from the surface of the thick filaments was found in X-ray diffraction of living muscle. Evidence of a high degree of order, rarely preserved when tissue is prepared for electron microscopy, was also demonstrated. Of great interest were comparisons between X-ray diffraction of intact muscle and light optical diffraction of electron micrographs, in the orderly muscles of an insect. These still represent the best evidence for a high order of arrangement of bridges and for changes in their positions at rest and in rigor.

In recent times, further improvements came with the introduction of electronic position-sensitive X-ray detectors in place of the far less sensitive photographic plates. With this technique, exposure times are greatly reduced, and appropriate synchronization allows time resolution of changes in intensity ratio of 1.0 and 1.1 equatorial reflections during the time-course of a contraction. Direct evidence for lateral movement of the bridges, as an event which precedes or coincides with onset of tension is thus obtained. A proportionality exists between the number of bridges that have shifted toward the actin filaments and tension produced (59).

Myosin, Actomyosin, ATP, and Bridge Cycles

Research on the biochemistry of the interaction of myosin (as an ATPase), ATP, and actin has reached very sophisticated levels. Interest in this research goes beyond muscle contractility. In most cells there are proteins which have intrinsic ATPase activity and a mechanochemical coupling role, e.g., dynein in cilia and all types of myosin in nonmuscle cells. Current interpretation of their function relies heavily on actomyosin models.

ATP has a dual effect on actomyosin. (*a*) It reduces affinity between the two molecules, thus allowing dissociation of actin from myosin. This step is considered equivalent to the detachment of bridges from actin and thus to the change between rigor and relaxation (60, 61). The physiological substrate for actomyosin ATPase in MgATP, which is present in the intact muscle fiber in the millimolar range. Binding of MgATP to actomyosin and dissociation of actomyosin both are very rapid steps (62). (*b*) ATP is the substrate for hydrolysis by myosin ATPase. The reaction involves several intermediate steps. The scheme outlined below was initially proposed by Lymn and Taylor (62) and has been confirmed and subsequently refined by the work of Trentham and co-workers (reviewed in references 60 and 63–66). Step 4 is rate-controlling (63):

$$M + ATP \overset{1}{\rightleftharpoons} M\cdot ATP \overset{2}{\rightleftharpoons} M^*ATP \overset{3}{\rightleftharpoons} M^{**}ADP\cdot P_i$$

$$\overset{4}{\rightleftharpoons} M^*ADP\cdot P_i \overset{5}{\rightleftharpoons} M^*ADP + P_i \overset{6}{\rightleftharpoons} M\cdot ADP \overset{7}{\rightleftharpoons} M + ADP.$$

Actomyosin ATPase has a much higher steady-state rate of hydrolysis than does ATPase in pure myosin. Hydrolysis of ATP by actomyosin is considered to be a simple extension of the myosin-ATPase cycle, as follows:

$$AM + ATP \overset{1}{\rightleftharpoons} AM\cdot ATP \overset{2}{\rightleftharpoons} A + M^*ATP \overset{3}{\rightleftharpoons}$$

$$A + M^{**}ADP\cdot P_i \overset{4}{\rightleftharpoons} AM\cdot ADP\cdot P_i \overset{5}{\rightleftharpoons} AM + ADP + P_i.$$

The rate-limiting step is bypassed, allowing for a much more rapid turnover. This scheme includes the dissociating effect of ATP on actomyosin, ATP hydrolysis, and the cyclic association and dissociation of the two proteins.

Interest in this research stems from the assumption that the biochemical steps are related to the cycle of activity of the bridges (Fig. 13). Fibrils deprived of ATP are in rigor, i.e., the bridges are all attached and the fibril is inextensible, but it does not produce active tension. After ATP is added, fibrils initially became extensible, i.e., they are in a relaxed state because the bridges are detached. Hydrolysis of ATP follows immediately. The bridges then bind to actin (step 4 in the kinetic scheme above) and release the reaction products and energy (producing either tension or movement). In the presence of ATP, detach-

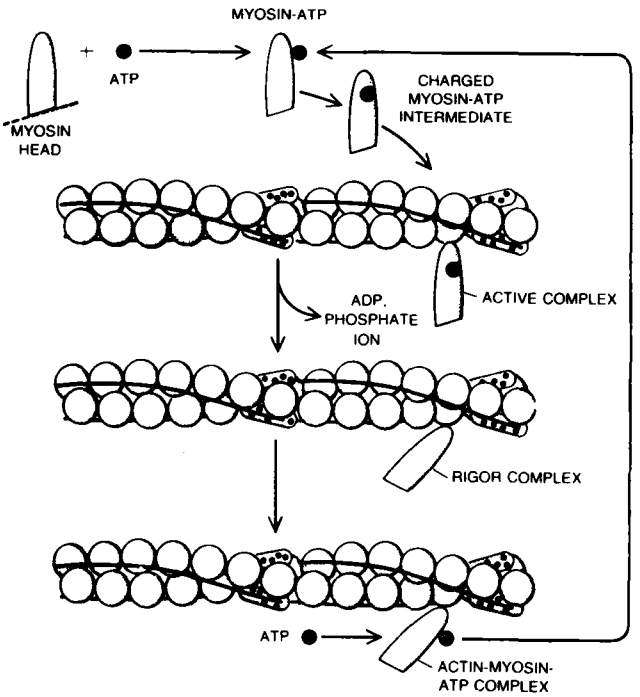


FIGURE 13 A schematic representation of the relationship between ATP splitting and the bridge-activity cycle. At top, the myosin head, in the presence of ATP, forms a charged intermediate and then binds to the actin filament (active complex). After release of the reaction products (middle), movement occurs, and the bridge is then a rigor complex until (bottom) it again binds ATP and releases actin. Reprinted from reference 67.

ment and another cycle quickly follow. In the absence of ATP, the bridges remain in rigor. The position of a bridge in the absence of ATP is thus considered equivalent to the position assumed by the bridge after having released its energy. Under physiological conditions, ATP is present in millimolar concentrations, and thus the bridges are continuously cycling as long as the calcium concentration is sufficient to remove the control proteins' inhibition of the activity.

Final understanding of mechanochemical transduction by the bridge requires information on the actual shape changes of the myosin molecule, which allow it to produce tension and/or movement. Interestingly, the most detailed theory available to date is derived from studies of the mechanical, rather than the biochemical, performance of the bridges. Precise experiments were designed to measure the transient force response of an isometrically contracting muscle, which is subjected to a very rapid, small change in length. The basic setup is the same as that used for the exact measurement of length-tension relationship (2, 68, 69). The response to a length decrease consists of four phases: (*1*) an initial, instantaneous tension drop; (*2*) rapid recovery to almost, but not quite, the initial tension; (*3*) a slower, plateau, or slight decrease in tension; (*4*) a slow redevelopment of tension to final values. Phase 1 is consistent with the existence of an undamped-series elastic element within the bridge, possibly in S2. The rapid recovery of phase 2 may be caused by an internal rearrangement in the position of S1 relative to actin. This is possible if the bridge can rock among three different positions (possibly attachment angles) that reflect different states of the myosin molecule. Phases 3 and 4 are explained as results of detachment of some bridges, those that have rocked to their farthest position, and their subsequent reattachment. In an overall scheme, the bridge (HMM) may comprise a hinge region at its junction to LMM (52), which allows it to acquire different positions relative to the thick filament shaft, an elastic segment (S2), and a head (S1), which, in turn, can take different angles relative to S2 and the actin filaments (68).

Regulatory Proteins

Extensively purified preparations of actin and myosin are fully turned on (hydrolyze ATP at a fast rate), as long as MgATP is present in sufficient concentrations. "Natural" actomyosin and intact myofibrils, on the other hand, have an ATPase activity that is regulated by the calcium concentration in the medium. At calcium concentrations below approximately 10^{-7}, natural actomyosin's ATPase is inhibited (turned off), and at higher calcium concentrations, it is turned on. The difference is caused by the presence of additional proteins called regulatory proteins. The regulation by "natural tropomyosin" of the interaction of actin and myosin was first described by Ebashi (70). On further analysis, this "natural tropomyosin of Bailey," was found to have two major components: tropomyosin (TM) and troponin (TN) (71, 72). TM mediates association of TN with actin.

TN is a globular protein with the highest affinity for calcium in the contractile system, and is composed of several fractions, with different properties. In time, three components were recognized (73), and they now have specific roles attached to each. Following Greaser and Gergely's nomenclature, these are TN-T, TN-I, and TN-C (74). TN-T is responsible for attaching the TN group to TM. TN-I inhibits actomyosin ATPase, in concert with TM and the other TN subunits. TN-C is the calcium-binding subunit (64). Occupation of all Ca-binding sites of TN-C results in a release of TN-I inhibition of the actomyosin ATPase and thus in activation of the contractile material (Fig. 14) (60).

Tropomyosin is a rod-shaped molecule, 40 nm long (75). Three-dimensional reconstruction of the density profile of thin filaments and thin filaments containing tropomyosin (76), indicate that tropomyosin is located in the grooves of the actin helix. A pair of tropomyosin molecules cover the length of seven pairs of actin monomers (Fig. 15). Troponin binds at a specific site along the tropomyosin molecule. In the intact fibril, this produces a "40-nm period," distinctly different from the period of the actin helix (58, 77). Because there is one mole of troponin for one of tropomyosin, two troponin complexes are attached at 400-Å intervals along the thin filaments, one for each tropomyosin (Fig. 15).

Three lines of experimental evidence have been consolidated into a scheme for the mechanism of interaction between contractile and regulatory proteins. (*a*) Optical reconstructions of decorated thin filaments indicate that S1 penetrates fairly deeply into the groove of the actin helix (78). Thus, the binding site between actin and myosin is thought to be located within the groove, close to the position occupied by the string of tropomyosin molecules. (*b*) Intensity changes in x-ray-diffraction reflections attributed to the thin filaments may indicate a movement of the tropomyosin nearer to the center of the groove in conditions where myosin bridges and actin filaments are interacting (79). (*c*) An apparent cooperative effect between actin monomers is revealed in the presence of "rigor complexes," i.e., in the presence of S1 bound to actin at low ATP concentrations (80, reviewed in reference 67). A rigor complex is able to override the inhibitory action of Ca-free troponin, possibly by obliging TM to move deeper into the actin groove and to reveal myosin-binding sites on the actin monomers. By this mechanism, the effect of rigor complexes and of Ca binding to troponin are both amplified by tropomyosin, to cover a range of 400 Å, or seven actin monomers.

A second mechanism of regulation exists in some invertebrate muscles: calcium acts directly on the myosin molecule to regulate its rate of ATP splitting (81). Finally, some muscles possess both myosin-based and tropomyosin-troponin-based regulation.

Electrical Parameters

Electrical parameters of muscle have been most extensively investigated for frog-twitch fibers (reviewed in references 50, 82–84).

A variety of equivalent circuits have been studied as representations of the contribution of the surface and transverse tubular membranes to the overall electrical properties of the muscle fiber. Initially, based on AC impedance analysis, the contribution of the T system was thought to be well represented by a single capacitance in series with a resistance (lumped model [42]). Later papers have demonstrated a tendency toward a more realistic representation of the T system as capacitance and resistance spread along the radius of the fiber (distributed model), or as a distributed system with an extra resistance ("access" resistance) in series with the tubules near the surface of the fiber (reviewed in references 82, 84, and 85). In general, there is agreement that the capacities per unit area of transverse tubules and surface membrane are the same and slightly less than 1 $\mu F/cm^2$.

More directly related to muscle function have been attempts to relate electrical potential changes in the T tubular membrane

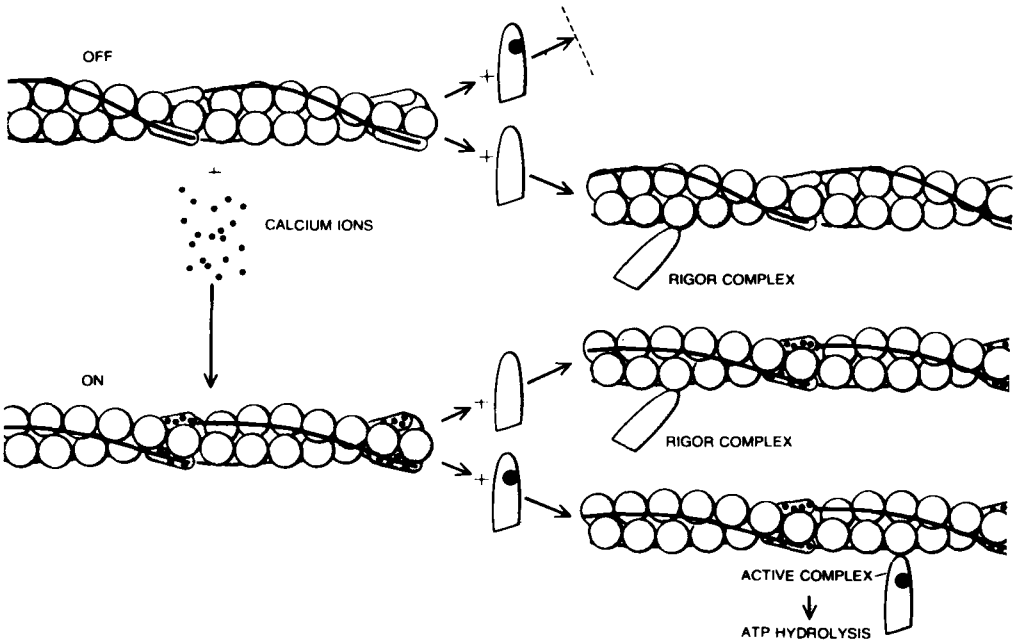


FIGURE 14 Both calcium ions and ATP are needed to turn on the complete contractile system, which is composed by myosin and actin-tropomyosin-troponin filaments. Myosin-ADP complex is indicated by a black dot on the bridge. In the absence of calcium ions, top, the bridge is either in the relaxed or rigor state in the presence or absence of ATP, respectively. In the presence of calcium, the bridge is in an active state in the presence of ATP (see Fig. 13), and in rigor in its absence. Reprinted from reference 67.

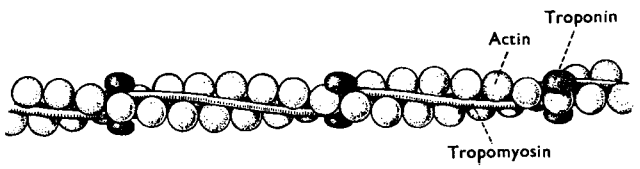


FIGURE 15 In a native actin filament, tropomyosin molecules occupy both grooves of the actin helix, providing a scaffolding for the periodic attachment of troponin molecules. Reprinted from reference 72.

to the activation of contraction. Adrian et al. (86) determined the decrement in potential along the tubules and, from this, a length and time constant for the network. These experiments were done on fibers in tetrodotoxin-containing solutions, to inhibit action potentials, and, therefore, dealt with a passive T tubule.

Subsequently, Costantin (87) showed that, in the absence of tetrodotoxin, depolarization sometimes resulted in the contraction of centrally located fibrils and not of the peripheral ones. From these experiments and from earlier measurements of the speed of inward spread of excitation by Gonzales-Serratos (reviewed in reference 80), it was concluded that, in frog-twitch fibers, an action potential is the normal mode in which the T system conducts depolarization into the fiber. A series of cleverly designed experiments followed, confirming the existence of an action potential in the transverse tubules of frog-twitch fibers (reviewed in reference 81).

Peachey and Adrian (84) computed action potentials spreading along the fiber surface and into the T network, using a Hodgkin-Huxley scheme for the active changes in membrane conductance. A visible hump at the beginning of the after potential in the computed action potential is a result of activity in the T tubules.

Excitation-Contraction Coupling

The term, as initially defined by Sandow (88, reviewed in references 46, 50, 89, and 90), indicates the steps between excitation of the surface membrane and contraction of the myofibrils. Similar concepts are now extended to coupling, in other cell types, between events at the surface membrane and intracellular events (i.e., excitation-secretion coupling at nerve endings and in secretory cells in general). In muscle, it includes several steps, only one of which still is totally unclear in its mechanism. Successive steps are as follows (see 49).

1. SPREAD OF DEPOLARIZATION ALONG THE SURFACE: In twitch-muscle fibers, a sufficiently large excitatory postsynaptic potential generated at one or two large (*en plaque*) end plates initiates a self-regenerating action potential. The action potential spreads without decrement along the muscle fiber, essentially as in an axon. The mechanical response to a normal action potential is an all-or-none twitch. Experimentally, the magnitude and time-course of muscle fiber depolarization can be varied and, under these conditions, a twitch fiber produces a mechanical response of variable magnitude and duration. From this, one concludes that the intact twitch fiber acts in all-or-none fashion because the action potential is all-or-none, but that later events in e-c coupling can be graded.

2. THE ROLE OF T TUBULES: Where present, T tubules are obligatory intermediates between external surface membrane depolarization and the subsequent steps in e-c coupling. This is most directly demonstrated by the local stimulation experiments and by glycerol treatment. The latter is a procedure that either disrupts the T tubules or interrupts their continuity (reviewed in references 40 and 83). In glycerol-treated twitch-muscle fibers, even though excitation of the surface membrane is unaltered, no contraction follows, because spread of depolarization along the T tubule network is impeded.

3. CHARGE MOVEMENTS: Schneider and Chandler (91, reviewed in reference 92) have explored the electrical properties of muscle fibers exposed to solutions designed to eliminate all voltage-dependent permeability changes, and depolarized to a

potential within the contraction threshold range. Under these conditions, a depolarizing step produces an extra, small outward current above that measured with a similar step performed at membrane potentials near the resting level. The current is most likely to be capacitative, i.e., caused by the movement of charge within the membrane's dielectric. This study is significant for two reasons. *(a)* It is the first measurement of a nonlinear capacitative (gating) current. *(b)* Because the measured charge movement occurs in the same membrane potential range as e-c coupling, it is speculated that it is a necessary step in the coupling (91).

4. T TO SR COUPLING: It is calculated that the amount of calcium needed for activation is far more than that entering the fibers during an action potential (50). The SR is an obvious source for this calcium. At triads, dyads, and peripheral couplings, a small gap separates SR and T tubules (or surface membrane). It is generally accepted that transmission from surface membrane or T tubules to SR occurs at those junctions, although the mechanism of this transmission is unknown.

Two current hypotheses of transmission at the triad take into account most of the data currently available. One (91) assumes that intramembranous charge movement within the T tubule's membrane facing the SR is the initiating step, and that its effect is directly transmitted to the SR membrane by links located in the junctional feet joining the apposed membranes. The effect of the transmission would be to open channels through which Ca can exit the SR, following its chemical gradient.

A second hypothesis (93) proposes that a small ionic current flows between the lumina of T tubules and SR during excitation. This is a result of the temporary opening of channels across the T-tubule membrane, which is strongly dependent on voltage. A regenerative change in SR's permeability to calcium and Ca release follows.

5. MECHANISM OF CA RELEASE: Even though little is directly known about transmission at the triad, a good deal can be deduced by observing the results of the coupling, i.e., the release of calcium. Autoradiographic studies of calcium distribution reveal the presence of two calcium pools in the SR: an uptake and a storage pool. The latter, located in the triad, may coincide with the release sites (94). Direct evidence for calcium release from the SR was obtained following recent refinements in the technique of electron microprobe analysis of frozen sections (95). Interestingly, the measured amount of calcium release exceeds that needed to saturate fully troponin.

Ca-sensitive probes are employed to detect appearance of calcium in the sarcoplasm. Murexide was the first to be used, whereas the bioluminescent protein, aequorin, and others were subsequently introduced (96). By use of calcium indicators, it was determined that a steep relationship exists between calcium transients and the intramembranous charge movements (97). After its initial use in muscle, aequorin has been effectively used in other cell types, to detect changes in intracellular calcium concentration at critical physiological phases of the cell's life (e.g., during activation in eggs).

In the skinned muscle fiber preparation, the surface membrane is removed (mechanically in the early preparations, more recently "chemically,") and the SR is accessible to solutions introduced in the medium. The preparation is particularly useful in probing for calcium release from the SR, because it has built-in fast, sensitive, and reliable calcium sensors: the myofibrils (reviewed in references 50 and 98). When exposed to Ca-EGTA solutions buffered to calcium concentrations below the contraction threshold, the SR of a skinned fiber will load calcium to a concentration 3–4 times the one in vivo. Under appropriate conditions, such loaded fiber will produce repetitive twitch-like contractions. These, it is thought, are caused by calcium cycling in and out of the SR, not unlike that occurring in the contraction-relaxation cycle of an intact fiber. On close scrutiny, however, it was found that this "calcium activated calcium release" does not occur under strictly physiological ionic conditions (reviewed in reference 98). In addition, it was shown by Armstrong, Bezanilla, and Horowicz (reviewed in reference 50) that muscle fibers are capable of normal twitches when exposed to a calcium-free solution, as long as the resting potential is not affected. Thus calcium entering the fiber during excitation is not the stimulus normally initiating calcium release from the SR.

A second stimulus capable of producing a rapid release of calcium from SR of skinned fibers is a sudden change in ionic composition of the medium (e.g., a change from the impermeant sulfate to the permeant chloride ion), which presumably produces a change in SR membrane potential (reviewed in reference 50). There is evidence that a change in SR membrane potential accompanies (but does not necessarily precede) calcium release. Results were obtained from the study of optical signals. These can be used to measure changes in electrical potential across membranes not accessible to microelectrodes. The technique is currently used in a variety of cell types. Baylor and Oetliker (99) have measured birefringence changes from muscle fibers, and Bezanilla and Horowicz (100) have measured a fluorescence change in fibers treated with Nile Blue A, a penetrating lipid-soluble dye. In both cases, in addition to signals assigned to surface and T-tubule membranes, a large component of probable SR origin was seen.

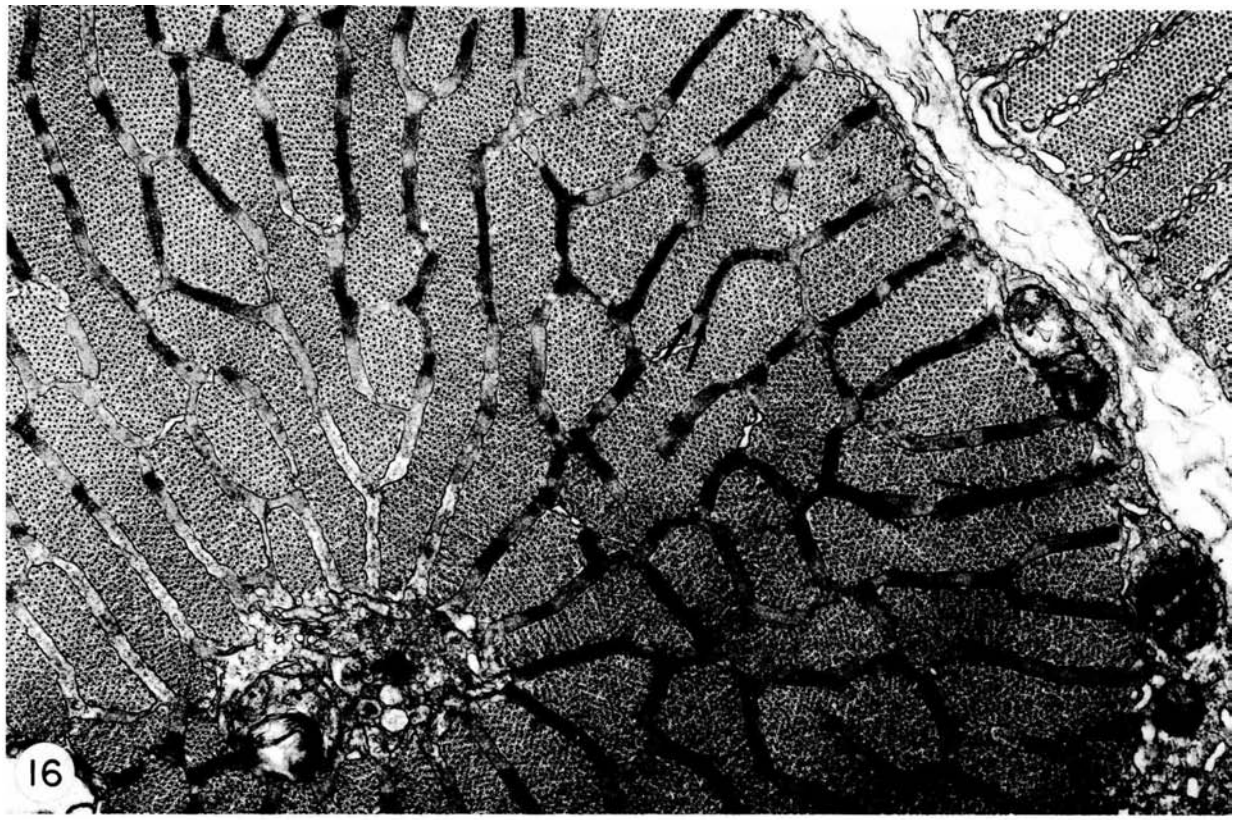
T Tubules and SR

GENERAL DISPOSITION: Different portions of SR and T tubules have special structural and functional characteristics, the former being detectable either in thin sections or in freeze-fracture replicas. In addition, both systems vary a great deal in size and distribution in different fiber types. Individual variabilities have been extensively described and reviewed in the past (40, 41, 51, 101–109). In general, a good correlation exists between content of SR and T tubules and the speed of a muscle fiber's activity cycle (Figs. 16 and 17). The relationship is quite complex, because there are several morphological parameters to be considered: amount and distribution of T tubules; number and disposition of dyads; triads, and peripheral junctions; amount of calcium-pumping SR and its content of Ca pump and Ca-binding proteins. Additionally, factors other than the membranes affect the overall speed of a muscle fiber.

High-voltage electron microscopy of thick slices of muscle fibers, in which either SR or T tubules, or both, are selectively "stained" by electron-dense substances has recently contributed the most complete views of the two membrane systems (Fig. 18).

T TUBULES: There are two basic components of the T tubules' network: (*a*) narrow-diameter tubules with a round cross section, which do not participate in junctions with the SR; (*b*) flat tubules and cisternae, which form junctions with the SR. The difference between the two is most obvious after glutaraldehyde fixation, and it is emphasized by infiltration of the network with electron dense tracers.

In vertebrate-twitch fibers (Figs. 18–20), the transverse net-



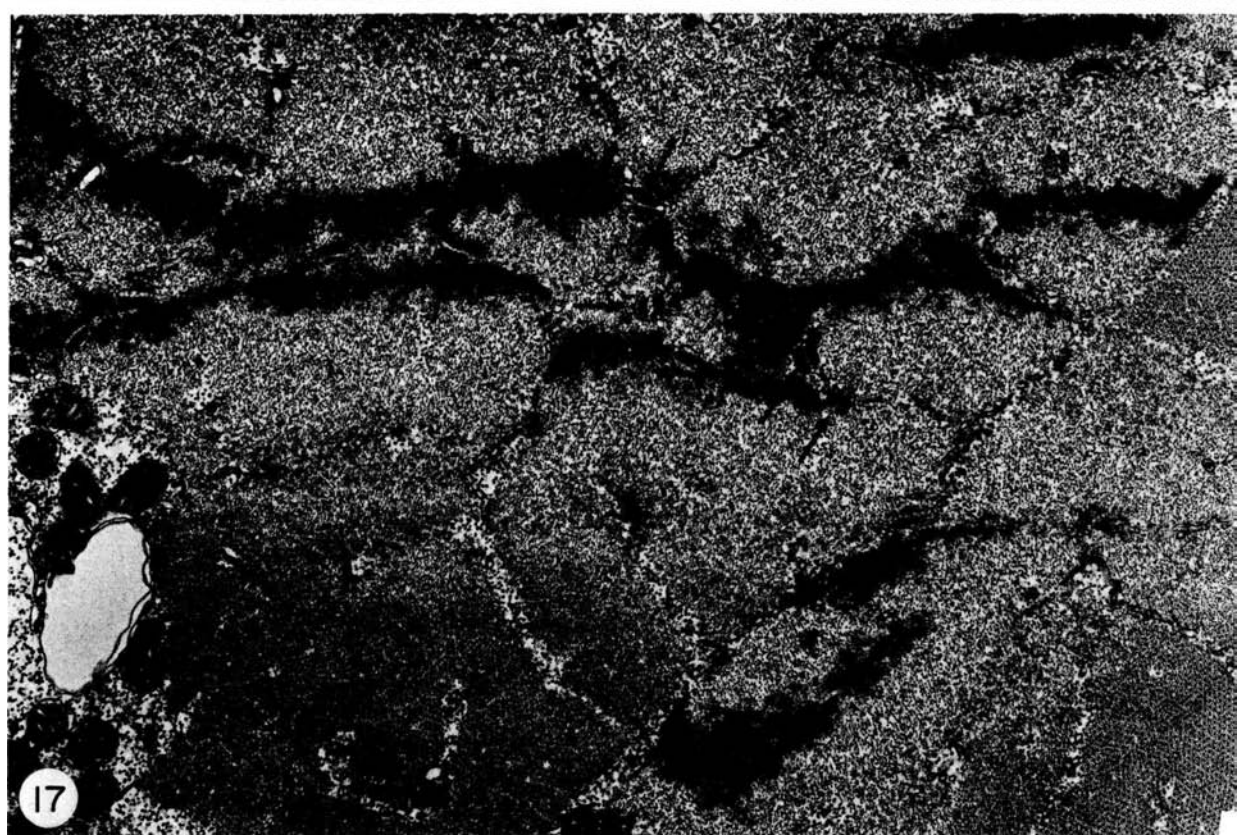


FIGURE 16 Cross-section through a fast-acting muscle from a fish. Fibrils are small and completely separated by SR and T tubules. Most of the section is at the level of triads, and the rows of junctional feet joining SR to T tubules are visible (arrows). × 18,000.

FIGURE 17 Cross-section of a slow, tonic fiber from the frog. Fibrils are large and often not entirely separated from each other a the A-band level. Triads (arrows) are fairly numerous, but they do not cover as much of the T network as in twitch fibers (se above). They also have a different orientation. × 23,000.

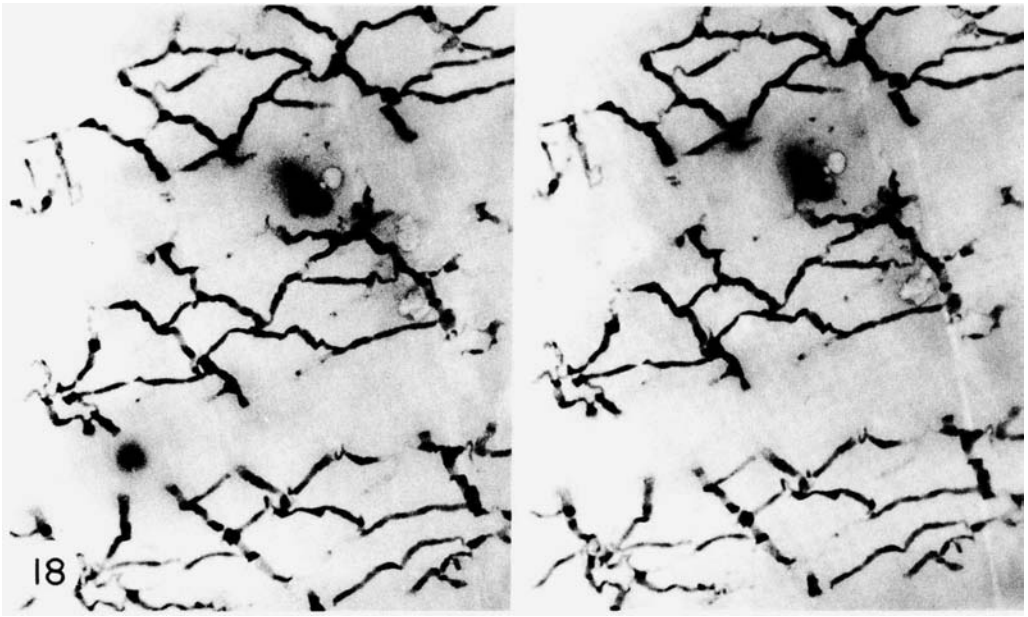


FIGURE 18 Stereo view from a thick (about 3 μm) slice of a frog twitch fiber. T tubules are infiltrated by lanthanum. Several rings of the T tubule network are visible at each Z line level. The fibrils run through the holes in the net. Note correspondence in the shape of the holes at different levels. Most of the network is composed of flat, ribbonlike T tubules, forming triads. Smaller, round tubules are nonjunctional. × 15,000.

works of tubules that penetrate across the fibers at periodic intervals are composed of alternate sections of flat tubules and round tubules. The fraction of round tubules within the transverse network varies among different fiber types. Longitudinal extensions of the network exist in all types of fibers. Network parameters have been quite precisely worked out in the case of frog twitch fibers.

In slow fibers of vertebrates and in most fibers from invertebrates, the T-tubule networks are less precisely arranged transversely, and in some cases run as frequently in the longitudinal as in the transverse direction. The networks are composed of alternate round tubules and flat, pancakelike cisternae (Fig. 21). The latter forms dyads and triads with the SR.

High-voltage electron microscopy has revealed a new feature of the fibers examined; an area of the fiber was found where the network is not transverse, but in the form of a helicoid, with a pitch equal to the sarcomere length (110). Helicoids also have been seen in this way in rat twitch fibers. At the center of each helicoid is a dislocation in the cross-striation, and in this region longitudinally oriented T tubules are most numerous. Even though the presence of helicoids does not alter the basic concepts of e-c coupling described above, it results in a rather more complicated T network than thus far envisaged.

SR: In twitch fibers from higher vertebrates, the SR is segmented by the penetration of the T-tubule network across the fiber. Generally, the SR is composed of distinct regions (Fig. 4): (*a*) the lateral sacs of the triad on either side of the T tubules; (*b*) intermediate cisternae and longitudinal tubules, which join the lateral sacs of the triad to (*c*) a fenestrated collar adjacent to the middle of the A band. In fibers with two triads per sarcomere, a fenestrated collar is situated opposite the I bands.

In slow and cardiac muscle fibers from vertebrates and in skeletal and cardiac muscle of invertebrates (Fig. 21), the SR is more continuous because junctions with the T tubules are in a longitudinal or oblique, rather than transverse, plane (dyads and triads). The fenestrated portion of the SR is more prominent and the longitudinal elements less so. Flattened regions of the SR participate in the formation of dyads. Other components of the SR form flat cisternae, which form junctions (peripheral couplings) with the surface membrane. Peripheral couplings are likely to be functionally equivalent to dyads and triads.

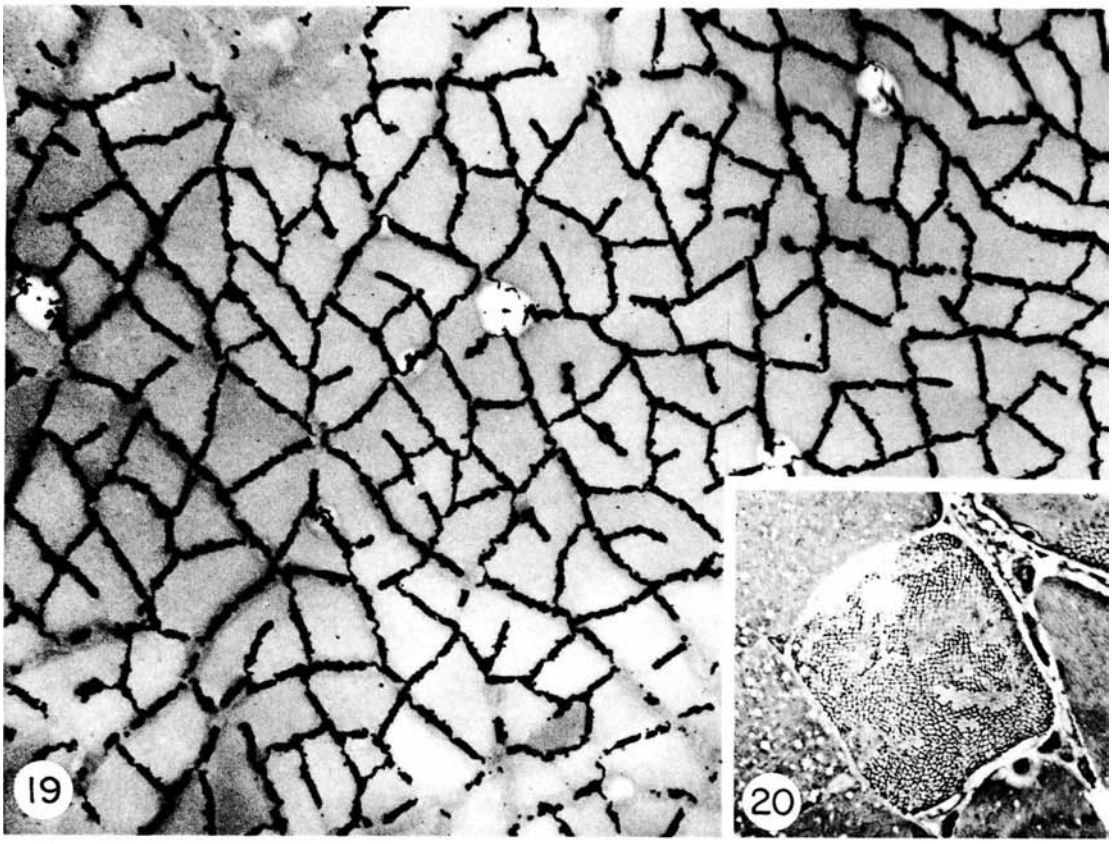
The amount of SR and T tubules has been estimated for a number of fibers (43, 44, 102). As an example, in a 100-μm diameter frog twitch fiber, the T-network surface area is approximately seven times the external surface area, and the total SR surface area is almost 20 times larger than that of the T tubules.

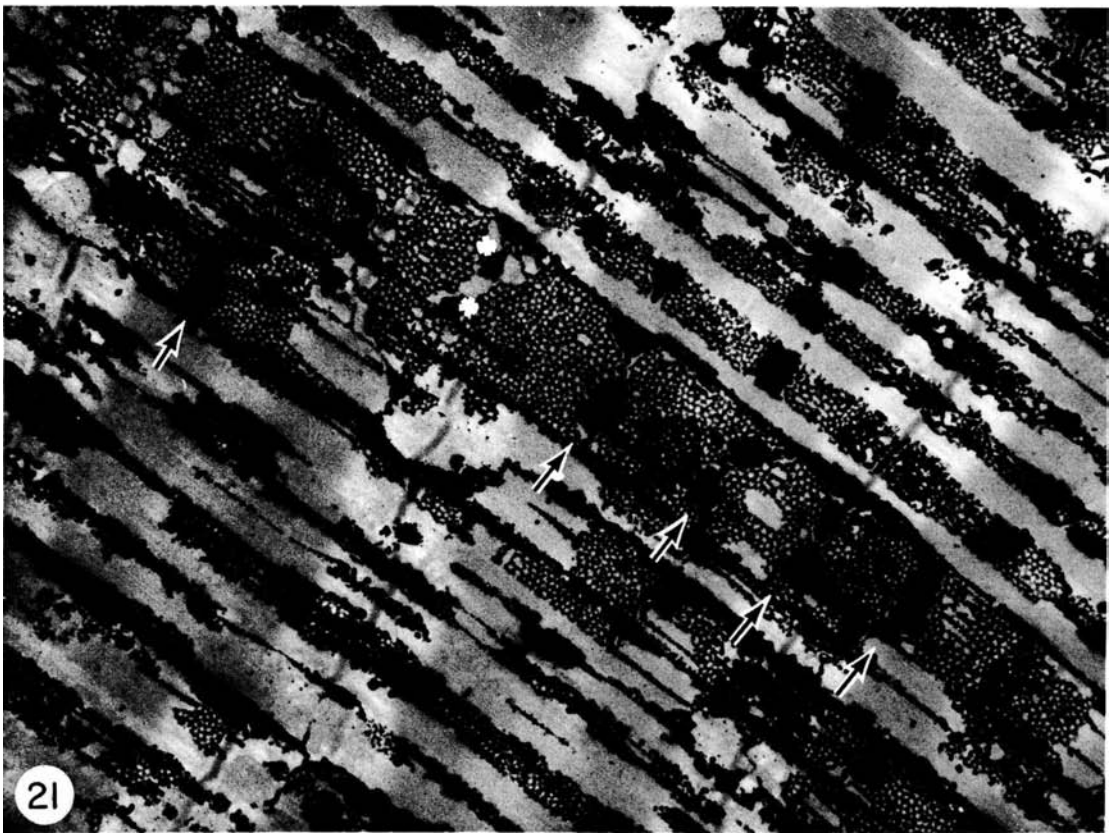
COMPOSITION: Purified microsomal fractions from muscle homogenate contain vesicles of SR origin and a small number of proteins. The most abundant are: a membrane protein, the calcium-activated ATPase, and an internal calcium-binding protein, calsequestrin (reviewed in reference 111). In smaller amounts are acidic proteins with high affinity for calcium (contained within the lumen), two intrinsic proteins of 30,000 and 34,000 mol wt, and a third small (M55) protein.

Vesicles with a dense content are identified as originating from the lateral sacs of the triad, and they contain the calsequestrin (Fig. 22).

The light SR fraction is identified with the longitudinal elements of the SR. It has an extremely simple protein composition. More than 90% of the protein is the calcium pump ATPase. M55 protein is also present. Because of this very pure composition, the light SR fraction is ideal for basic studies on the structure and interactions of functional intrinsic proteins with their surrounding lipids.

MEMBRANE ARCHITECTURE: It is postulated that the SR membrane facing directly toward the T tubules (junctional SR, or jSR) must be the sensor that detects the level of T-tubule membrane depolarization (112). The remainder of the SR membrane, facing toward the fibrils and called free SR (fSR), does not participate in the formation of any junction with other membrane systems. In twitch fibers of the frog, there is approximately 10 times more free than junctional SR. By





FIGURES 19 AND 20 Cross-section of rat twitch fibers, stained by the black reaction of Golgi, seen by light and electron microscopy. Both images show the completeness of the T tubule. In Fig. 19, wider and thinner sections of T tubules correspond to junctional and nonjunctional regions of the network, respectively. Fig. 19, × 11,000. Fig. 20, × 340.

FIGURE 21 In this muscle fiber from a crab, both T tubules and SR have been "stained" by the black reaction of Golgi. The T tubules (arrows) are mostly located over the A bands and consist of alternated thin-diameter tubules, and flat, junctional cisternae. The SR forms a longitudinally continuous fenestrated network, with intercalated flat cisternae (white asterisk). SR and T tubule cisternae are closely apposed to form dyads and triads (not seen in this illustration). × 11,000.

contrast, amounts of fSR and jSR are about equal in insect fibrillar flight muscles.

Structural differences between jSR and fSR membranes are visible after freeze fracture. The free SR is a markedly asymmetric membrane (Fig. 23). It contains intramembraneous particles in its cytoplasmic leaflet (P face), and it has a luminal leaflet (E face) which shows barely visible indentations (presumably a negative image of the particles). The particles are aggregates of Ca pump proteins (reviewed in references 111 and 113). On the average, there are three to four ATPase molecules for each intramembraneous particle (see reference 112). The Ca ATPase is located deep within the SR membrane, and it extends into the lumen and into the cytoplasm. The free SR has a remarkably uniform distribution of particles along its entire surface although the specific density of particles per unit area of SR varies in fibers of different types (reviewed in reference 114). This suggests that the calcium-pumping capacity per unit surface area of SR is uniform throughout a muscle fiber.

In proximity to the T tubules, the fSR shows an abrupt transition into jSR.

Two regions of the membrane delimiting the T tubules are defined. One faces toward the SR (jT) and the other away from it (fT). fT membrane has very few particles on its cytoplasmic leaflet and has either none or very few particles (depending on species) on the luminal leaflet. Its structure is quite different from that of jT membrane to be described below.

STRUCTURE OF TRIADS (AND HOMOLOGOUS ORGANELLES): At triads and dyads, the membranes of transverse tubules and SR are separated by a narrow (about 10 nm) junctional gap. The surface of the SR is dimpled, and the

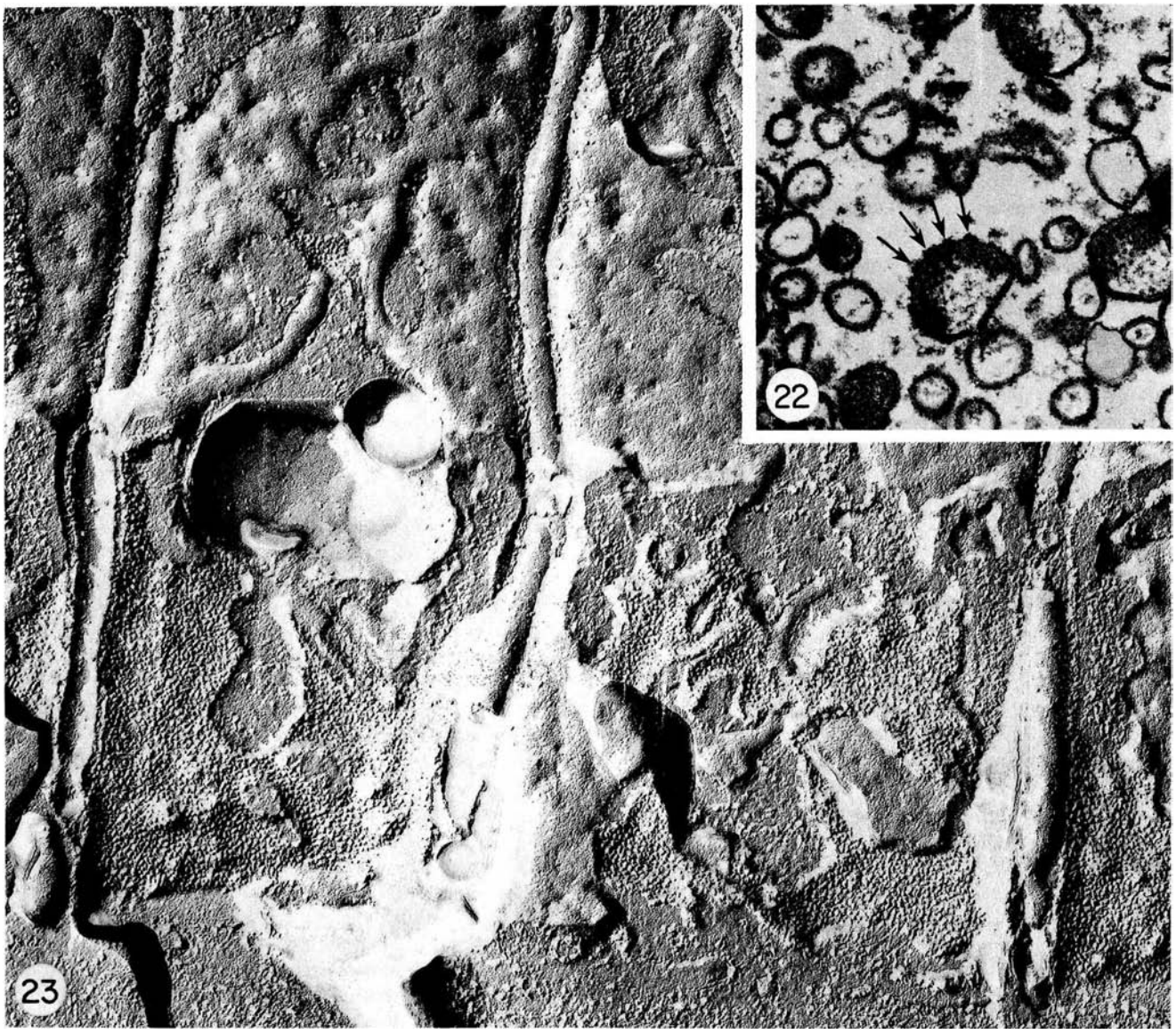


FIGURE 22 Microsomal fraction from rabbit muscle, containing lateral sacs of the triad. These are identified by the dense content (probably the calcium-binding protein, calsequestrin) and by the junctional feet (arrows) on the cytoplasmic surface of the membrane. Reprinted from Campbell, K., C. Franzini-Armstrong, and A. D. Shamoo. Manuscript submitted for publication. × 30,000.

FIGURE 23 Freeze-fracture of a twitch fiber from a fish muscle. Most of the membranes shown belong to the SR. The cytoplasmic leaflet of the SR is occupied by a large number of intramembranous particles, which probably represent aggregates of the Ca-pump protein. The protoplasmic leaflet is smooth. × 11,000.

dimples are attached to electron-dense "feet," which cross the junctional gap and join SR and T tubule membranes (reviewed in reference 114). The feet have variable appearances within the same section, and this has resulted in a variety of descriptions in the literature. There is, however, general agreement that they are arranged in a tetragonal disposition, forming either two or multiple parallel rows (Fig. 16). In freeze-fracture replicas, specializations are found both in the jSR and jT membranes. However, the structure of these two membranes is somewhat difficult to describe because they usually do not present the striking arrangement of intramembraneous particles that are characteristic of membranes forming special junctions between cells (i.e., gap and tight junctions). The T to SR junction is unique in that the two apposed membranes have different structure from each other, thus making the junction asymmetric. So far, descriptions of the junction have provided no obvious clue to its function. There is, however, some evidence that intramembraneous components bear some relationship to the junctional "feet" and thus that the feet may have some direct role in the coupling of the two membranes.

Special Types of Fibers

Special biological requirements have resulted in the evolution of types of fibers with distinctive structural and functional characteristics. As examples from two extremes, this section describes the very fast asynchronous, fibrillar flight muscles of insects and the slow fibers of vertebrates.

FIBRILLAR FLIGHT MUSCLES: Fibrillar flight muscles are found in selected insect orders. They are recognizable as a distinct fiber type on the basis of several unusual functional and morphological details (reviewed in references 41 and 109). The name fibrillar derives from the appearance in cross sections, where relatively large fibrils (1–5 microns diam) are easily seen in the light microscope because they are separated by numerous, large mitochondria. Mitochondria, as in all insect flight muscles, occupy a large percentage of the fiber's volume.

The structure of the sarcomere is highly organized. Alignment of thick filaments is precise, and thin filaments lie rigidly halfway along the line joining two adjacent thick filaments. This disposition results in a 3:1 thin-to-thick filament ratio, different from the 2:1 ratio of vertebrate muscles. Because of this precise arrangement, this muscle has proved to be ideal for the study of bridges.

The most striking morphological feature of the membrane system is the scarcity of selected components of the SR. It consists of little more than the vesicles forming dyads with the T tubules. Thus fSR, the calcium-pumping portion of the SR membrane, is reduced relative to most other types of fibers, whereas jSR is not. T tubules are numerous, and indeed their openings were first observed in this type of muscle (38). Adequate oxygen supply is insured by the penetration of tracheolae into deep folds of the surface to all areas of the muscle fiber.

Under the appropriate mechanical conditions, fibrillar flight muscles produce an oscillatory contraction of high frequency when stimulated (reviewed in references 109 and 115). Frequency of motor-nerve impulses and electrical events at the surface membrane do not correspond on a one-to-one basis to the mechanical events (hence the name "asynchronous," by which these muscles are known). Special properties of the fibrils, when activated, are responsible for the oscillation. Briefly, the effect is caused by a delayed change in tension after a step change in length. Necessary small amplitude length changes are imposed on the muscles by the wing and elastic ligaments in the insect's thorax. Nerve impulses simply activate the muscle (presumably, by conventional e-c coupling). A high-frequency oscillatory contraction then follows, which lasts as long as the fibrils are activated. Thus, in this muscle a sufficient sarcoplasmic concentration of calcium must be maintained for a prolonged period of time. Paucity of Ca-pumping fSR is consistent with this requirement. Adequate supplies of T tubules, jSR, and mitochondria are consistent with requirement for a rapid turn-on and for an adequate supply of ATP.

SLOW FIBERS: Function of slow fibers is thought to be the maintenance of a prolonged state of contraction with the least expenditure of chemical energy. In the frog, for example, such a contraction is needed during the mating season, when the male's amplexus may last uninterrupted for many days.

The following is a description of slow fibers in frogs. Others may vary slightly from this prototype (reviewed in references 104, 106, 107, and 116). Slow fibers differ from fast fibers in the following properties. They do not conduct an action potential, and they respond to application of acetylcholine or potassium with a slow prolonged contracture. The latter phenomenon suggests that prolonged depolarization does not result in inactivation of some step in e-c coupling, as in twitch fibers. The response to stimulation through the nerve is also graded and slow.

Two details of structure and function of slow fibers may be directly correlated to the lack of an action potential: (*a*) multiple innervation, which allows postsynaptic potentials to be produced at frequent intervals along the length of the muscle fiber; (*b*) large membrane resistance, which makes the fiber a more effective, if slow, cable.

The morphological differences between slow and twitch fibers in the frog are quite prominent. End plates are small and multiple (*en grappe*). Myofibrils are large and often incompletely separated by a layer of SR. Z lines are wider than in twitch fibers, and with a different structure; M lines are apparently absent. The thin filaments have variable lengths. T tubules, SR, and their junctions are present, but in amounts and disposition different from twitch fibers (see Fig. 17). The T tubules mostly course at the level of the Z line, but not as precisely as in twitch fibers. Longitudinal extensions are more numerous. The SR is less abundant than in twitch fibers, mostly because it forms an incomplete cuff around the fibrils. In addition, it has far fewer intramembranous particles per unit area of membrane. Triads and dyads are irregularly oriented, but they are composed of essentially the same structural elements as triads in twitch fibers. Peripheral junctions between SR and plasmalemma are numerous.

The morphology of the membrane system indicates a basic similarity of function with that of twitch fibers. This conclusion is largely confirmed by the following physiological data. In skinned fibers, the contraction produced by direct application of calcium to the fibrils has a slow time-course, indicating that the slow onset of tension production is mostly caused by slow bridge cycling rather than by a large delay in e-c coupling (reviewed in reference 50). Activation kinetics, as indicated by strength-duration curves that correlate pulse duration with membrane potentials in voltage-clamped fibers, imply a similar mechanism of calcium release in slow and in twitch fibers (reviewed in reference 117). Slow onset of tension and metabolic economy are thus mostly results of the slow rate of bridge cycling.

Types of Twitch Fibers

In this section, we consider the differences within a restricted group of fibers: the twitch-type fibers that compose the voluntary musculature of mammals. In the study of "fiber types," various techniques are integrated. Initial approaches included histochemistry, electron microscopy, and physiological studies of mechanical properties and how innervating axons affect them. More recently, various combinations of sophisticated physiological studies of individual motor units, quantitative electron microscopy, immunocytochemistry, microbiochemistry, experiments with cross innervation and chronic stimulation in vivo and in vitro, and the study of differentiation during development have brought us to the threshold of a complete understanding of the factors controlling differentiation of fiber types and of the close match between the properties of a motor neuron and the fibers it innervates. Muscle pathology has greatly benefited from these studies, and, in turn, it has contributed clarifying information, in the form of diseases whose expression depends on the type of fiber affected.

Twitch fibers are all capable of a propagated action potential, to which they respond with an all-or-none twitch. Repetitive stimulation at appropriate frequency produces a tetanus. Innervation is at one or two large (*en plaque*) end plates. Within this general scheme, there are variations in details, such as the intrinsic speed of shortening, the frequency of stimulation necessary to produce a fused tetanus, and fatigability, i.e., failure of the muscle fiber to produce tension as a result of repetitive stimulation. A fiber possessing a particular set of characters is assigned to a "fiber type." Such classification is very useful, as long as it is kept in mind that the boundaries between different fiber types are not always clear-cut. Fibers with virtually any possible mixture of characters may exist. In addition, many of the characters do not vary in stepwise fashion, but form a continuum between two extremes.

In the leg muscles of cats, three distinct types of fibers are defined on the basis of interrelated morphological biochemical and functional characters (118): fast fatigable (FF), fast fatigue resistant (FR), and slow fatigue resistant (SR). Fast fatigable fibers have high myofibrillar ATPase, a high content of glycolytic enzymes, and few mitochondria. They produce a fast twitch, have a high fusion frequency, and fatigue rather rapidly. FR fibers have a high, acid-unstable ATPase, a good supply of glycolytic enzymes, and fairly numerous mitochondria. They have a fast twitch and a moderately high fusion frequency, and are innervated by axons of fast conduction velocity. They are relatively resistant to fatigue. SR fibers have low ATPase, a large number of mitochondria, and low glycolytic enzyme activity. Their twitch is definitely slower than that of fibers in either fast category. They are relatively resistant to fatigue. SR motor units are used for tonic, postural movements, FR for rapid movements of some duration, and FF are probably used only for very rapid movements, such as those involved in an escape reaction.

It is likely that the above classification can be applied to leg muscles of most mammals, even though details vary with species (reviewed in reference 106, 119, and 120), and further subdivisions may need to be included. When one examines muscles responsible for more delicate and complex movements, classification becomes far more difficult. On the basis of morphology alone, for example, at least five categories of fibers are described in extraocular muscles.

There are three metabolic categories of fibers: predominantly oxidative, oxidative glycolytic, and glycolytic. These correlate very well with the relative content of mitochondria (highest in oxidative fibers, Fig. 24). Content of oxidative and glycolytic enzymes varies in a continuous, not a stepwise fashion, and so does the relative volume of mitochondria (102). Fibers rich in mitochondria contain the oxygen-carrying pigment myoglobin and are associated with a rich network of capillaries. For that reason, the portions of muscles containing them have a deeper color. Fibers rich and poor in mitochondria are often referred to as red and white, respectively (reviewed in reference 121). No direct correlation exists between the pattern of metabolic activity and the speed of contraction. Muscles that are purely fast may contain red as well as white fibers. On the other hand, a correlation exists between mitochondrial content and fatigability: red fibers are more resistant to fatigue than are white fibers. Metabolic properties are, at least partially, under the influence of exercise.

Intrinsic speed of shortening is a property of the myofibrils and is related to the rate of ATP splitting by actomyosin ATPase (122). There are two genetically distinct types of myosin, one contained in fast-twitch fibers and the other in slow-twitch fibers. Fibers of intermediate speed contain a mixture of the two (Figs. 25 and 26). The two myosins differ in the number and type of light chains associated with S1: "slow" myosin has only one type of alkali light chain with a molecular weight different from either of the two alkali light chains of "fast" myosin. The two types of myosin are antigenically distinguishable, and thus they can be located by immunofluorescence (see Figs. 25 and 26). The study of the distribution of myosins in different fiber types in the adult and developing muscles and in experimentally altered muscles is one of the most active current fields of muscle research. A good collection on papers on this subject has been published recently (123).

A muscle's well-being depends on innervation. A motor neuron and the muscle fibers it innervates comprise a motor unit, which is composed of the same types of fibers. A motor neuron that innervates a slow-twitch motor unit produces a steady, low-frequency discharge (10–20 per second). A fast motor axon fires isolated bursts of action potentials at a higher frequency (30–60 per second). When the motor nerve that serves a muscle predominantly composed of fast twitch fibers is crossed with that of a predominantly slow-twitch fibers, the mechanical properties of the two muscles are, at least in part, reversed, as is the myosin ATPase. If, as is often the case, the muscles used for cross-innervation have fibers with different mitochondrial contents and calcium-pumping activity of SR, then this also is reversed. It is now established that, following cross-innervation, the fibers gradually change their content of myosin (and other proteins) from the fast to the slow type and vice versa. Under the influence of the steady activity of a slow motor neuron, a fast fiber supresses the genes responsible for the synthesis of fast myosin. Over a period of time, which is presumably dependent on the turnover rate of the myosin molecule, the fiber then gradually acquires a content of slow myosin.

Chronically implanted electrodes, producing direct excitation of the muscle at a steady rate of 10 per second over a period of 20–40 days, induce the change of a fast to a slow muscle as effectively, or more so, than does cross-innervation. Muscle fibers can be caught in the process of transforming, at a stage when they possess both types of myosin simultaneously.

In early stages of differentiation, all muscle fibers synthetize fast myosin. If left alone, or if innervated by a fast axon, they will continue to do so. Appearance of slow myosin in future

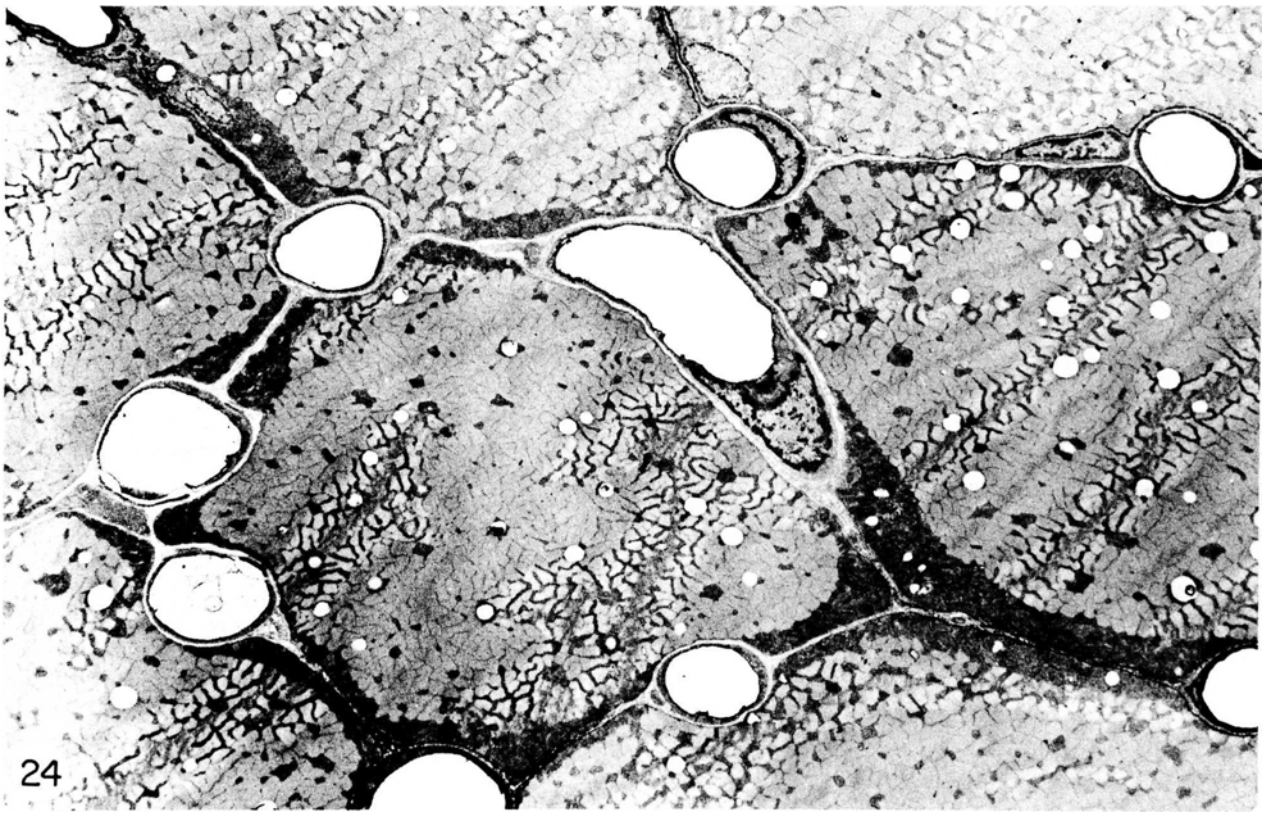
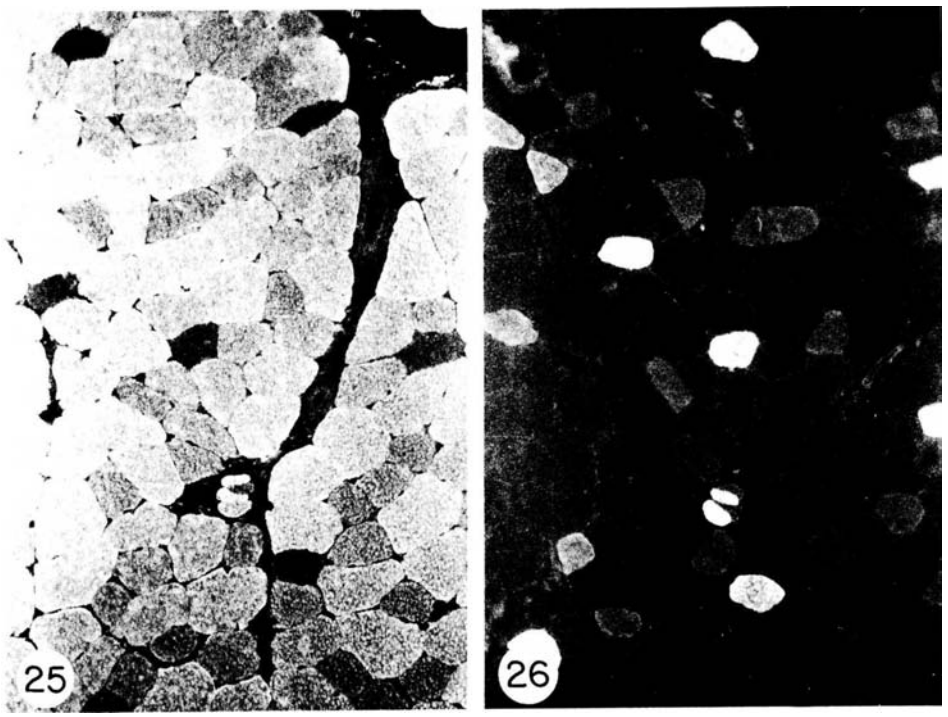


FIGURE 24 Cross section of the "red" portion of a rat muscle. The small-diameter muscle fibers, probably of a nonfatigable type, have a large content of mitochondria located between the fibrils and in aggregates at the periphery of the fiber. Numerous capillaries surround each muscle fiber. × 3,200.



FIGURES 25 AND 26 Serial sections of the rat extensor digitorum longus muscle, stained with affinity purified antibodies against rabbit "fast" and "slow" myosin, respectively. Some heterogeneity in staining with antifast is observed, and few fibers are not stained. In Fig. 26, those fibers which fail to stain with antifast do stain intensely with antislow. Additionally, a number of fibers that stain with slightly less than maximum intensity with antifast also stain lightly with antislow. These fibers probably are intermediate in speed of shortening between the other two. Reprinted from Rubinstein, N., and A. M. Kelly. Manuscript submitted for publication.

slow-twitch fibers is detectable at stages when muscle becomes innervated. Thus, either the steady firing of the slow motor neuron, some trophic factor, or a combination of the two, are responsible for inducing the synthesis of slow myosin. This may be at the basis of the higher sensitivity of slow-type fibers to denervation and for the prevention of their atrophy by activity.

Conclusion

This chapter considers only those aspects of the skeletal muscle fiber that distinguish it most from other cells: its elaborate contractile machinery and the accessory structures responsible for the control of activity. Great progress has been made in the understanding of these structures. Most of the steps in excitation-contraction coupling are well identified. Remaining questions on the basic mechanism of contraction are at the molecular level. Factors responsible for the exquisite adaptation of fibers to varying functional demands and for the integration of motor neuronal and muscle-fiber properties are established. We are at the threshold of a complete understanding of what makes us move—and how.

It should not be forgotten that, even though highly specialized, a muscle fiber shares properties with other cells, and that much that has been learned in the study of muscle applies to other cell types, as well. The muscle fiber should be thought of as an extreme state of differentiation—a modulation and augmentation of basic cellular structure and functions.

So that the reader should not be misled into thinking we have reviewed all that is known about muscle, we should mention that several broad areas of past and current interest in the study of the cell biology of muscle have not been covered at all: among these are structure and function of smooth and cardiac muscle; muscle metabolism, structure, function, and development of end plates; the integration of nerve and muscle function and their feedback control; response of muscle to training and disuse; differentiation and regeneration; and muscle pathology.

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IV. The Cell and Its Environment

Membrane Structure

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This chapter surveys selected highlights of the evolution of modern ideas about the molecular organization of biological membranes. The survey is in no sense complete, and references 1–14 may be consulted for more details on the topics covered here. Many important topics left almost or completely untouched include membrane transport (15–21), black lipid films (22–31), and many aspects of membrane biochemistry (32–35).

Historical Background Before the Electron Microscope

EARLY IDEAS: The existence of some kind of membrane structure that bounds cells was implicitly recognized as soon as the cell concept was defined by Schleiden and Schwann in 1839 (36). Bowman in 1840 (37) was one of the first to depict such a structure as an anatomical entity in his drawings of the sarcolemma. The earliest intimations that the membrane contained lipid came from the work of Overton in 1895 (38, 39). The essential point was the discovery that lipid-soluble molecules penetrated into cells more easily. J. Bernstein developed the hypothesis, definitively presented in 1902 (40) but intimated as early as 1868 (41), that living cells consisted of an electrolyte interior surrounded by a thin membrane relatively impermeable to ions. He also postulated that there was an electrical potential difference across the membrane at rest, and that during activity the ion permeability barrier was reduced to a relatively low value. The proof of the essential correctness of Bernstein's main point came in 1910–1913 with the experiments of Höber (42–44), who measured the electrical resistance to an alternating current of a mass of red blood cells centrifuged in sucrose. He found that at 1 kilocycle/s the resistance was high (~1,200 Ωcm) but that it became much lower (~200 Ωcm) at 10 megacycles/s. The latter is the resistance of a 0.4% NaCl solution. After hemolysis and treatment with saponin, the same low resistance was found at both low and high frequenices.

Fricke in 1923 (45, 46) measured the capacitance of the red blood cell membrane to be 0.81 $\mu F/cm^2$. He supposed the membrane to be an oil film with a dielectric constant of 3 and so calculated the thickness to be 33 Å. This was the first indication that a membrane might be of molecular dimensions. Many membranes, though not all, were subsequently found to have a capacitance of ~1 $\mu F/cm^2$, a first intimation of the existence of some kind of unitary structure.

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MONOMOLECULAR FILMS: Studies on monomolecular films were of fundamental importance. Lord Rayleigh in 1890 (47) measured the thickness of a film of olive oil to be 1.63×10^{-7} cm. Devaux (48) did much pioneering work on oil films on water as well as protein monolayers at both air-water and oil-water interfaces. Langmuir (49) in 1917 (cf. Harkins [50–51]) showed that some lipid molecules were amphiphilic (52), in having a polar head and a nonpolar carbon chain. When spread at an air-water interface, they formed a monolayer on the surface and affected the surface tension. When a surface barrier was moved so as to reduce the area while measuring force, a characteristic force/area curve was obtained. A minimal area was reached at which the force was maximal and then the film collapsed, as evidenced by a break in the curve. Langmuir interpreted these findings correctly as showing that the lipid molecules were amphiphilic. At a low surface pressure they were randomly arranged in the water surface, but upon being pushed close together by the moving barrier, they formed a structure like a picket fence with their polar heads in the water and their nonpolar carbon chains pointed into the air. He calculated the area/molecule of a variety of fatty acids and other substances.

THE LIPID BILAYER: In 1925 Gorter and Grendel (53) extracted the lipid from a known number of red blood cells, calculated the total cell area, and found the measured minimal area of the total lipids compressed on a monolayer trough to be twice this value. This led to the bilayer concept. The extraction procedure did not extract all of the lipids, but this was compensated by underestimation of the cell area. This work provided the first suggestion that a lipid bilayer might be a fundamental feature of biological membranes, but no effort was made to generalize.

Schmitt et al. in 1937–1938 (54, 55) studied erythrocyte ghost membranes in polarized light and concluded that they contained lipid molecules oriented perpendicular to the plane of the membrane as would be expected if a lipid bilayer were present.

BIOPHYSICAL PROPERTIES OF MEMBRANES

Fluidity

During the 1930s, Chambers and Kopac (56, 57) showed that an oil droplet applied to the surface of a denuded marine egg quickly passed through the surface and appeared as a droplet on the cytoplasmic side. They also noted that, if two such droplets were applied on different areas and the seawater

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was agitated, the droplets moved relative to one another, indicating that the membrane was fluid (58).

Surface Properties

Mudd and Mudd in 1931 (59) did a revealing experiment on red blood cells (cf. 60). They examined microscopically a droplet of blood on a glass slide in contact with an oil droplet under a coverslip. The white cells remained in the water phase, but some red blood cells entered the oil phase, showing that their surfaces were relatively hydrophobic. These observations indicated that the external surface of the erythrocyte membrane was covered by material, probably protein, which could become predominantly hydrophobic. This means that hydrophobic bonding of a protein to a membrane surface does not necessarily require bonding to lipid.

In 1932 Cole (61) measured the surface tension of starfish egg membranes. He determined the force required to compress an egg between two glass coverslips and calculated surface tension values of ~0.1 dyn/cm. Harvey and Shapiro (62) found similar values by measuring the surface tension of oil droplets within cells with the centrifuge microscope. These low values seemed strange because people were thinking of cell membranes as thin, oily films and the surface tension values of oils were much higher. Danielli and Harvey in 1934 (63) studied oil droplets from mackerel eggs and found that after extensive washing they gave surface tension values of about 9 dyn/cm. When a cytoplasmic extract was added to the oil, the surface tension was lowered and they identified the agent responsible as protein.

The Devaux Effect

In 1938 Langmuir and Waugh (64) gave the name "Devaux effect" to a phenomenon related to the above. Devaux (65) simply shook a solution of albumen in water with benzene and noted that at a certain albumen concentration the benzene formed droplets and the albumen spread at the oil-water interface into a monolayer. The surface tension at the resulting interface was obviously very low because the droplets spontaneously assumed peculiar shapes. Similarly, Danielli (66) found that proteins spread at an oil-water interface showed an initial marked fall in surface tension, which rose with time to a final value less than that of the oil-water interface alone.

Kopac (67–68) reported that a droplet of oil injected into a protein solution soon became crenated, indicating the development of low surface tension because of the Devaux effect. Droplets microinjected into the cytoplasm of marine egg cells remained smooth and spherical. However, if the cell was pricked with a needle to cause cytolysis, the oil droplet immediately became crenated. Later Trurnit (69) made the relevant point the proteins in solution generally adsorb and spread at the air-water interface as well as any other high-tension surface. The fact that no such interfacial spreading occurred in the intact egg indicated that the egg cytoplasmic matrix did not contain protein molecules in simple solution.

All these experiments were important in the early evolution of thinking about membrane structure. Interestingly, the important point was missed that some natural phospholipids, e.g., phosphatidyl choline (PC), in monolayers give quite low surface-tension values of <5 dyn/cm (70). Synthetic dipalmitoyl lecithin at an air-water interface gives a surface-tension value that is hardly measurable (0–2 dyn/cm) (70, 71). Phospholipids are the dominant lipids of biological membranes and one of their functions may be to confer low surface-tension properties on membrane lipids. This could be important in preventing the denaturation of membrane proteins.

Membrane Capacitance

In 1950 Cole and Curtis (72) tabulated the known values of membrane capacitance, ranging from 0.81 $\mu F/cm^2$ for the human erythrocyte membrane through 9.0 $\mu F/cm^2$ for cow erythrocytes to 0.012 $\mu F/cm^2$ for frog peroneal nerve. Cole in 1935 (73) made the surprising observation that sea urchin eggs, normally having a capacitance of 1 $\mu F/cm^2$, displayed lower values when swollen. He expected the reverse because of the thinning of the membrane. Instead, the lower values suggested thickening. His findings were confirmed by Iida (74, 75) who showed the phenomenon to be reversible. High capacitance values were also obtained for skeletal muscle fibers by Katz (76).

The reason for some of the variations in membrane capacitance was elucidated by Lord Rothschild in 1957 (77). He showed that there was an error in the calculation of the area of egg membranes. By electron microscopy he found that the surface membrane was thrown into minute folds not apparent by light microscopy, thus increasing greatly the actual surface area. Similarly, with our understanding of the T system in skeletal muscle fibers (78–81), it became known in the late 1950s that the actual measured areas of muscle fibers used in calculating membrane capacitance were wrong because the T system was not taken into account.

THE DANIELLI-DAVSON MODEL: In 1935, Danielli and Davson (82) presented a model of cell membrane structure which they later generalized (58) into the "pauci-molecular" theory that stated that all biological membranes had a "lipoid" core bordered by monolayers of lipid with the lipid polar heads pointed outward and covered by protein monolayers (Fig. 1). In 1943 (Fig. 16*b* in reference 58) they presented a detailed molecular diagram showing hydrophobic amino acid side-chains penetrating between lipid headgroups and lying between the carbon chains of lipid molecules in a relationship that would result in hydrophobic bonding.

The pauci-molecular theory provided the major membrane paradigm into the 1950s. The original theory did not specify the bilayer as a general structure, although Danielli clearly favored it and, in one of his later diagrams (83), he drew transmembrane polypeptide chains arranged with their polar groups apposed so as to make a polar transmembrane channel.

In a paper published in 1935 on the thickness of the red blood cell membrane, Danielli (84) discussed Fricke's choice of a value of 3 for the dielectric constant. He noted that the value depends on the orientation of the dipoles in the film and indicated that higher values, up to 6.7, could occur. This would require a larger thickness value for the membrane for a given measured capacitance. Perhaps this is why the model was thicker than one bilayer. In 1936, Danielli (85) drew a number of models varying from a single lipid monolayer to the thicker original model. The cautious way in which this problem was treated illustrates the uncertainty about membrane thickness of the period.

In 1949, Waugh and Schmitt (86) measured the thickness of erythrocyte membranes with the "analytical leptoscope." Their results, though compatible with a single bilayer, were not unambiguous and were restricted to erythrocyte membranes. Thus, it seems fair to say that the single bilayer model, which

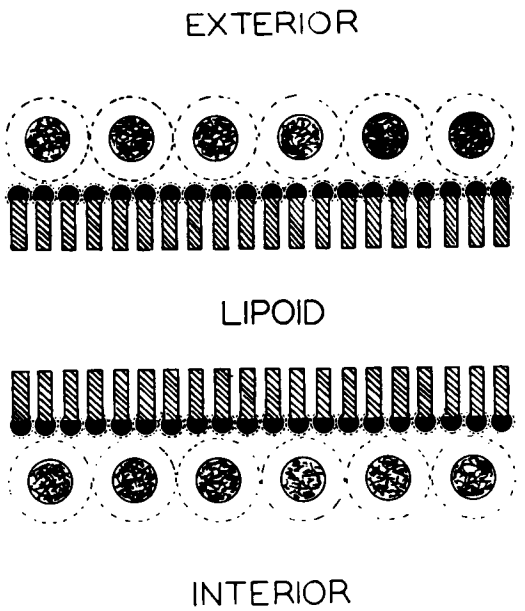

FIGURE 1 Schematic diagram of the molecular conditions at the cell surface published by Danielli and Davson (82) in 1935.

Danielli clearly favored, was based on good evidence for the erythrocyte membrane, whereas no hard evidence for a generalization of the bilayer existed.

The Early Electron Microscope Period

THE UNIT MEMBRANE MODEL: In the 1950s the electron microscope made it possible to look profitably at sectioned cells at resolutions better than 50Å. The introduction of potassium permanganate as a fixing agent by Luft (87) and epoxy resins as embedding materials by Glauert et al. (88) led to visualization of the cell membrane as a triple-layered structure ~75Å thick consisting of two dense strata, each about 25Å thick, bordering a light central zone of about equal thickness. This triple-layered pattern was observed in nerve fibers (89–92) and in other tissues (93–97).

Figure 2 is an electron micrograph of a human erythrocyte membrane fixed with glutaraldehyde, embedded in polyglutaraldehyde by the glutaraldehyde-carbohydrazide (GACH) method (98), and stained with uranyl and lead salts. The overall thickness is ~100Å. The bilayer core is the clear stratum ~40Å thick between the two dense surface strata. The core of the bilayer does not take up heavy-metal strains because it is hydrophobic. It consists mainly of the hydrocarbon chains of lipid molecules and the hydrophobic polypeptide chains of integral membrane proteins. The surface strata are hydrophilic and take up the stains avidly. The hydrophilic structures are the polar heads of lipid molecules, protein molecules, and, in the outside surface, carbohydrate residues. Asymmetry is not revealed by this method.

A similar, but thinner, triple-layered pattern was also observed in model systems consisting of smectic fluid crystals of phospholipids (94–96). When these were hydrated, the individual bilayers appeared as pairs of dense strata separated by a light central zone, in this way looking very much like cell membranes but definitely thinner. This finding meant that heavy-metal atoms accumulated in the polar head regions of the lipid molecules although the primary reaction of OsO_4 was with the double bonds of the lipid carbon chains (99). Single bilayers did not appear as single, dense strata but always as a pair of dense strata making a triple-layered structure. This was rationalized (5, 94–97) by assuming that the primary reaction product of the Criegee reaction, OsO_3, because it is more polar than OsO_4, must be driven out of the hydrophobic interior of the membrane and become adsorbed in the polar regions, increasing the relative density there by adding to density caused by direct reaction of head groups constituents. Stoeckenius (100) later performed some experiments based on the work of Luzatti and Husson (101) on model lipids, and confirmed this interpretation.

In 1954, Geren (102) postulated that the nerve myelin sheath might consist of a spirally wrapped mesaxon; in 1955 (103), both outer and inner mesaxons were observed to connect a fully developed myelin sheath with the inner and outer surfaces of the Schwann cell, proving her theory. The application of permanganate fixation and epoxy embedding to developing mouse sciatic nerve fibers showed the mesaxon clearly as two triple-layered membranes united along their external surfaces, and also showed that compact myelin resulted from the close apposition of the cytoplasmic surfaces of the membranes of the mesaxon. At the time, there was a tentative molecular model of the radially repeating unit of the myelin sheath (104–106). Inasmuch as the mesaxon was obviously the repeating unit, it was possible to identify the strata within compact myelin in molecular terms (92–95). This analysis indicated that the Schwann cell membrane was a lipid bilayer covered by monolayers of nonlipids on either side, as in Fig. 3. The validity of this analysis was confirmed later by X-ray diffraction studies when the phase problem was solved at a resolution of ~30Å by Moody (107) and by Caspar and Kirschner (108) at higher resolution.

It was also possible to deduce from the structure of the myelin sheath, as well as from its staining characteristics observed by electron microscopy, that the outer surface of the membrane was chemically different from the inner surface. Two unit membranes were included in one radial repeating unit, which showed that there had to be a difference between the inside and outside surfaces of the membrane, Finean's "difference factor" (105). There was a good reason for generalization of the idea of membrane asymmetry. The external

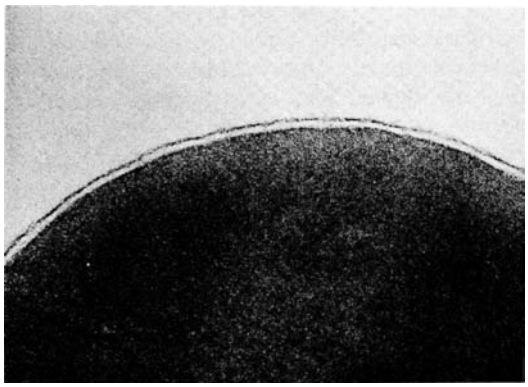

FIGURE 2 Electron micrograph of a human erythrocyte membrane fixed with glutaraldehyde embedded in polyglutaraldehyde by the GACH method (98) and stained with uranyl and lead salts. The membrane consists of two dense strata separated by a light central zone. The overall thickness is about 130–140 Å in this preparation. This is higher than is seen after OsO_4 fixation and the high value is probably due to some displacements due to sectioning as is common in GACH embedded specimens not postfixed with OsO_4 and RuO_4. × 100,000

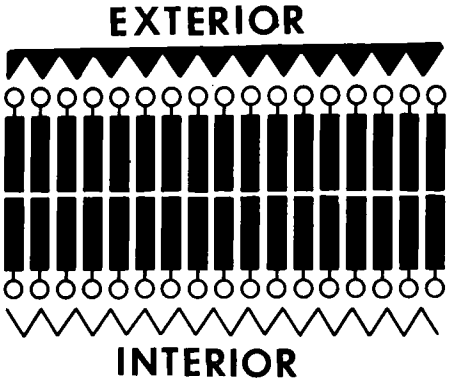

FIGURE 3 Original unit membrane model. The lipid bilayer is indicated very schematically by the bar and circle figures. The nonlipid monolayers at the polar surfaces are indicated by the zigzag lines. The chemical asymmetry produced by the presence of carbohydrate in the external surface is indicated by the partial filling in of the zigzag representing the external monolayer.

stratum of the Schwann cell membrane in OsO_4-fixed myelin often appeared to be fragmented. At the free surface of the Schwann cell, as well as other cells, OsO_4 alone usually preserved only the cytoplasmic dense stratum, whereas $KMnO_4$ preserved both strata. Revel et al. (109) reported that glycogen granules in liver cells were not well fixed with OsO_4 but were well preserved with $KMnO_4$, fitting the view that the outer surface of membranes contained carbohydrate (93–97).

A survey conducted of many different tissues, in several different animals in different phyla and bacteria, showed that the triple-layered pattern could be demonstrated with $KMnO_4$ in all cellular membranes whether at the surface or in membranous organelles (93). It was concluded that all biological membranes consisted of the same kind of fundamental structural pattern, i.e., a lipid bilayer arranged with the polar heads of the lipid molecules pointing outward and covered by monolayers of nonlipid with a preponderance of carbohydrate in the external surface, as in Fig. 3. In that this structure was the repeating unit of myelin and of all membranous structures of cells, it was called a "unit" membrane. The unit-membrane theory (89, 93–97, 110) introduced a new paradigm that was useful for about 15 years. The model built on the earlier Danielli-Davson model by adding two new concepts: it proved the universality of the single bilayer and introduced for the first time the idea of chemical asymmetry, neither of which were features of the earlier model. To be sure, Danielli clearly believed the bilayer to be the dominant structure and he deserves credit for this. He even guessed the existence of transmembrane proteins.

In 1966, the unit-membrane paradigm came under attack (111) because it was believed that the structure of cell membranes must be more complicated than the theory seemed to imply. In pointing to the fact that all membranes had the same kind of basic structural plan, the impression was given that all membranes were molecularly identical. This was, of course, a complete misunderstanding (96). The unit-membrane model was incomplete in that it did not deal with membrane fluidity nor with the idea of penetrating proteins. It was deficient in that it implied that membrane proteins were unfolded in the same manner as proteins at air-water interfaces. However, its major features—the universality of the lipid bilayer and chemical asymmetry—are generally accepted today.

THE SUBUNIT MODELS: Various alternative models in which the bilayer was altered or interrupted in a variety of ways were proposed in the 1960s by Sjöstrand (112, 113), Lenard and Singer (114), Green and his colleagues (115, 116), and Benson (117, 118). The essence of these models was that the bilayer was not the dominant structure, but that lipid molecules were arranged in various patterns in the membrane. The Benson model was the most extreme; the membrane consisted of a thin layer of protein with lipid molecules simply intercalated in a variety of ways. The present-day Sjöstrand and Barajas models (119–121) are somewhat similar. These all fall more or less into the general rubric of "subunit" models, and the earlier ones were dealt with quite thoroughly in a review in 1969 by Stoeckenius and Engleman (14), in which they concluded that the bilayer model was the only reasonable one. The basic fact here was that it was found impossible to break up any membrane structure into subunits of uniform composition that would reassemble into a functional membrane. Another problem was that this concept implied that membrane biogenesis occurred by additions of aliquots of components as aggregates of many molecules that served as building blocks for the whole structure. Studies of membrane biogenesis should then have shown evidence of parallel increases of at least some set of related components. No such increases were found. For example, Siekevitz, Palade, and coworkers (122–128) showed that individual nascent membrane proteins are inserted into developing membrane systems at different times, and that proteins in membranes turn over at different rates, independent of one another and independent of the turnover of membrane phospholipids.

The Modern Period

FREEZE-FRACTURE-ETCH (FFE) ELECTRON MICROSCOPY: Freeze-fracture-etch electron microscopy has become important in structural studies of membranes. Steere introduced the FFE technique in 1957 (129), and it was developed further in 1961 by Moor and Mühlethaler (130), who noted that membranes fractured transversely give the same triple-layered unit membrane appearance found in sections. They believed that when the fracture plane followed the plane of the membrane it ran along its surface. Branton and his associates later (131–133) proposed that frozen biological membranes tend to fracture centrally, and noted that the fractured replicated surfaces displayed particles about 50–100Å in diameter (Fig. 4); da Silva and Branton (134) proved that the particles were located inside the erythrocyte membrane by labeling the external surfaces with ferritin and identifying it in the external etch (ES) faces in a plane outside the particulate fracture faces. The particles were much more numerous on the protoplasmic (PF) than on the external fracture (EF) faces of erythrocyte membranes. Almost all fractured membranes showed this distribution of particles, but they were not usually observed in the nerve myelin sheath and in pure lipid model systems (135–140). Pits in the complementary fracture faces, although sometimes seen, were usually absent. Generally, retinal rod outer segment membranes also failed to show discrete particles (141–145).

THE FLUID MOSAIC (FM) MODEL: Singer (11, 12) and Singer and Nicholson in 1972 (146) proposed a new membrane paradigm which they called the "fluid mosaic" model. This retained the bilayer concept, but introduced a new way of looking at the distribution of protein. Both the outer and inner surfaces were depicted as largely naked lipid. The

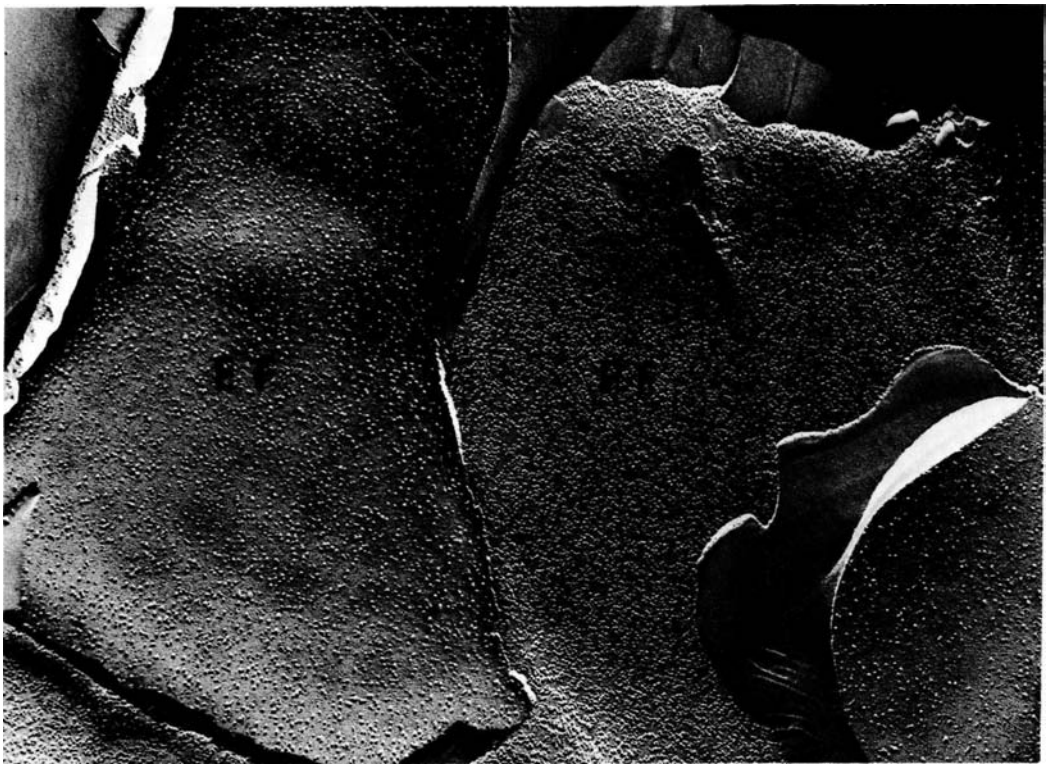

FIGURE 4 Freeze-fracture micrograph of portions of several human erythrocyte membranes. The large area of concave membrane to the left center represents the external fracture (EF) face of an erythrocyte membrane. The convex fracture face to the right represents the protoplasmic fracture (PF) face of another erythrocyte membrane. Note the particles 50–100 Å in diameter that are scattered irregularly all over both kinds of fracture faces. These are more numerous on the PF face than on the EF face. Micrograph from H. P. Beall. × 47,500.

protein was visualized as macromolecules embedded in the bilayer in an iceberglike fashion, penetrating either half or all the way through (Fig. 5). The protein molecules traversing the bilayer were visualized by Singer (12) as having water-filled holes in their center that subserved membrane transport functions. The emphasis in this model was on an extreme degree of fluidity, based on the work of Frye and Edidin (147), demonstrating fluidity in membranes by a fluorescent dye-labeling technique. The protein molecules were visualized as being completely free to translate laterally in the liquid bilayer. The model offered a ready explanation for the presence of 50–100Å intramembrane particles (IMPs) in FFE preparations, and it rapidly became the generally accepted membrane paradigm.

Some features of the original model need to be revised. For example, Singer has recognized the inadequacy of depicting the bilayer as a virtually naked structure. If membranes in vivo were generally naked lipid bilayers without continuous layers of protein on either surface, they would have the mechanochemical properties of lipid bilayers. Evans (148) and LaCelle (149) independently compared the mechanochemical properties of several different kinds of cell membranes with lipid bilayers and found them to be radically different. In 1974, Singer (12, 150) proposed that spectrin (151) made a meshwork on the cytoplasmic surface of the red cell membrane and restricted the motion of integral proteins. This added feature made the FM model compatible with the mechanochemical properties.

But an important problem still remained, because even today the model calls for the external surfaces of membranes to be mostly naked lipid bilayers. Conceivably, in some special cases like the purple membrane (2) with closely packed transmembrane proteins, the lipid is naked in patches in vivo, but there is no firm evidence for this and certainly no basis for generalizing such a feature. To take the erythrocyte as an example, if the lipid were naked externally, one would expect to see essentially no differences in the susceptibility of intact red blood cells, ghosts, or lipid bilayers to phospholipases. This is not the case. Zwaal and Roelofson (152) reported that some phospholipases are active on intact erythrocyte membranes, some have little effect, but all are active on ghosts. Ottolenghi (153) has prepared a highly purified phospholipase A_2 and found no effect at all on intact human erythrocytes, although ghosts were attacked readily. Adamich and Dennis (154) found

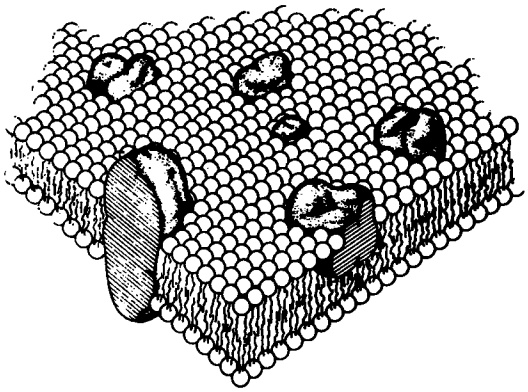

FIGURE 5 Diagram of the fluid-mosaic model of membrane structure from Singer and Nicholson (146). The bilayer is represented here by circles for the head group with two lines for each hydrophobic tail. Protein is represented by the cross-hatched particles embedded in the bilayer.

that less than 1% of the phospholipids in intact erythrocytes were hydrolyzed by phospholipase A_2, whereas 38% of the total phospholipids of ghosts were hydrolysed under the same conditions. It seems clear, then, that the intact erythrocyte membrane is definitely more resistant to phospholipases than are ghosts or lipid micelles. The lipid accessibility is much less than the FM model implies.

We shall now turn to a more detailed consideration of the currently accepted concepts of membrane structure.

PRESENT CONCEPTS OF THE MOLECULAR ORGANIZATION OF MEMBRANES

General

It is now generally agreed that all biological membranes contain a lipid bilayer, as described above. The protein: lipid: carbohydrate ratios vary considerably by weight from membrane to membrane, ranging from 75:25:0 with the purple membrane of *Halobacterium halobium* at one extreme, through 49:43:8 for human erythrocytes, to 18:79:3 for myelin at the other extreme (6). The lipids are mainly PC, phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), sphingomyelin, and cholesterol. Some membranes are high in glycolipids, phosphotidylinositol, or cardiolipin. One thing that all the lipids have in common is amphiphilicity (52).

There are two kinds of membrane proteins: peripheral and integral (12). The former are operationally defined as ones easily removable by ionic manipulations and the latter by the need for detergents or other chaotropic agents, because they are hydrophobically bonded. Some are confined to one side of the bilayer (ecto- or endo-[7]), and some penetrate it partially or completely. The dominant mass of this integral protein in most membranes is located in the polar regions of the bilayer. For example, in a recent neutron diffraction study of retinal rods Yeager et al. (155) estimated that the total mass of the rhodopsin molecule that can be in the anhydrous hydrocarbon region is 15–20%. The band-3 protein of erythrocyte membranes has only 19% of its mass in the penetrating component (156). The bacteriorhodopsin molecule is an exception; more than half its mass is in the hydrocarbon region (2, 157, 158).

The operational definition of peripheral and integral membrane proteins does not hold strictly, as Singer noted in 1974 (12). For example, ligatin (159–163), an ~10,000 *d* membrane-binding protein, although hydrophobically bonded to lipid and hence integral by this criterion, can be removed by 10–40 mM Ca^{++}, taking with it a complement of lipid, mainly triphosphoinositol plus some PC and cholesterol. It is a highly negatively charged glycoprotein, which does not have a high complement of hydrophobic amino acids. Exactly how it is bound is not clear, but it resides in the external surface of certain membranes, where it functions to bind certain ectoproteins.

The Erythrocyte Membrane as an Example

Despite its specialization, the erythrocyte membrane has been more widely studied since 1971 than any other membrane. It is about 60:40 protein:lipid by weight and contains at least a dozen well-defined proteins (10, 35, 164–170). These are generally referred to by the numbers of the positions they assume as electrophoretic bands in polyacrylamide gels, following the terminology of Fairbanks et al. (164). Chemical labeling experiments, first by Bretscher in 1971 (171–173) and later more definitively by Whiteley and Berg (174), and proteolytic dissection experiments first done in 1971–1972 by Steck et al. (10, 175) and others (156, 176, 177) have resulted in the location of most of these proteins as peripheral or integral (see reference 35 for bibliography). Bands 1, 2 and 4–6 are peripheral endoproteins. Bands 1 and 2 represent spectrin (10, 35, 178–180), also called tektin A (181, 182); band 4 is uncharacterized; band 5 is actin (10, 35); and band 6 is glyceraldehyde-3-phosphate dehydrogenase (35, 183, 184). Actin and spectrin are associated (35, 185). Periodic acid-Schiff (PAS) positive 1 and 2 and band 3 are the major glycoproteins. PAS 1 and 2 are interconvertible sialoglycoproteins (35) identified with glycophorin A, a blood-group substance (186) that makes up 75% of this group although only ~2% of the total protein (187). Following earlier work by Winzler in 1969 (188) and Morawiecki in 1964 (189), Marchesi and his colleagues (190–192) studied this protein extensively. Its amino acid sequence has been determined (35, 192). It contains a stretch of 20 hydrophobic amino acids that transverses the lipid bilayer. In common with all the glycoproteins, the carbohydrate moiety is external (35). Band 3 is a transmembrane glycoprotein containing no sialic acid that makes up 20–25% of the total membrane protein (169). It functions in anion transport (169) and can be chemically crosslinked with spectrin (193). A number of other proteins, such as Na^+K^+ ouabain-sensitive adenosine triphosphatase (ATPase) (194, 195) and acetylcholine esterase (AChe) are present in lesser amounts (35). The former is believed to be a transmembrane protein. The latter is externally located (196).

The Intramembrane Particle

IMPs are clearly associated with proteins in membranes. In 1971, for example, Branton (133) found that the number of IMPs are reduced in red cell membranes treated with proteases. Pure lipid bilayers do not normally contain IMPs, although they may display some patterned substructure under some conditions (197). Hong and Hubbel (198) showed in 1972 that addition of rhodopsin to bilayer vesicles cause the appearance of IMPs. However, in 1975 Deamer and Yamanaca (199) found that sarcoplasmic reticulum membranes treated with proteases to the extent that all their protein components were reduced to polypeptide fragments of 10,000 *d* or less still contained about the same number of IMPs, although they lost their dominant PF face orientation. Verkleij et al. (200) have shown that a pure mixed lipid system may, in the presence of Ca^{++}, display typical ~100 Å IMPs with corresponding pits in the complementary fracture faces. Attempts have been made to correlate the numbers of IMPs with the known numbers of copies of certain integral proteins. Although never exact, such correlations sometimes have appeared to be close (10) but in other cases no correlation at all was found (201). Two groups—da Silva et al. in 1971 (202) and Tillack et al. in 1972 (203)—showed that there was a relationship between the external surface components of glycophorin and band-3 protein in erythrocytes and the intramembrane particles seen in FFE preparations. da Silva in 1972 (204), Elgsaeger and Branton in 1974 (205), and others more recently (206, 207) have presented evidence of IMP aggregation phenomena. These are altered when spectrin is extracted from erythrocyte ghosts, which is interpreted as indications of the transmembrane connections of glycophorin and band 3 with spectrin. The work of Tilney and Detmers (185) suggests how spectrin, actin, and the glycoproteins might interact, and it is believed (208) that under the control of an endogenous kinase and phosphatase these

proteins interact with adenosine triphosphate to regulate the shape of the cell.

Weinstein et al. (156) studied erythrocyte membranes treated, before fracturing, by mild proteolytic digestion to remove some external protein and leaving the band-3 protein intact. Surface projections were seen in the ES face after etching related to underlying ~66 Å IMPs in the PF faces. They were interpreted as the surface ~38,000 *d* components of the band-3 protein. Inside-out erythrocyte membrane vesicles depleted of spectrin and actin with the band-3 protein intact showed, in etched preparations, granulofibrillar components having an average diameter of 90 Å. Protease digestion under conditions that resulted in release of the 40,000 *d* component resulted in loss of these granulofibrillar components. The pattern of disposition and relative numbers of these components before removal was consistent with their being the 40,000 *d* cytoplasmic surface component of the band-3 protein. In that the whole molecule has a molecular weight of 95,000 *d*, the transmembrane component is 17,000 *d* in weight. Even as a dimer plus the glycophorin chain, this is too small to correspond exactly to the ~ 66 Å IMPs.

Glycophorin A does not appear to be important in the production of IMPs, because it is absent in a rare blood type En(a-) (186, 209), and there is no effect on the IMPs except for changes in the dynamic aggregation properties that suggest association of the cytoplasmic component of glycophorin with spectrin.

Because of the known association of some IMPs with protein there is a tendency to regard any IMP literally as a metal-plated protein molecule. This oversimplification has led to much confusion in the literature. The exact nature of IMPs is not yet resolved.

The Erythrocyte Lipids

It has been known since 1971 that the distribution of the lipid constituents of the bilayer in erythrocytes is asymmetric (154, 171–173, 210–216), with amino lipids located primarily in the internal monolayer and choline and sphingo lipids localized mainly in the external monolayer. This was first suggested by labeling experiments conducted in 1971 by Bretscher (171–173), who showed that the relatively impermeant agent FMMP does not react with the amino phosphoglycerides in intact cells, whereas both PS and PE react in open ghosts. These experiments were somewhat inconclusive because they depended on the assumption that no major molecular rearrangements occur in ghosts. However, Gordesky and Marinetti (211) found in 1973 that trinitro-benzene sulfonate, a nonpenetrating reagent, did not label PS and only partially labeled PE in intact cells, thus agreeing with Bretscher's conclusions. Van Deenen (204) has reviewed the evidence from selective degradation of the phospholipids of intact erythrocyte and ghost membranes relating to this problem (215, 216), and Adamich and Dennis (154) have reported similar findings with cobra venom phospholipase A_2. The enzymatic degradation and double-labeling experiments agree in general. Presumably, glycolipids are also localized in the outer monolayer because there are no sugar residues on the cytoplasmic surface.

Bretscher (210) pointed out in 1973 the significance of observations of Rouser et al. (217) on the fatty acid composition of the phospholipid of human erythrocytes in relation to the above. The amino lipids PE and PS contain much more 20:4 and total polyunsaturated fatty acids than do the choline-containing phospholipids. Sphingomyelin contains much 16:0, 24:0, and 24:1 fatty acids. PS is highest in 18:0 acids. Thus, the inner half of the bilayer contains more unsaturated lipids, whereas the outer half contains more saturated and longer chain lipids.

Molecular spectroscopic studies in 1971 by Kornberg and McConnell (218) have shown that lipids do not become translocated spontaneously from one side of a model bilayer to the other (flip-flop) within a time span of hours, but they may do so fairly frequently within a time span of minutes in excitable membranes by special unknown mechanisms mediated by protein. In contrast, these authors (219) showed that lateral diffusion in PC bilayers is eight orders of magnitude more rapid. Van Deenan (214) noted that the flip-flop rate of phosphatidyl choline, which is virtually undetectable in bilayers, is speeded up by the addition of glycophorin.

FLUIDITY

Lipid

One kind of fluidity involves motion of the carbon chain relative to the head group; cholesterol influences this greatly. There is some evidence that cholesterol is localized to the outer half of the myelin membrane (108) and the erythrocyte membrane (220, 221). It has been shown (222–224) that cholesterol has a condensing effect on phospholipids in monolayers or bilayers, decreasing the average area per lipid molecule. This implies that it makes the membrane more rigid. However, it also has the function of converting lipids in the stiff, extended (L_β) conformation to the more liquid (L_α) conformation (139, 140), a function it shares with double bonds in the lipid carbon chains and higher temperature. This is due to the production of potential spaces in the center of the bilayer that result from cholesterol being shorter than most membrane lipids.

The term fluidity is used in another sense, in which whole lipid molecules diffuse laterally. An increase in the localized mobility of the individual hydrocarbon chains is not necessarily implicated. Still another kind of fluidity involves rotation of lipid molecules. Special techniques beyond the scope of this article are used to detect each of these various kinds of fluidity.

Lipid and Lipid-Protein Domain Fluidity

In still another kind of fluidity, aggregates of lipid molecules may diffuse as units either as free domains or together with protein constituents with which they are specifically associated. The ileum membrane of suckling rats (160–163) is a good example of lipid-protein domain fluidity.

There is also evidence that separate lipid domains may exist in membranes without necessarily being associated with protein. In 1978, Marinetti and Crain (213), using penetrating and nonpenetrating crosslinking probes, provided good evidence for the asymmetric distribution of phospholipids in erythrocyte membranes as well as for mosaic associations of groups of specific phospholipids and specific phospholipid-protein complexes. Shimshick and McConnel (225) found in 1973 that if two lipids differing in chain length by at least two carbon atoms are mixed and kept at a temperature above the phase transition of one and below that of the other, the lipids separate into two phases, one in the L_α and the other the L_β state. Ranck et al. (226) and Costello and Gulik-Krzywicki (197) showed that different lipid domains produce different textures in fracture faces in FFE preparations. Klausner et al. in 1980 (227) used fluorescent probe studies to produce evidence that lym-

phocyte membranes contain separate lipid domains. FFE studies of urinary bladder epithelial cell membranes (228–231) also suggest that such lipid domains exist and that they may be related to protein as well as to the production of artifactual IMPs.

The spreading and mixing of fluorescent surface markers first described in 1970 by Frye and Ediden (147), as well as studies of capping phenomena (232–237) of surface markers such as ferritin or fluorescently labeled lectins or antibodies represent a kind of mosaic fluidity that probably involves both lipids and proteins.

THE PURPLE MEMBRANE: The purple membrane of *H. halobium* has been studied extensively since 1968 by Stoeckenius et al. (2, 238–243). This highly differentiated membrane, which pumps protons from the cell (241, 243), appears in patches in the plasma membrane of *H. halobium* that contain a purple protein pigment molecule of 26,000 *d* called bacteriorhodopsin (BR) (240), and unusual phospholipids (244). Unwin and Henderson (157–159) showed that BR is arranged in the lipid bilayer core as a transmembrane protein in a hexagonal lattice with P_3 space group symmetry and a lattice constant of 62 Å. Seven alpha helices traverse the lipid bilayer for each molecule.

The isolated purple membrane probably should be regarded as a highly specialized residual skeletal membrane from which peripheral proteins have been removed during isolation, because there is no evidence that it exists in vivo as a naked bilayer. Similarly, Reynolds and Trayer (245) found that erythrocyte membranes could be stripped of up to 90% of their protein by treatment with dilute ionic solutions containing EGTA, producing degraded erythrocyte membranes. Thus, in terms of general membrane models, the isolated purple membrane is relevant only in the sense of being an extreme example of concentration of hydrophobic transmembrane protein in the bilayer.

Fisher and Stoeckenius (246) in a FFE study of the isolated purple membrane noted the absence of the usual globular IMPs. They observed much smaller particles in the PF faces. They regarded these as aggregates of 9–12 BR molecules. Kuebler and Gross (247) and Usukura et al. (248) reported a lattice of ~50 Å-diameter particles in the PF faces. Robertson et al. (249) have observed similar particles and identified them as aggregates of three BR molecules displayed mainly by decoration. Thus the purple membrane contains a transmembrane protein with transmembrane mass comparable to that of other transmembrane proteins, but the individual molecules have not been resolved.

Engelman et al. (250) derived a theoretical molecular model (Fig. 6) of BR *in situ* primarily from the known amino acid sequence determined by Ovchinikov et al. (251; see also 252 and 253). The model contains nine charged residues buried in the hydrophobic core, but at least six are neutralized. BR is an inside-out protein, since there is a higher concentration of hydrophobic residues next to the lipid, with the hydrophilic residues tending to be more concentrated in the interior of the molecule (254, 255). However, the part of the molecule embedded in the hydrocarbon region is dominantly hydrophobic. If one counts the hydrophobic and hydrophilic residues drawn in Fig. 6 within the hydrocarbon region of the bilayer, 73% are hydrophobic. The cytoplasmic surface stratum is 50% hydrophobic and the outside surface stratum is 58% hydrophobic. The average hydrophobicity [H_ϕ (ave)] as defined by Bigelow (256) is 1613 for the core and 728 and 754 for the cytoplasmic and external surface strata. There is thus a strong gradient of

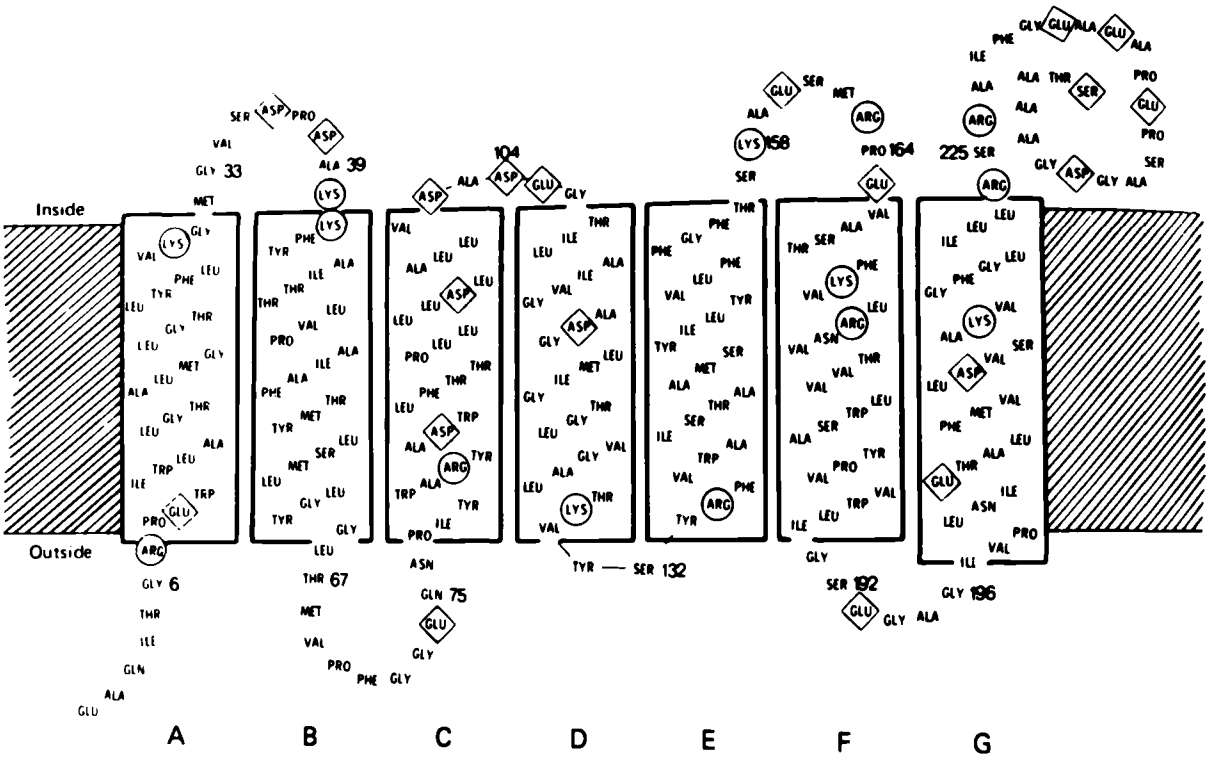

FIGURE 6 Model taken from Engleman et al. (250) of the arrangement of the polypeptide of bacteriorhodopsin across the membrane of *Halobacterium halobium*. The seven alpha helices are labeled A–G starting from the amino terminus. The hatch marks indicate the approximate location of the lipid hydrocarbon regions ○ and ◇ indicate positive and negative charges respectively. The sequence is taken from Ovchinikov et al. (251).

hydrophobicity toward the center of the membrane. This explains the distribution of heavy-metal stain in the sectioned membrane in which the protein is not stained (5, 249). According to Zaccai and Engleman (254), who studied the membrane by neutron diffraction, there is no suggestion of a hydrophilic channel that could support a column of water across the membrane through the molecule. They stated that no pockets in the protein contain 12 or more water molecules, and that their results exclude the possibility that passive transport occurs via a bulk water channel in the protein.

We shall now turn to the gap junction, the study of which has led to a much better understanding of membrane structure.

THE GAP JUNCTION: In the early 1950s Sjöstrand et al. (257–259) obtained some of the first electron micrographs of sections of epithelial cells in which surface membranes could be seen. They saw intercellular boundaries as dense lines <100 Å thick next to cytoplasm separated by a clear interzone ~160 Å wide (258). They proposed that the clear zones represented lipid and the dense zones protein, based on Sjöstrand's sections of nerve myelin in which the constituent membranes were first visualized (260), and in which he correctly interpreted the dense strata to represent protein and the light strata lipid. In 1958, he and his colleagues (261) studied cardiac muscle and resolved the previously observed single dense lines that border the intercellular clear zones into two triple-layered structures, each ~75 Å thick. However, they interpreted these as monolayers of protein. They observed narrowing in the widths of the clear intercellular zones in some places which we can recognize today as gap junctions, but they evidently believed that these simply represented variations in the thickness of the intercellular lipid layers. They also observed desmosomes, and here there was some material between the membranes that stained. This led them to postulate that the desmosome was the site of electrotonic coupling. This is interesting historically because it illustrates particularly well the importance of the paradigm in the development of the field. The unit-membrane concept cleared up this matter by identifying each of the triple-layered structures seen at intercellular boundaries as a complete cell membrane, including a lipid bilayer and the clear ~100–150 Å gap between the membranes as highly hydrated extracellular space that could be varied in thickness experimentally. About the same time (in 1959) it was demonstrated that the gaps normally present between the Schwann cell membrane and the axon membrane in the internodal regions of myelinated nerve fibers were closed in the juxta-terminal myelinated region at nodes of Ranvier (262). The gaps were present between the Schwann cell nodal processes and the axon membrane. It was recognized that the gap closures at the node would function to prevent lateral flow of ions along the surface of the axon, thus facilitating saltatory conduction. The closure of the intercellular gaps in the myelin sheath was also necessary for saltatory conduction in the same sense. The term "external compound membrane" (92) was proposed for two such membranes in close contact. The development of the above concepts laid the basis for the understanding in the next decade of the functions of both the occluding junction and the gap junction.

Close contacts of membranes were first reported in the crayfish median-giant-to-motor synapses in 1953 (263), but were not understood. Later, in 1961, micrographs of the membranes in this synapse showed the unit membranes with complete closure of the synaptic cleft (264). Furshpan and Potter (265) showed in 1959 that this synapse was an electrical rectifying one, and the significance of the closure of the cleft as a possible morphological basis for electronic coupling was immediately apparent. Karrer and Cox in 1960 (266) described membrane contacts in intercalated disks in cardiac-type muscle in mouse thoracic and lung veins, and referred to them as "external compound membranes." They recognized their probable function as sites of transmission of excitation between muscle cells. They were thus the first to publish clear electron micrographs of thin sections of what are now called gap junctions and to deduce their function correctly.

In 1962, Furshpan and Furakawa (267) found evidence in the Mauthner cell of the goldfish medulla of the presence of electrical synapses, and Furshpan presented evidence (268) that these were the club endings of Bartelmez (269) on the lateral dendrite. Thin sections of these endings showed membrane contacts ~0.3–0.5 μm in diameter in each club ending. In frontal view, these junctions showed a hexagonal array of subunits with a lattice constant of ~80–90 Å which had not been seen before (Figs. 7 and 8). These contacts were called "synaptic disks" (270–272). Figure 9 (273) is a FFE micrograph of such a junction. Dewey and Barr (274, 275) independently found evidence of electrical contacts between smooth and cardiac muscle fibers and discovered a structure very similar to the synaptic disk that they related to electrical transmission, although, like Karrer and Cox, they did not observe substructure in the membranes. They called these contacts "nexuses." In 1965, Bennedetti and Emmelot (276) observed membranes with patterns like the synaptic disk in negatively stained membrane fractions isolated from liver. Later they identified these as tight or occluding junctions (277).

Farquhar and Palade in 1963 (278) proposed a set of terms for the various junctions seen in epithelial tissues. In earlier work with sections of intestinal epithelium (Fig. 34 in reference 96) and in mesaxons of myelinated nerve fibers (Fig. 34 in reference 264), regions of focal partial fusion were noted in which unit membranes came into close contact and the overall thickness was reduced to well below twice the thickness of one unit membrane. At the same time, the two external dense strata disappeared focally and the light central zones merged for distances of ~100 Å or so. These were regarded as zones of focal partial molecular fusion. Farquhar and Palade (278–280), in surveying various epithelia in sections, saw these structures

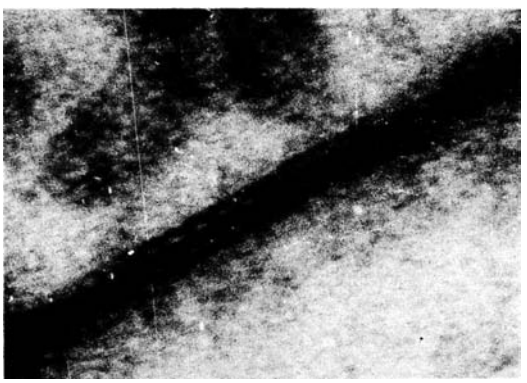

FIGURE 7 Transverse section of gap junction from a club ending on the lateral dendrite of the Mauthner cell in a goldfish brain. The two membranes are closely apposed and a beading is seen in the region of contact repeating in a period of about 80 Å. This preparation was fixed in potassium permanganate and embedded in Araldite. Under these conditions the gap in the junction does now show up. This structure was called a "synaptic disc" in the original publication in which it was presented in 1963 (271). × 443,500.

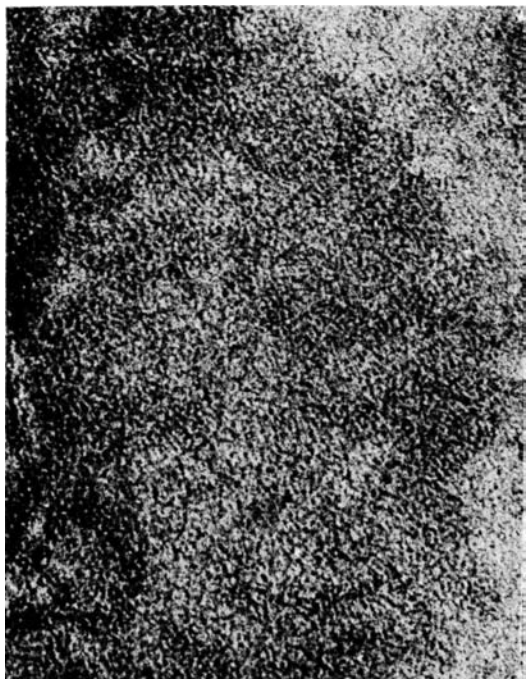

FIGURE 8 Frontal view of a gap junction similar to the one presented in Fig. 7 reproduced from the same paper (271). Note that a fairly regular hexagonal array of facets was seen. Each facet has a dense border about 25 Å wide surrounding a clear zone in the center of which is a spot about 20–25 Å in diameter. × 134,000.

and correctly deduced their function in limiting lateral diffusion of material between cells. They visualized them as beltlike structures around the junctions of epithelial cells designed to limit passage of material between cells. They called them "zonula occludens," but also used the older term "tight junction." They noted the punctate regions of partial membrane fusion, and referred to them as membrane "pinches." Figure 10 is a FFE micrograph of an occluding junction (281). The ridges correspond to the punctate regions of partial fusion in sections. Farquhar and Palade (278) emphasized the close contact of the intervening membranes, which they called regions of membrane "fusion," using fusion in the sense of close apposition. They included the "nexus" (274) in the same class as the "tight" junction. They distinguished two other types of junction, the zonula adhaerens and the macula adhaerens, to both of which they assigned an attachment function. The zonulae occludens and macula adhaerens had been described by light microscopists and called respectively "terminal bars" and "desmosomes."

A matter related to the evolution of this field is the use of lanthanum as a tracer. Lettvin et al. (282) noted that La^{+++} acted in the peripheral nervous system like a "super Ca^{++}." W. F. Pickard synthesized $La(MnO_4)_3$ and Doggenweiler and Frenk (283) used it as a fixative in 1965. They noted that La^{+++}, either introduced in this way or added by incubation in $La(NO_3)_3$ before fixation, imparted great density in the intercellular substances of the nervous system. Revel and Karnowski (284) in 1967 then developed an extracellular tracer technique based on this work, by combining lanthanum salts with glutaraldehyde. Extracellular spaces were stained generally and the techniques showed up regions of close contact between epithelial cells in a variety of different tissues which resembled very much the synaptic disc after $KMnO_4$ fixation. In order to distinguish these junctions sharply from the "tight" junctions or occluding junctions described earlier by Farquhar and Palade (278–280), they applied to them the term "gap junction." The term is now almost universally used despite the suggestion by Siminoescu et al. (285) that these junctions be called "maculae communicantes," or communicating junctions.

In 1968, Kreutziger (286) produced the first electron micrographs of FFE preparations of gap junctions. He observed on one fracture face particles in a roughly hexagonal array with a center-to-center spacing of ~80–90 Å and, on the other, a corresponding pattern of pits (see Fig. 9). Unfortunately, he misidentified the fracture faces and placed the particles in the EF face. This was carried on by others (273, 287, 288) until Chalcroft and Bullivant (289) in 1970 and Steere and Sommer (290) in 1972 independently produced complementary double replicas and correctly identified the fracture faces. It is now generally agreed that in vertebrates the particles are always in the PF faces. McNutt and Weinstein in 1970 (291) presented a model showing transverse channels crossing the junctional membranes in each face.

Peracchia (292, 293) has shown that in the crayfish lateral giant septal gap junctions the IMP localization in the fracture faces is reversed; i.e., the particles are in the EF faces and pits in the PF faces. The particles, though in rough hexagonal array, are spaced about twice as far apart (~200 Å) and the gap is distinctly wider (40–50 Å). Peracchia and Dulhunty (294) also found that the particles are much more tightly and regularly arrayed (150–155 Å spacing) if the junctions are uncoupled by treatment with Ca^{++}- and Mg^{++}-free solutions with EDTA, or by dinitrophenol.

Peracchia extended these studies to rat gap junctions (295) and again found that coupled junctions have looser, less regular particle arrays spaced at ~103–105 Å, whereas uncoupled ones have more tightly packed particles spaced at ~85 Å.

Gap junctions are involved in intercellular communication in many different animals and tissues. A large literature has developed around combined microelectrode and structral studies of intercellular communication by use of fluorescent dyes and other substances, dating back to early studies by Lowenstein from 1966 onwards (296), Potter et al. (297), Pappas and Bennett (298), Sheridan (299), and others. It is beyond the scope of this article to review this literature, but articles by Lowenstein (300) and Warner (301) in a recent symposium volume may be consulted for key references.

Several groups, following the pioneering work of Benedetti and Emmelot, isolated gap junctions from various sources for detailed chemical and structural analysis. Goodenough and Stoeckenius (302) reported a method using collagenase and hyaluronidase digestion after treatment with the detergent sarcosyl, which, as Evans and Gurd (303) also found, selectively dissolved nonjunctional membranes. The literature is confusing in that a number of different molecular-weight proteins were isolated as follows: by Goodenough in 1974 (304)—34,000 *d*, 18,000 *d*, and a doublet at 10,000 *d* called connexin A and B; by Gilula in 1974 (305)—10,000 *d* and 20,000 *d*; by Dunia et al. in 1974 (306)—34,000 *d*, 13,000 *d*, and 26,000 *d*; by Goodenough in 1976 (307)—9,000 *d* and 18,000 *d*, the latter called "connexin" and used as the basis for a model (308); by Duguid and Revel in 1975 (309)—26,000 *d* and 36,000 *d*; by Benedetti et al. in 1976 (310)—34,000 *d* and 26,000 *d*; by Culvenar and Evans in 1977 (311)—38,000 *d* and 40,000 *d*; by Zampighi and Robertson in 1977 (312) and Zampighi in 1978 (313)—25,000 *d*; by Ehrhart and Chauveau in 1977 (314)—34,000 *d*; by

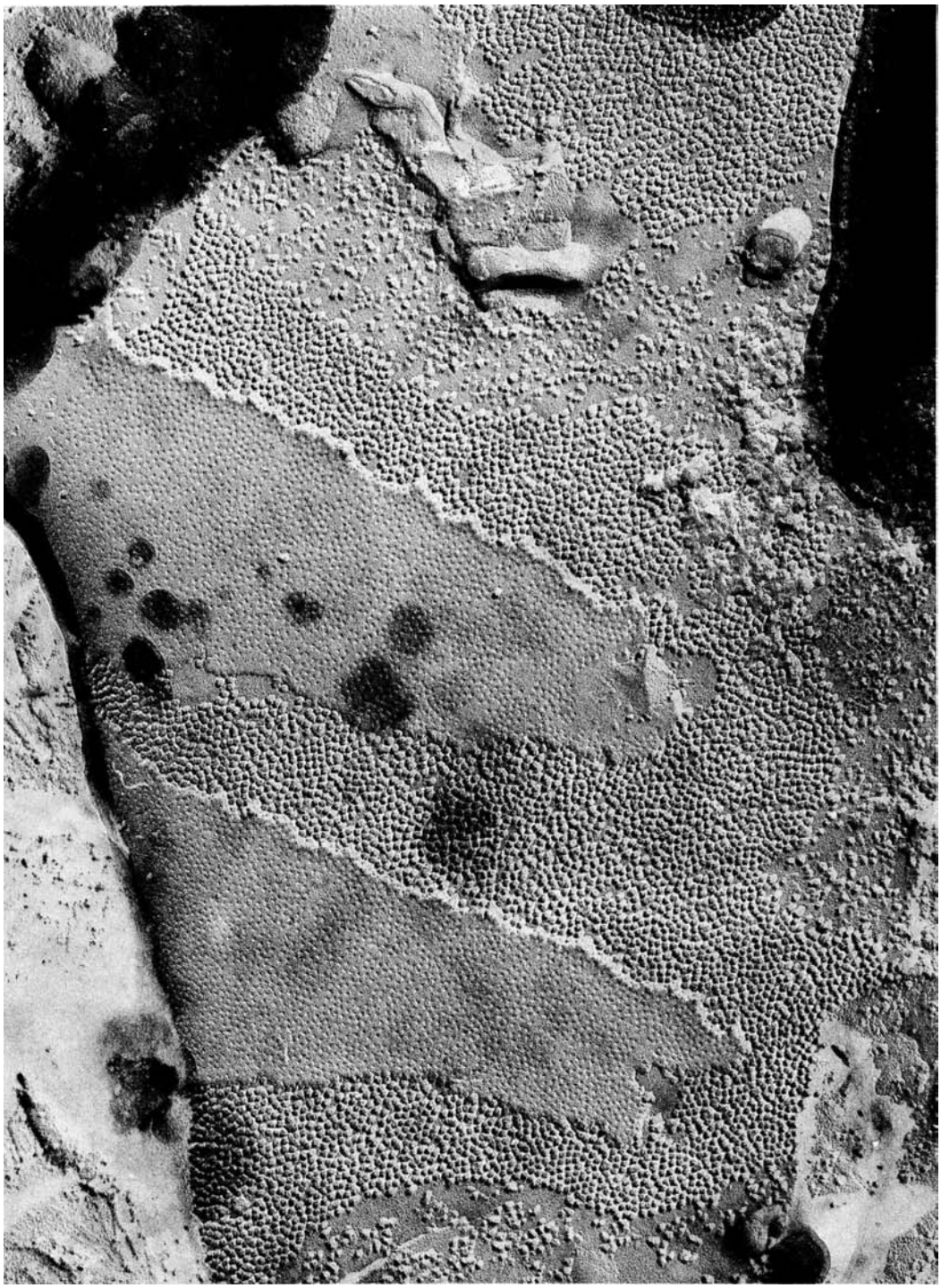

FIGURE 9 Freeze-fracture preparation of a gap junction taken from Goodenough and Revel (273). Note that the junction consists of rather irregular arrays of particles alternating with regions in which pits are seen. The particles are located on the PF face of one of the junctional membranes and the pits are located in the EF face. × 102,500.

Gilula in 1978 (315) and Hertzberg and Gilula in 1979 (316)—47,000 *d* and 27,000 *d*.

In 1979, Henderson et al. (317) clarified some of these conflicting reports. They avoided enzyme treatments for purification, used 6 M urea (310) in the isolation procedure, and avoided boiling in sodium dodecyl sulfate (SDS). They concluded that there were only two molecular species present at 26,000 *d* and 21,000 *d*, and that the smaller one was probably a degradation product. The higher molecular-weight components were considered to result from aggregation of the hydrophobic 26,000 *d* components in boiling SDS. This component was reduced to 13,000 *d* by trypsin treatment. They performed amino acid analyses on the 26,000 *d* fragment and noted that it had a hydrophobicity index discriminant function, $Z = 0.322$, referring to the classification of Barrantes (318), who found that integral membrane proteins all have Z values in excess of 0.317. The trypsin-treated protein showed a distinct increase in Z value to 0.589, due to a reduction in the total content of hydrophilic amino acids. They concluded that the 13,000 *d* trypsin-resistant component was probably buried in the lipid

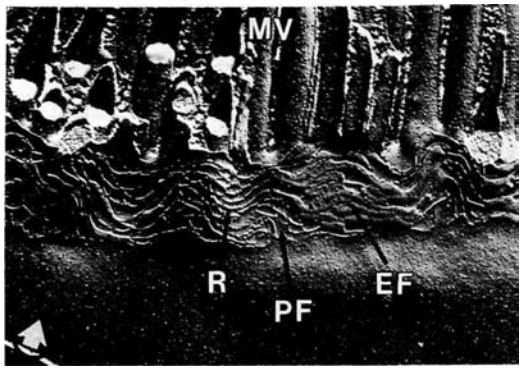

FIGURE 10 Freeze-fracture of zonnula occludens between glutaraldehyde fixed epithelial cells in the ileum of an adult rat. After fixation the junction features a characteristic belt like network of branched and anastomosing ridges (R) on the PF face and corresponding furrows on the EF face. Microvilli (MV) are seen above. An extensive PF face is seen below. × 42,380.

as the transmembrane part of the molecule. They also noted that there is a very high molar ratio of cholesterol in the junctions despite the detergent treatment, and suggested that it plays a structural role.

Finbow et al. (319) have reported an independent line of evidence which also suggests that the major gap-junctional protein is the 26,000 *d* component. This group had shown (320, 321) that partial hepatectomy caused gap junctions to disappear during a postoperative period of 24–28 h with a return to normal within 48 h. They noted that the 26,000 *d* component was absent in the 24–28-h period and reappeared at 48 h. There is now general agreement that the principal gap-junctional protein has an apparent molecular weight of 26,000 *d*.

Caspar et al. (322) and Makowski et al. (323) conducted a combined chemical, electron microscope, and X-ray diffraction study of isolated gap junctions which led them to propose a model. They relied heavily on a micrograph published by Goodenough in 1976 (307, 324), interpreted as showing PTA filling an ~20 Å transverse hydrophilic channel in an isolated junction. This led them to postulate the existence of aqueous ~20 Å -diameter, protein-lined channels completely traversing the junctions. They supposed that flow through these channels was regulated by variations in the diameter of the channel in the region between the two membranes. They applied the name "connexon" for the complete channels that run across each of the two membranes of a given junction.

It has long been clear that some sort of transverse channel structure is present in the unit membranes of the junctions. Lowenstein, for example, has shown that molecules up to 20 Å in diameter can pass through (300). However, electron micrographs failed to show direct evidence of any such pore. Thus, Zampighi and Robertson, in 1974, (325) found that it was possible to degrade the isolated junction selectively by treating it with EDTA or EGTA. After treatment, the junction broke up into fragments that consisted of only a few of the repeating units, some of which were found to lie on their side in negative stain. No evidence of transverse channels was found. A channel ~20 Å in diameter would be expected to fill and be seen under these conditions, because there is a comparable hole in tobacco mosaic virus that fills readily with PTA (326). It was concluded that the channel must be smaller than the ~20 Å suggested by the size of the stain accumulation between the two membranes. This brought into focus the problem of the nature of the channel. Obviously, one possibility would be a tubular structure consisting, perhaps, of something like a β-pleated sheet of polypeptide chains rolled into a cylindrical form with hydrophobic residues on the outside and hydrophilic residues inside that form a channel ~20 Å in diameter. However, this would be seen in electron micrographs. To be sure, others claim to have seen the expected structure, but the evidence presented (306, 307, 324) was not acceptable (see reference 5). Thus, it seemed unlikely that the earlier models (308, 322, 323) were correct.

Zampighi et al. (327) have reported recently on a gap-junction fraction (313) studied in thin sections and by negative staining using stereo-image analysis techniques. The major conclusion reached was that transverse channels could not be seen clearly in sections nor in edge-one views of the junctions in negative-stain preparations. Tilt studies showed clearly that the ~20 Å pools of strain seen in frontal views of negative-stain preparations did not exist as columns running through the two junctional membranes.

Zampighi and Unwin (328, 329) pursued these studies by employing a minimal-dose electron microscope technique (157, 158). They worked out the three-dimensional (3-D) structure to a level of 18 Å resolution. They concluded:

(*a*) The junctions may exist in two forms, A and B depending on detergent content.

(*b*) The connexon consists of six slightly twisted protein subunits asymmetrically disposed across each membrane with considerable mass protruding from the bilayer surface on the outside but essentially none on the cytoplasmic side. Twist is greater in the A form.

(*c*) The negative stain was concentrated in two regions between the two junctional membranes.

(*d*) A barely detectable amount of stain penetrated the channel through the two adjacent membranes.

(*e*) The regulation of size of the channel was most probably localized to the core of each bilayer. These results led them to postulate a heuristic model for the junction with the six subunits arranged diagonally across the bilayer. Fig. 11 from Zampighi and Unwin (328) shows contour maps of cross sections of one membrane of junctions in each of the two states.

From the above it is clear that the channel in the closed state is a hydrophobic structure overall. It reacts to heavy-metal stains like BR in the purple membrane. How can this be reconciled with the function of the channel? We know that the channel must be able to pass hydrophilic molecules up to ~20 Å in diameter (294). However, in isolation the channels are hardly penetrable by much smaller heavy-metal stain molecules. Clearly, the core part of the channel must be a dynamic structure capable of marked changes. There must be more hydrophobic amino acid residues in the core than hydrophilic ones, as in the purple membrane, but there must be some way for the hydrophilic residues to be arranged to make a transverse hydrophilic channel up to ~20 Å in diameter in the open condition, but still able to return to a very different arrangement in the closed state. It will be exciting to see how the evidence develops as we learn more about this fascinating structure and are able to arrive at a precise understanding of how it is constructed and functions.

Conclusion

This chapter has attempted to trace the evolution of our ideas about the molecular structure of cell membranes as embodied in various paradigms. The FM model provided a

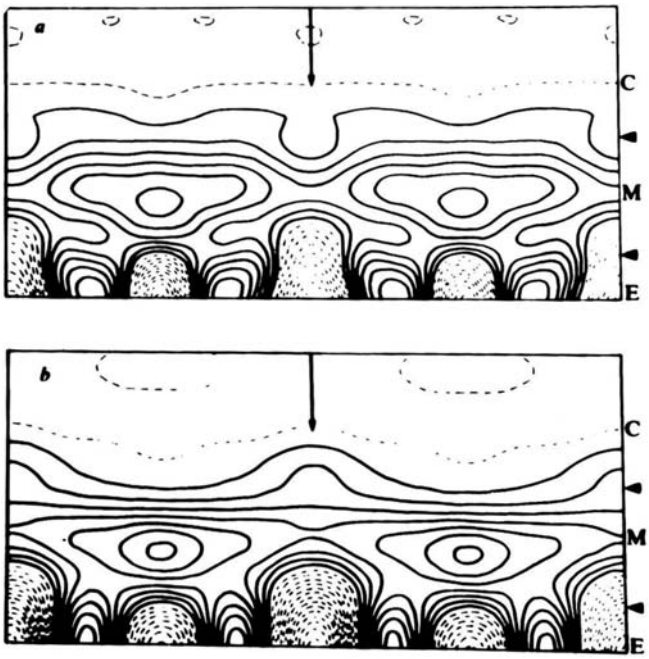

FIGURE 11 Contour maps of two perpendicular sections through one membrane of two different gap junctions showing two different states in which the junction can exist. Arrowheads point to the approximate locations of the cytoplasmic (upper) and extracellular (lower) membrane surfaces. The zones (C, M and E) refer to the cytoplasm, membrane, and extracellular space, respectively. Contours corresponding to stain excluding regions (negative contours) are drawn as continuous lines. Contours corresponding to stain-filled regions (0 and positive contours) are drawn as broken lines. The sections contain two unit cells in the horizontal direction and half of the junction in the vertical direction. Note the concentration of stain in the central region along the connection axis mainly between the two membranes. A smaller concentration of stain occurs at the periphery of each subunit. Two states are shown. There is a slight opening in the center of the connexon to the top state (*a*). In the transition between the two states matter moves toward the connexon axis (vertical arrow) to close the slight opening in the cytoplasmic surface in going from *a* to *b*.

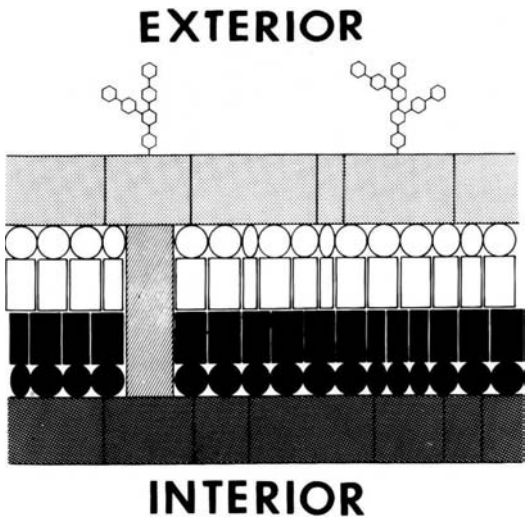

FIGURE 12 Highly schematic diagram of model of a cell membrane. The lipid bilayer core is represented by the joined circle and rectangular figures. The asymmetry in the lipid bilayer discussed in the text is represented by filling in the nonpolar carbon chain regions (rectangles) and head groups (circles) of the lipid molecules in one half of the bilayer. The protein constituents are cross-hatched differently to indicate the asymmetry of the inner and outer protein components and the existence of transmembrane protein components is indicated by a third cross hatch pattern. The presence of sugar residues in the external surface of the membrane is represented by the branched chains of joined hexagons in the external surface. The molecules are drawn approximately to scale but very schematically. The bilayer is about 50 Å thick and each protein monolayer is about 20 Å thick. No effort is made to show different kinds of lipid molecules but the fact that the lipid molecules are in a relative liquid state is indicated by showing different projections as seen in different states of rotation. Fluidity owing to flexing of the hydrocarbon chains is not shown.

very useful membrane paradigm for the 1970s. It focused attention on transmembrane proteins and membrane fluidity at a time when these features of membranes were coming to the forefront of membrane research. The model served a very useful purpose in emphasizing the importance of transmembrane proteins. However, as with all models, oversimplifications inevitably occurred. It became obvious almost immediately that the fluidity element had been greatly exaggerated and that depicting much of the bilayer as naked was incorrect. The model also has been misleading in suggesting that there is quantitatively more protein in the hydrophobic core of the bilayer than in most membranes. Also, the model has led some to regard the ubiquitous IMPs as metal-plated protein molecules. It is now apparent that IMPs are more complex. Even when related to transmembrane proteins, they do not give a faithful representation of those proteins. Polypeptides are present in the bilayer core in much smaller quantity than the number and size of the IMPs suggest. Finally, the FM model has supported the concept of permanent hydrophilic, water-filled, transmembrane channels (12, 316), for which there is no structural evidence. It is thus quite clear that the FM model needs to be revised in significant ways, although some of its features remain valid. Fig. 12 presents in a highly schematic fashion a model, referred to as the hydrophobic barrier model, incorporating features of all contemporary membrane models.

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Extracellular Matrix

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The advances during the past 25 years in our understanding of the biology of the extracellular matrix seem particularly remarkable in the context of a review volume such as this one. If we begin by taking stock of the situation in 1955 and merely look at a few of the most significant aspects of this new knowledge, it becomes apparent immediately how much a well-funded and appropriately staffed effort in unprogrammed basic science can accomplish in a short space of time. From 1951 to 1955, Highberger, Gross, and Schmitt were the first workers to use the electron microscope to analyze the "needlelike" precipitates that Orekhovich et al. (1) had produced from citrate extracts of connective tissues. By varying the in vitro precipitating conditions, they created fibrous-long spacing (FLS) and segment-long spacing (SLS) configurations, as well as "native" collagen fibrils with the 67-nm repeat period seen in tissues; from their electron microscope and X-ray diffraction data, they deduced the dimensions of the molecule, which they called "tropocollagen," to be about 300 nm long and less than 2 nm wide (2), a figure Boedtker and Doty (3) soon confirmed with physical chemistry. In 1956, a supplement to the second volume of this journal, heralding recent advances in the field of biophysical and biochemical cytology, included a now-classical article by Gross (4) summarizing the new "quarter-stagger" model of native fibril structure (Fig. 1) and predicting the implications of these in vitro collagen "self-assembly" experiments for cell and developmental biology.

The advances in collagen biology since then that I shall summarize in the ensuing pages include discovery of (*a*) the three polypeptides (α-chains) comprising "tropocollagen"; (*b*) the precursor "procollagen" molecules; (*c*) the enzymes that posttranslate, cross-link, and degrade the molecules; (*d*) the cell pathways and cell types involved in collagen biosynthesis; (*e*) the effects of collagens on cell differentiation; and (*f*) the genetic diversity of collagen structure. The implications of such discoveries for clinical medicine are summarized by Gross (5) in his 1973 Harvey Lecture and more recently by Lapiere and Nusgens (6), Prockop et al. (7), and Bornstein et al. (8, 8a). We are now arriving, for example, at an understanding of diseases characterized by excess procollagen (and the concurrent absence of procollagen peptidase), birth defects deficient in the cross-linking of collagen molecules, and the abnormalities of collagen degradation that occur in wound healing and disease.

In 1955, the ground substance surrounding the collagen fibrils of the extracellular matrix was conceived as consisting of neutral and acid mucopolysaccharides that were best visualized in frozen sections stained by the periodic acid–Schiff method or by basic dyes (9). The so-called "neutral heteropolysaccharides," whose sugar moieties are reduced to aldehydes by periodic acid, must have included collagen—some forms of which are highly glycosylated—fibronectin, and some of the other structural glycoproteins that have recently been characterized; the term fortunately has now been discarded. The "acid mucopolysaccharides" consist of hyaluronate (HA), chondroitin, chondroitin-4 and -6 sulfates (CS), dermatan sulfates, keratan sulfate (KS), heparan sulfate (HS), and heparin (Fig. 2); they are now called glycosaminoglycans (GAG). With the exception of HA, GAG in tissues are always linked covalently to protein as proteoglycans (PG).

For the most part, GAG biochemists in the first decade covered by this review worked independently without much interaction with cytologists (10, 11). One of their most important recent breakthroughs has been the discovery that, in cartilage, CS and KS are linked to core proteins which are attached to HA to form polymers after the monomers leave the cell. GAG and sugars in general are not well preserved by aldehydes and osmium, but in the past 5 years, electron microscopists applying Luft's (12) ruthenium red (RR) fixation have been routinely visualizing GAG in sections and correlations with biochemical (13) and morphogenetic (14) studies are progressing rapidly. Other matrix proteins such as elastin, fibronectin, and laminin are being characterized biochemically and by immunohistochemistry. These structural and developmental studies will undoubtedly have payoffs for the health sciences. Recent advances in our understanding of PG degradation and immunology are already beginning to shed light on the mechanisms that may be involved in osteoarthritis, rheumatoid arthritis, corneal macular dystrophy, and various abnormalities of inflammatory processes (13, 15).

In this review of the past 25 years of extracellular matrix, emphasis will be placed on the discoveries of particular relevance to cell and developmental biology. The original articles that seem, to this author, to have been the most significant in the unfolding of the story will be cited together with review articles that provide a more comprehensive analysis. With this selective overview, the author invites the cell biologist to sample the exciting progress in knowledge of the extracellular matrix that has occurred since the founding of *The Journal of Cell Biology*.

Collagen

MOLECULAR AND SUPRAMOLECULAR STRUCTURE: The "quarter-stagger" model of the native collagen fibril

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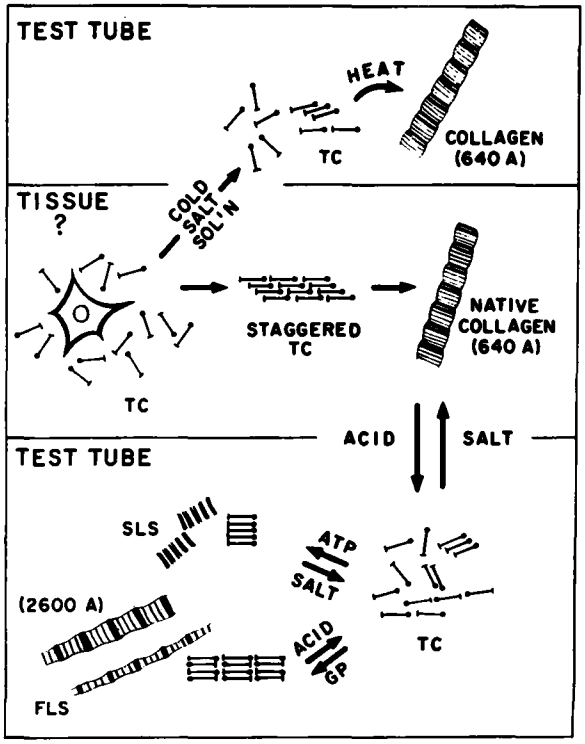


FIGURE 1 Tropocollagen (TC) polymerizes into different structures under different conditions. If extracted from the tissue with cold salt solution (SOL'N) and heated to 37°C in a test tube, it forms collagen fibrils with the native striation period (~640 Å) like those found in tissues in vivo. These can be dissolved with acetic acid and reprecipitated with salt (e.g., 0.1 M NaCl). Addition of ATP to an acid solution of tropocollagen produces SLS crystallites. In the presence of α1-acid glycoprotein (GP), tropocollagen forms FLS fibrils with a repeating period of 2,600 Å. From Gross (4), courtesy of The Rockefeller University Press.

(Fig. 1) was modified by Hodge and Petruska (16) to include a "hole" region 40 nm long between the ends of the linearly arranged molecules. As result of this gap, the front end (N terminal) of a quarter-staggered molecule overlaps the back end (C terminal) of a laterally associated molecule only by 27 nm, instead of by the whole 67-nm repeat unit; the repeat unit is thus only approximately one-quarter of the length (290 nm) of the molecule (Fig. 3). The hole can be visualized by negative staining as a dark region 0.6 of a period in length (Fig. 4). Positive staining with metal salts can be used to correlate loci of charged residues with the overlap region (Fig. 4); the characteristic *a–e* bands in the repeating period thus demonstrated have now been renumbered I–XII (17).

In the remarkable SLS configuration, the collagen molecules are aligned in parallel with their C and N terminals in register (Fig. 1). In the decade following its discovery, the SLS collagen configuration has been used to splendid advantage by Hodge and Schmitt (18) and others (19, 20) to analyze the axial band structure of the fibril, by Gross and Nagai (21) to localize the action of animal collagenase, and by a number of investigators to help in sequencing the polypeptide chains of collagen (5, 20) and to characterize the telopeptide extensions of procollagen (5, 22). Moreover, inasmuch as the relation of the SLS bands to native fibril bands is known and the position of the charged amino acids in SLS is also known, the amino acid sequence can actually be related to the striated pattern in native collagen fibrils (20). The SLS pattern can also be used to distinguish

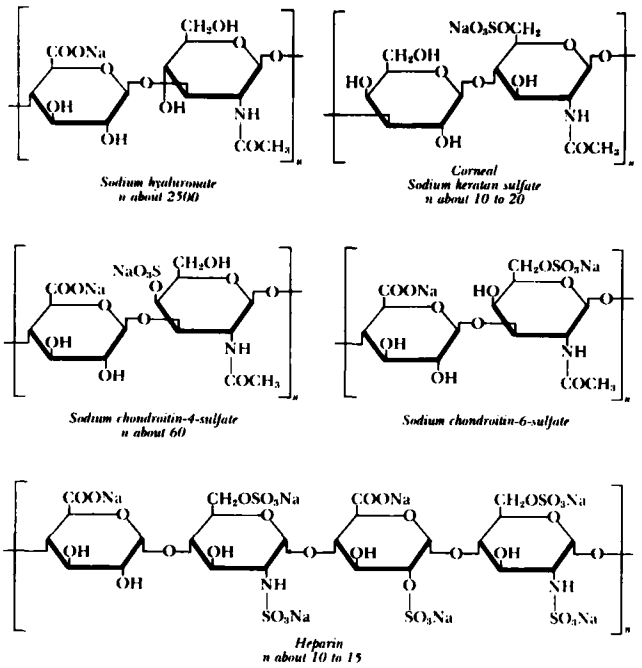


FIGURE 2 Structure of the repeating disaccharide subunit of hyaluronate, keratan sulfate, chondroitin sulfates, and heparin. The glycosaminoglycans contain numerous such subunits. The molecular weights of sulfated GAG are usually in the range 1–2 × 10^4, but hyaluronate may be as large as 1–2 × 10^7. The saccharides shown here are connected by alternating β1–4 and β1–3 bonds (hyaluronate, chondroitin, and keratan sulfates) or α1–4 bonds (heparin). From Schubert and Hamerman (10), courtesy of Lea & Febiger.

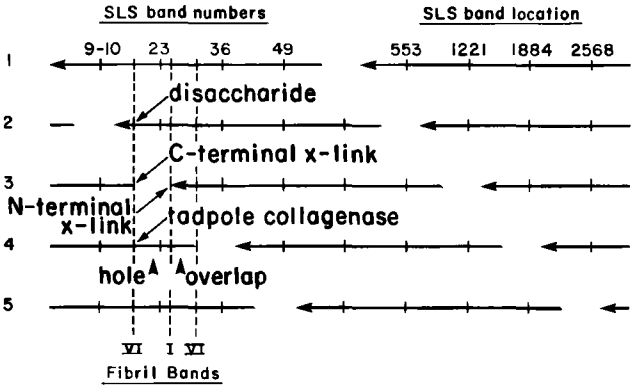


FIGURE 3 The approximate arrangement of tropocollagen molecules in a collagen microfibril. The molecules are staggered in such a way as to create a hole between the C terminal of one and the adjacent N terminal of the next molecule. The SLS band location is given in angstroms from the N-terminal end. The sites of covalent bonding of disaccharides to α-chains, of C-terminal cross-linking, and of tadpole collagenase cleavage are all near the amino terminal edge of the hole zone, whereas the site of amino terminal cross-linking is near the carboxyl terminal edge of the hole zone. From Bruns and Gross (17), courtesy of John Wiley & Sons, Inc.

type II collagen from type I collagen (23). These studies provide some of the most interesting applications of electron microscopy, using positive and negative staining, to the bridging of the invisible link between molecular and supramolecular structure.

Chromatographic procedures using carboxymethyl-cellulose developed in the early 1960s by Piez and his co-workers (24) made it possible to separate the three polypeptides (α-chains)

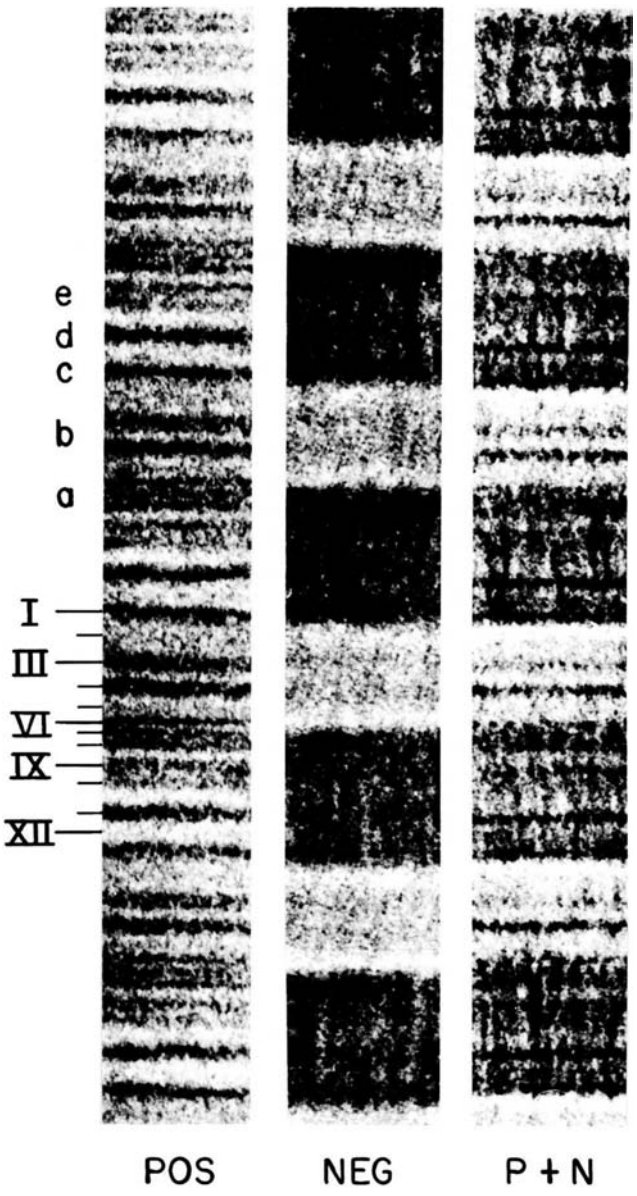


FIGURE 4 The native banding pattern of the collagen fibril is shown here as revealed by positive (POS) staining using phosphotungstic acid, by negative (NEG) staining using uranyl nitrate, and by a combination of positive and negative (P + N) staining. The old nomenclature for bands a–e has been replaced by a newer nomenclature (I–XII); all 12 bands are marked to the left of the positive-stained fibril, although only 5 are numbered. In the negative-stained fibril (center), the hole zone is the dark area which has filled in with stain. The repeat period is 67 nm. Courtesy of R. R. Bruns.

composing the collagen molecule ("tropocollagen") into two kinds; one chain ($\alpha 2$) migrates more slowly than the other ($\alpha 1$) because it contains more basic amino acids. The collagen of bone and skin, now known as type I collagen, was shown to consist of two $\alpha 1$-chains and one $\alpha 2$-chain, designated $[\alpha 1(I)]_2$-$\alpha 2(I)$. Each α-chain is coiled in the form of a polyproline type II helix and the three α-chains in turn are wrapped around each other to form a superhelix, except for the two short nonhelical regions at the C and N terminals which are important in intermolecular cross-linking.

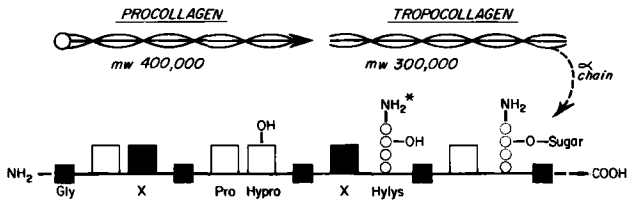
It soon became apparent that not every collagen molecule contains two $\alpha 1$-chains and one $\alpha 2$-chain. In 1969, Miller and Matukas (25) found a new $\alpha 1$-chain in cartilage and postulated the structure of cartilage collagen to be $\alpha 1(II)_3$. Isolation of the intact molecule from the cartilage of lathyritic chicks by Trelstad et al. (23) made further characterization possible. Type II collagen has now been isolated from a number of ocular tissues, including the embryonic avian cornea (26). Limited pepsin digestion of the nonhelical ends of the molecules allows still a third type of collagen $\alpha 1(III)_3$ to be extracted, mainly from reticular connective tissue (27), and extensive degradation releases type IV collagen, a rather complex molecule containing intermittent nonhelical regions (28). Kefalides and his co-workers have shown that type IV collagen is a major constituent of basal laminae or the so-called basement membranes (28–30). Type V (AB_2, ABC) collagen seems to derive mainly from basement membranes. An $\alpha 1(I)$-trimer has also been described. This nomenclature for the collagen types, which is now based on the chronology of discovery, is in need of revision to become more functionally meaningful in the future (see reference 8 for additional discussion of variants in collagen types).

The α-chains obtained by the carboxymethyl-cellulose chromatography method (24) have now been extensively analyzed biochemically. The $\alpha 1$-chains of the different collagens differ from each other and from $\alpha 2(I)$ in amino acid composition. We know the entire sequence for the 1,000 or so amino acids of the $\alpha 1(I)$-chain (31). Cyanogen bromide cleavage of the molecule, electron microscopy of SLS patterns of renatured segments, and metabolic experiments using isotope incorporation played important roles in these analyses (5, 20). It is beyond the scope of this article to discuss these chemical studies of the molecule. Suffice it to say that collagen, which has attracted the attention of electron microscopists for three decades because of its highly patterned supramolecular structure, is also a distinct and exciting protein chemically. Every third residue is glycine and about every fourth residue is proline or hydroxyproline; of the remaining residues, hydroxylysine is of particular importance (Fig. 5); the role of these residues in the helical structure of the molecule, in cross-linking, and in glycosylation is a fascinating story (5, 7, 8, 32, 33). Not only is the amino acid sequence (primary structure) unique, but also the number of posttranslational steps involved in the synthesis of collagen is highly unusual. At least seven different enzymes operate on the molecule after its release from the polyribosome; these will be discussed later in more detail.

Of the discoveries relating to collagen structure in the past decade, few have attracted more attention than the demonstration of its high molecular-weight precursor, procollagen. Schmitt (34) in 1960 had postulated that enzymatic removal of a terminal peptide might be needed before monomeric collagen could polymerize into fibrils and Speakman (35) predicted the existence of telopeptides that function as a "registration" mechanism. A high molecular-weight precursor, in triple helical form, that might fulfill these functions was found in 1971 in cultures of skin fibroblasts by Layman et al. (36) and in homogenates of rat calvaria by Bellamy and Bornstein (37). These studies were quickly extended in a number of laboratories (see references 5 and 7 for review). The nonhelical procollagen "extension" peptides are cleaved by procollagen proteases during or after secretion from the cell, leaving short nonhelical telopeptides on the final molecules.

Unexpected progress has been made in the past decade in the immunology of collagen, and these studies have confirmed and extended our knowledge of the genetic diversity of collagens. At the beginning of the 25-year period under review, Watson et al. (38) reported the first unequivocal evidence that

collagen is an antigenic protein. Schmitt et al. (39) called attention to the importance of tyrosine in the antigenic activity of the protein, an idea that has been partially substantiated by subsequent studies by Timpl (40) and others. Collagen is a weak immunogen. The major antigenic sites that have now been characterized in the collagen molecule per se are helical determinants (conformation-dependent), central determinants (sequence-dependent), and terminal determinants (in the short, nonhelical ends). Procollagen is more antigenic than collagen because of the increased number of terminal determinants. Most of the earlier studies by Timpl, Dehm, Furthmayer, von der Mark, and others (40) usually employed rabbits as hosts, but other animals have been used, and recently Linsenmayer and co-workers (41, 42) have obtained monoclonal antibodies to type I and type II collagens.



	INTERSTITIAL COLLAGENS				BASEMENT MEMBRANE		
	∝1(I)	∝2(I)	∝1(II)	∝1(III)	TYPE IV	TYPE V A	TYPE V B
3OHPro	0	1	2	1	11	7	9
4OHPro	99	90	100	115	125	110	109
Pro	117	115	115	95	62	99	119
Gly	333	333	333	350	340	346	334
Ala	125	120	105	90	33	53	45
Hylys	5	5	20	7	45	23	37
Sugar (galactose, glucosylgalactose)	5%	1%	5%	1%	10%	2%	5%

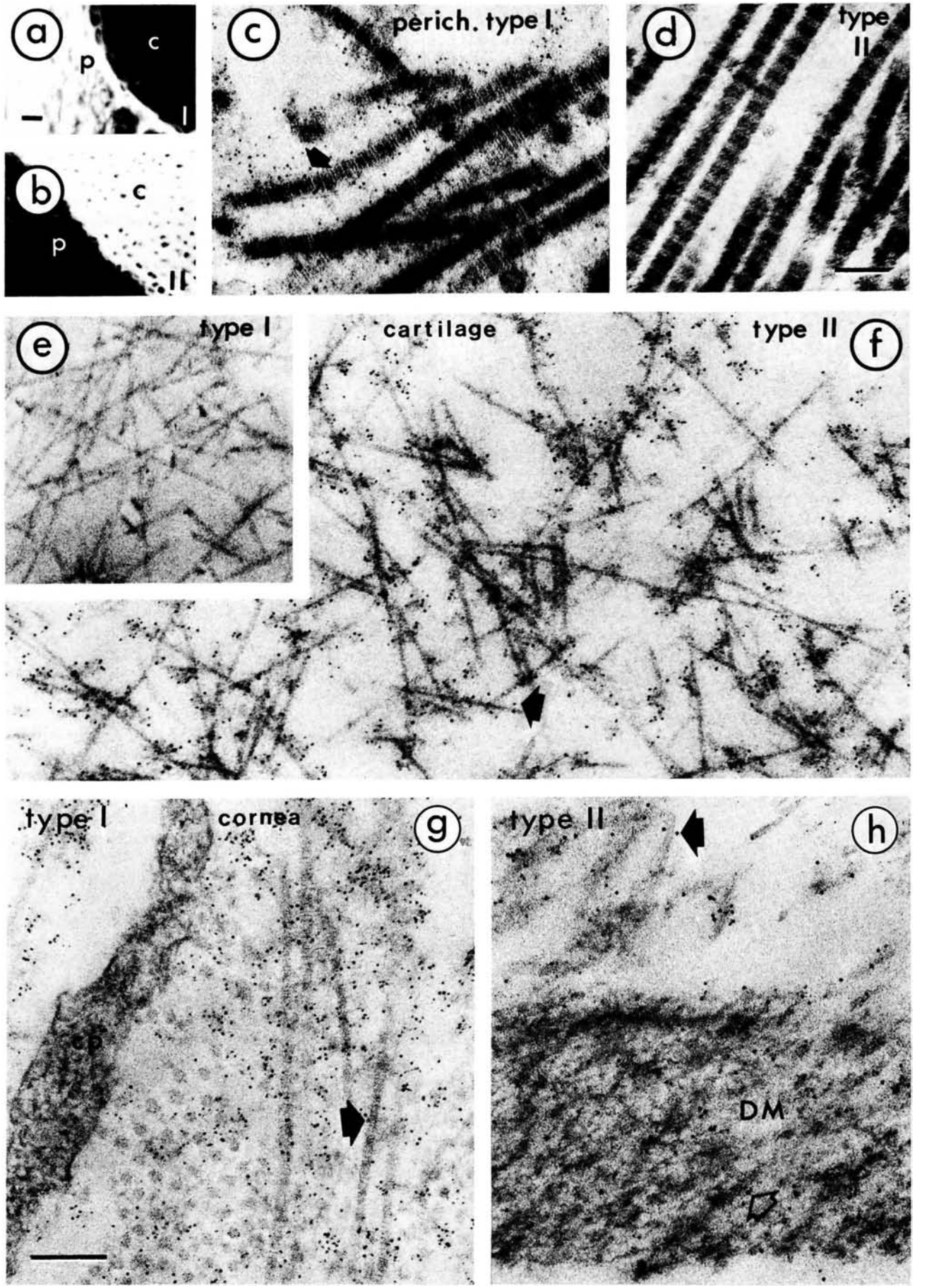
*covalent crosslink

FIGURE 5 Some major chemical features of collagen molecules. There are many more helices in the tropocollagen molecule than indicated here. Part of the amino acid sequence of one of the three component α-chains is shown in the center of the diagram. Glycine (Gly) occurs in every third position, and proline (Pro) and hydroxyproline (Hypro) comprise 20–25% of the remaining residues. 3OHPro is more abundant in basement-membrane-type molecules than in interstitial collagens, but 4OHPro is the major form of this imino acid in all collagens. Hydroxylysine (Hylys), although outnumbered by alanine (Ala), glutamine, and others not shown (X, middle diagram), is a very important amino acid because it binds the sugars and also plays a role in cross-linking (*). Type I collagen is the only interstitial collagen depicted here that contains an α2-chain [α2(I)]. Although type V collagen is a component of basement membranes in some tissues, it may also prove to be an interstitial collagen. From Hay et al. (26), courtesy of Academic Press, Inc.

The applications to immunohistochemistry are obvious and only a few can be listed here. Type III collagen has been shown by immunofluorescence to be a component of reticulum (43). Type II can be localized in cartilage matrix and type I in the perichondrium by antibodies at the immunofluorescent level (44). Extended to the electron microscope, both rabbit and monoclonal antitype II antibodies can be seen with ferritin conjugates to label the nonstriated fibrils of hyaline cartilage matrix (Fig. 6). The immunoferritin technique applied at the electron microscope level confirms the codistribution of types I and II collagens in the embryonic chick cornea revealed by immunofluorescence (45) and raises the interesting possibility that types I and II collagens can copolymerize in the same fibril. The distribution of collagens in the corneas of mammals as studied by immunofluorescence (46) reveals the unexpected presence of type III collagen, which is absent in the avian cornea (45). These and other immunohistochemical studies of embryos and adults (40) fail to reveal, in the distribution of the collagen types, any fundamental physiological or developmental reason for the genetic diversity that has been demonstrated. With the exception of type IV collagen, which seems to occur exclusively in basement membranes (30, 47), all of the collagens that have so far been isolated may be capable of forming striated fibrils. Striated fibrils of type III (reticular connective tissue) and type II (articular cartilage, cornea) are generally smaller (25–50 nm) than those of type I (50–200 nm), but the distinctions are minor and may be related to the PG composition of the tissue (14, 48, 49).

The supramolecular organization of collagens in the tissues and cuticles of vertebrates and invertebrates (50–52) is impressive, and we have much to learn about how it is brought about. One of the striking discoveries of the newly invented electron microscope, reported in 1954 by Jakus (53) and by Weiss and Ferris (54), was the fact that striated collagen fibrils are arranged in a remarkable orthogonal array, each layer at right angles to the next, in the dermis of lower vertebrates (54) and in the cornea (53). These orthogonal layers may be displaced in an angular fashion that reflects the organization of the overall body, rather than the specific organ (55, 56). In Descemet's membrane, collagen is arranged in an elaborate series of nodes (57, 58), and in cartilage, collagen may form a meshwork of overlapping fibrils (Fig. 6) or polymerize into striated fibrils (at joints). In bone, the striated collagen fibrils become mineralized and the "hole" region of the fibril may serve to anchor the apatite crystals (59). The basic unit of native collagen fibrillogenesis seems to be a microfibril 4–5 nm wide, composed of several collagen molecules, which aggregates laterally with other fibrils in a spiraling pattern, much as a rope is assembled (see references 26, 49, and 60 for further discussion of the molecular organization of collagen fibrils and variations in length of the repeat period).

FIGURE 6 An immunohistochemical analysis of collagen distribution in the perichondrium (p, perich.), cartilage matrix (c), and cornea of 12-day-old chick embryo. By immunofluorescence, the perichondrium and cartilage matrix are seen to stain coexclusively with monoclonal antibodies to type I collagen (*a*) and type II collagen (*b*). At the electron microscope level, ferritin-labeled antibodies reveal type I, but no type II, collagen in the striated fibrils of the perichondrium (*c, d*). The nonstriated fibrils that characterize cartilage matrix contain type II collagen (*f*) and do not label with anti-type I collagen (*e*), whereas the striated fibrils of the cornea contain both type I (*g*) and type II collagen, which are codistributed in the young cornea. At 12 days, type II collagen (*h*) is intermingled with type I collagen only in the anterior and posterior (*h*) stroma. The nodes and strands (open arrow, *h*) comprising Descemet's membrane (DM) are labeled by antibodies to type II collagen. Staining patterns with conventional antibodies are similar to those obtained with monoclonal antibodies, whereas controls show no staining. Closed arrows indicate ferritin on collagen fibrils. cp, cell process. *a, b,* × 100 (bar = 40 μm); *c–f,* × 100,000 (bar = 100 nm); *g, h,* × 150,000 (bar = 100 nm). From Hendrix et al. (58), courtesy of the C. V. Mosby Co.


a
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BIOSYNTHESIS OF COLLAGEN: Collagen secreted from fibroblasts may polymerize along the cell membrane or in cellular recesses (61). The close association of collagen with the cell surface, together with the abundant subplasmalemmal cytoplasmic filaments that they observed in fibroblasts and chondrocytes, led Porter and co-workers in 1959–1960 to conclude that collagen is secreted from the cell in part by "excortication" or "ecdysis," that is, by direct shedding of the cytoplasmic filaments (which they presumed to be collagenous) through the plasmalemma into the extracellular space (62–64). Because chondrocytes and fibroblasts contain abundant ergastoplasm (granular endoplasmic reticulum) and Golgi elements (63–65), some investigators argued that collagen secretion must follow the same pathway Palade and others had demonstrated in the pancreas (65). In one of the first autoradiographic studies to be carried out at the electron microscope level, Revel and Hay (66) in 1963 demonstrated that [^{3}H]proline is incorporated in the granular endoplasmic reticulum of chondrocytes into a proline-rich product, some of which moves 20–30 min later into the Golgi zone where it accumulates in the large Golgi vacuoles (Fig. 7); 1–2 h later the vacuoles begin to discharge their contents into the extracellular matrix where the labeled proline-rich products polymerize some distance from the cells. Because the principal extracellular protein produced by chondrocytes is collagen and collagen is uniquely rich in proline, this product was certainly collagen in large part; other secreted proteins that would be labeled by proline include proteoglycans and the telopeptide extensions on collagen.

The idea that collagen is synthesized by polyribosomes (Fig. 7*a*) and accumulates in the granular endoplasmic reticulum has been supported by the quantitative autoradiographic studies of Ross and Benditt (67) and Weinstock and Leblond (68), by isolation of microsome fractions (69), and by the immunohistochemical studies of Olsen and co-workers (70–72). Although the cytoplasmic ground substance incorporates [^{3}H]-proline in growing cells (73), the intracytoplasmic filaments formerly in question are now generally agreed to be cytoskeletal in nature (74, 75). The immunohistochemical (Fig. 7) and autoradiographic data, moreover, leave no doubt that a major part of the content of the Golgi vacuoles can be collagen. Morphologically, the Golgi product may appear amorphous (72, 76), fibrillar (66), or condensed into segments that are somewhat FLS-like in character (61, 68). In chondrocytes, Golgi vacuoles contain collagen-like filaments and/or PG granules (13, 66).

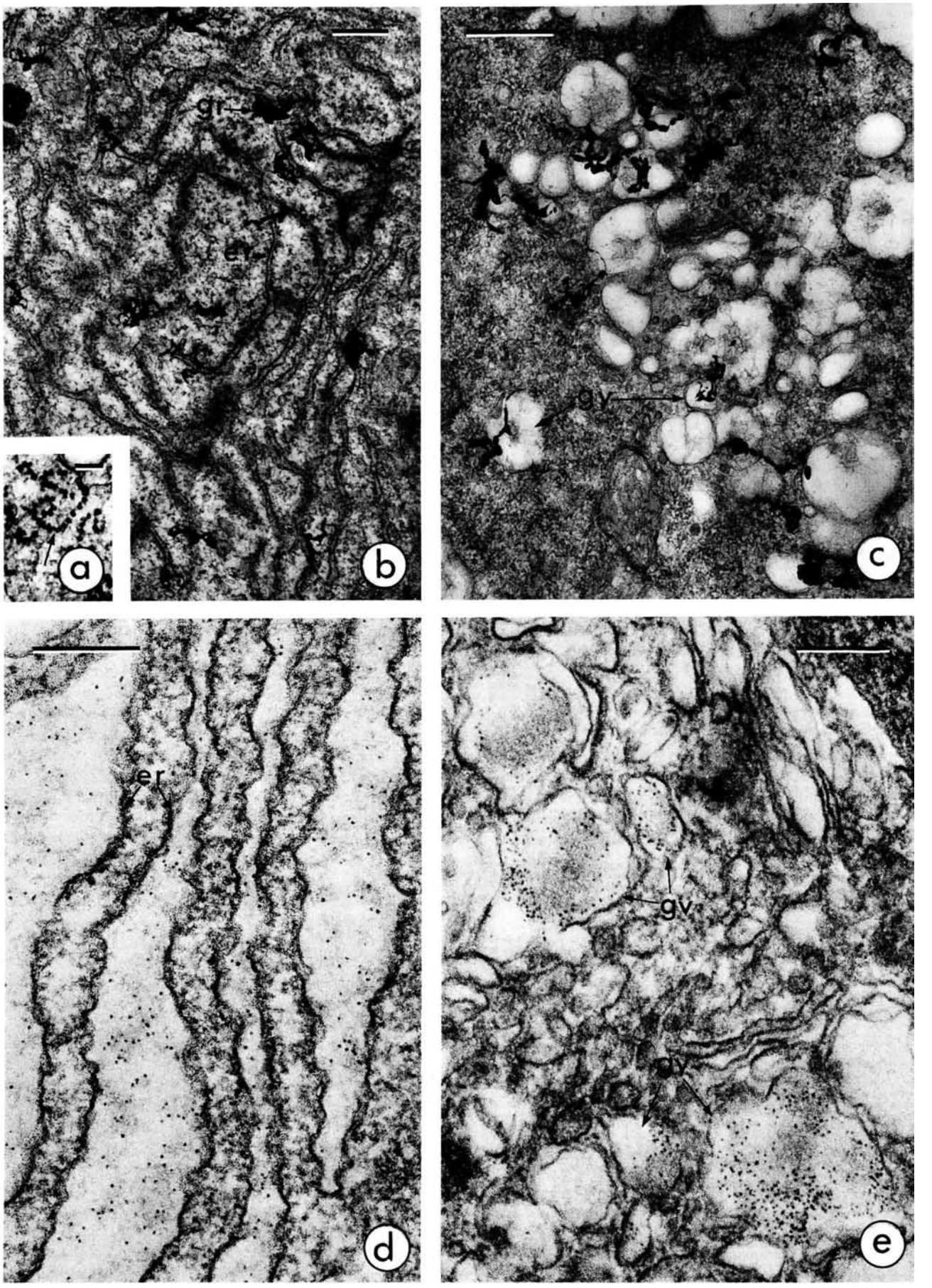
The question that cannot be answered with certainty is whether or not all of the collagen passes through the Golgi zone. Some might go directly to the extracellular space in non-Golgi vesicles (64, 67, 72). Quantitative autoradiography indicated that more label is taken up in the endoplasmic reticulum than seems to move to the Golgi zones of fibroblasts and chondroblasts, but it is difficult to achieve a sharp pulse with proline (67, 73). Weinstock and Leblond (68) argued that, although autoradiography cannot rule out some excretion from the cell via non-Golgi elements, in the polarized odontoblasts they studied, the proline-rich product moves from basal cytoplasm rich in ergastoplasm to an apical cytoplasm that contains mainly secretory vacuoles and granules. The Golgi elements of the odontoblast contain collagen as judged by immunohistochemistry (77). Certainly the fact that collagen does occur in Golgi vacuoles makes it seem reasonable to conclude that the Golgi vacuole is the only final element involved in collagen excretion (69).

The collagen revealed by immunohistochemical studies in the endoplasmic reticulum and in the Golgi vacuoles of fibroblasts is in the form of the high molecular-weight precursor, procollagen (70, 72, 77). Recent biochemical studies of cell-free systems containing collagen messenger RNA have demonstrated an even higher molecular-weight protein, pre-procollagen, which seems to have a hydrophobic "signal peptide" attached to it that carries procollagen from the polyribosome across the membrane of the endoplasmic reticulum (see 7, 78). Membrane-bound processing enzymes presumably immediately remove these peptides from the luminal side of the membrane. The longer-lived procollagen molecule, however, can be secreted into the medium by cultured fibroblasts, along with procollagen proteases, where it seemingly is cleaved extracellularly and is now free to polymerize (69). The exact location of polymerization of secreted collagen, and possibly also of procollagen proteases, whether next to the cell surface or some distance from the cells, undoubtedly varies from tissue to tissue depending on the pattern of growth (51, 61, 66, 68, 79).

Biochemical studies of the posttranslational changes that occur in collagen after it leaves the ribosomes have progressed rapidly in recent years. In addition to the protease actions described above, the primary structure of collagen is modified by hydroxylation of proline and lysine, synthesis of interchain disulfides, glycosylation, and formation of lysyl cross-links. Gross (5), Prockop and co-workers (7, 32, 69), Bornstein and co-workers (8, 80), Miller and Matukas (33), Fessler and Fessler (81), and others have elegantly reviewed this subject in recent years and should be consulted for details regarding methods used and cells that have been studied. In brief, the findings of particular interest to cell biologists are as follows.

The imino acid, hydroxyproline, is not directly incorporated by cells. An enzyme, prolyl hydroxylase, seemingly begins to hydroxylate proline as the amino-terminal ends of the pro-α-chains pass into the lumen of the endoplasmic reticulum (Fig. 8). Further hydroxylation occurs after the product has been released into the lumen of the reticulum, at which time inter-

FIGURE 7 Autoradiographic and immunohistochemical analysis of the cell organelles involved in collagen synthesis by chondrocytes (*a–c*) and fibroblasts (*d, e*). (*a*) Tangential section of the cytoplasmic surface of a cisterna of the endoplasmic reticulum. The circular polysome at the arrow contains over 30 ribosomes. (*b*) Autoradiograph (gr, exposed silver grain) of a section of cartilage fixed 15 min after the animal received [^{3}H]proline and (*e*) 30 min after injection of [^{3}H]proline. At the early time interval, newly synthesized, proline-rich product (presumably collagen in large part) is found in the endoplasmic reticulum (er), but not in the Golgi vacuoles. (*c*) By 30 min, some of the product has moved into the Golgi vacuoles (gv). (*d, e*) Sections of cells stained in block with antipro-α1(I) followed by ferritin-conjugated antibody. The contents of the endoplasmic reticulum (er in *d*) and Golgi vacuoles (gv in *e*) are labeled by the anticollagen ferritin-IgG complex. In *e*, the bounding membranes of the Golgi vacuoles appear broken in places; this is the result of partial homogenization of the cells prior to staining, which is necessary to permeabilize the organelles to the antibodies. *a*, × 50,000 (bar = 100 nm); *b*, × 20,000 (bar = 500 nm); *c*, × 30,000 (bar = 500 nm); *d*, × 200,000 (bar = 100 nm); *e*, × 170,000 (bar = 100 nm). (*a*) from Hay (65), courtesy of the Ronald Press Co.; (*b, c*) from Revel and Hay (66), courtesy of Springer-Verlag; (*d, e*) from Nist et al. (72), courtesy of The Rockefeller University Press.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chain disulfide bonds are also forming among the carboxy-terminal extensions (Fig. 8). Proline hydroxylase has been localized in the endoplasmic reticulum by immunohistochemistry (71, 82); it seems to be absent from Golgi vacuoles. There are two types, one forming 4-hydroxyproline and one, 3-hydroxyproline (7). A disulfide catalyst has not yet been identified. The interchain disulfide bonds are necessary for helix formation, and hydroxyproline is required for helix stability and for secretion of procollagen. The exact intracellular site of triple helix formation is unknown (78).

The importance of hydroxylation for transport of collagen out of the cell has been demonstrated by Prockop and his coworkers (see 7, 32, 69). Collagen secretion is inhibited if fibroblasts are incubated without O_2 (necessary for hydroxylation) or with $\alpha\alpha$-dipyridyl (which chelates iron needed for hydroxylases to act); the cells accumulate an unhydroxylated form of collagen called "protocollagen" in the endoplasmic reticulum (69). Proline analogs, such as L-azetidine-2-carboxylic acid, cause the synthesis of aberrant collagen chains that are not secreted normally. Ascorbate and lactate are involved in activating prolyl hydroxylase and, here again, a deficiency leads to abnormalities in collagen synthesis and secretion (69).

Lysyl hydroxylase seems to act on the molecule at the same time as proline hydroxylase (Fig. 8) and to require the same cofactors (ascorbate, etc.). Only lysine that precedes glycine in the helical part of the chain is subject to hydroxylation, but in the peptide terminals, where interchain cross-links later form, nonglycine-associated lysines are hydroxylated. Hydroxylysine can undergo subsequent enzymatic modifications. Glycosylation of hydroxylysine (78, 83) is catalyzed by the sequential action of galactosylhydroxyl-transferase and glucosylgalactosylhydroxylysl-transferase. The content of the two sugars, galactose and glucosylgalactose, varies among different collagens, as does also hydroxylysine (Fig. 5). Glycosylation occurs in the endoplasmic reticulum and possibly also in the Golgi complex (69). Inhibition of glycosylation by tunicamycin interferes with transport and secretion of collagen (84).

Lysine and hydroxylysine in the NH_2 and COOH terminal extensions can be converted to aldehydes by lysyl oxidase, an enzyme that acts extracellularly. Cross-links may form between the highly reactive aldehydes or between complex condensation products of the aldehydes (7, 85). All seem to originate with the formation of aldehydes catalyzed by lysyl oxidase. The lathyritic agent, β-aminopropionitrile (BAPN), prevents cross-linking by inhibiting this enzyme; reduced cross-linking results in loss of tensile strength of a tissue (5, 86). BAPN has been a useful agent not only in the study of cross-linking mechanisms, but also in the investigation of collagen production by tissues. The newly synthesized uncross-linked collagen fibrils appear normal, but the component collagen molecules are readily extracted in neutral salt.

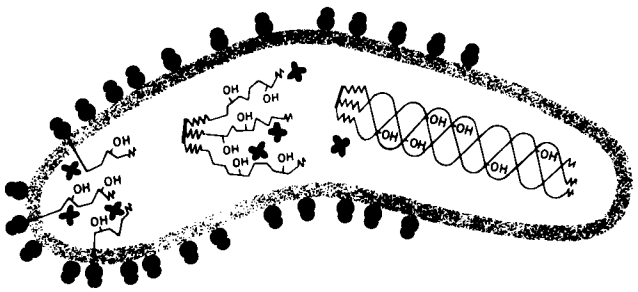

FIGURE 8 Diagram depicting the assembly of procollagen in the endoplasmic reticulum. As the amino terminal ends of the pro-α chains pass into the cavity of the reticulum, hydroxylation by proline hydroxylase (x) and lysyl hydroxylase begins. Hydroxylation continues as the partially assembled macromolecules pass along the cisterna. It is possible that triple helix formation begins in the lumen of the reticulum as shown here, but the exact intracellular site is not known. From Prockop et al. (69), courtesy of Plenum Press.

In 1955, it was an accepted dogma that only cells of the fibroblast family (mesenchyme) were capable of producing "true" collagen, an idea that persisted through the next decade (64). It was easy to believe the evidence (87, 88) that basement membranes were produced by epithelial cells, but claims that connective tissue proper might originate from epithelia remained buried in the literature (79, 89). We became interested in the issue when our electron micrographs of autoradiographs of larval amphibian skin indicated that the epidermis was secreting a proline-rich product through its basement membrane onto the underlying striated collagen fibrils (90). Our conclusion, that the proline-rich product is collagen, was challenged so strongly that we undertook a series of experiments in the late 1960s to prove the point. Isolated embryonic corneal epithelium (76, 91) and neural epithelium (92) produce typical native collagen fibrils in culture that can be easily identified morphologically by their striation pattern. Subsequently, biochemical studies of BAPN-treated cultures confirmed the production of type II and/or type I chains by these isolated epithelia (93, 94). At the present time, examples of almost every tissue type (except blood-borne cells) have been shown to be capable of producing collagen (and GAG) at some time in their history. Endothelium as well as epithelium secretes collagen and other connective tissue components including GAG (30, 95, 96), as does muscle (8, 97, 98), retina (99), and some components of nerves (100).

An interesting issue that arises as a result of these and other studies is the question as to whether or not the same cell in, for example, the corneal epithelium (76, 91) secretes more than one type of collagen. All of the basal corneal epithelial cells seem to be actively secreting, and inasmuch as the epithelium is producing both types I and II collagen (94), it would not be unreasonable to expect the same cell to be making two genetically distinct collagens. In monolayer culture, chondrocytes stop making type II and start making type I collagen. Some of the cells in transition can be shown by immunofluorescence to contain both types I and II and thus must have been making two types of collagen almost simultaneously (101). Interestingly, the dedifferentiating chondrocytes start to make type III, $\alpha 1(1)$ trimers, A and B collagen chains (101). Corneal fibroblasts, which do not make type III collagen in vivo, produce it in vitro (102). Bone matrix-induced transformation of muscle to cartilage turns on type II synthesis (103). *In situ,* normal genetic mechanisms cause limb mesenchymal cells to stop making type I collagen and to start making type II (101).

These and other aspects of the genetic control of collagen biosynthesis are gaining increasing attention. Church et al. (104) used corneal stroma-mouse fibroblast somatic cell hybrids to identify human chromosome 7 as responsible for the synthesis of human corneal type I procollagen. In that the gene for human skin type I procollagen is found on chromosome 17 (105), separate genes and control mechanisms seem to exist for skin and corneal type I procollagens. There are also species differences in type I procollagens (104) and it is possible that there are subclasses within the collagen types even in the same animal. Progress is being made in cloning collagen genes (7, 78). The pro-α2-gene has been shown to be large (400,000 base

pairs) and complex (106); it contains about 50 exons (coding regions) interrupted by introns (noncoding sequences). The genetic control of collagen biosynthesis is an intriguing area of future study because, in addition to the genes that determine primary structure, other sets of genes must be responsible for producing the enzymes that act after translation, and all of these activities must be coordinated in the cell. Moreover, following polymerization of the secreted molecules into fibrils, enzyme action and cross-linking continue extracellularly (85).

Collagen, once polymerized, is a stable component of the extracellular matrix. Its turnover rate is insignificant except in areas where remodeling is taking place. In the early 1960s, Gross and Lapiere (107) investigated the mechanism of resorption of the tadpole tail during amphibian metamorphosis. Certain of the tadpole tissues grown on collagen gels in the absence of serum digest the substratum. The omission of serum in these experiments was, according to Gross (5), a fortunate happenstance. It is now known that serum contains a collagenase inhibitor, α-2-macroglobulin. As a result of this combination of luck and intuition, studies on animal collagenases were launched and have progressed rapidly ever since (5, 108).

Similar collagenases have been found in regenerating amphibian limbs, postpartum rat uterus, healing wounds, ascites cell carcinoma, and rheumatoid synovial tissue (5, 108). They are not all identical biochemically, but they all seem to cleave the triple helical collagen molecule in solution or fibrillar form at a locus three-fourths the distance from the NH_2 terminal end, producing a long "A" fragment and a smaller "B" fragment (5). Bacterial collagenase, in contrast, chops the molecule in a number of places. Recently, preferential activity for one collagen type or another by certain animal collagenases has been reported (8). There has been some debate about the ability of animal collagenase to attack insoluble collagen fibrils without prior protease exposure, but the bulk of the evidence says that it can (5, 108). Protease activity is probably involved *in situ* in removing the A and B fragments, but before collagenase cleavage the triple helical collagen molecule is remarkably resistant to protease attack, with the possible exception of type III collagen (8). In the resorbing tadpole tail, epithelium, but not mesenchyme, makes collagenase (109), whereas in healing wounds of mammals, epithelium and mesenchyme (110) or mesenchyme alone (111) produces collagenase. In the next decade, it may be possible to localize enzymes such as collagenase (and hyaluronidase, to be discussed later) in embryonic and adult tissues by immunohistochemistry and thus come to a fuller appreciation of their distribution and effects during development and disease.

Proteoglycans and Hyaluronic Acid

In an authoritative book published in 1968, Schubert and Hamerman (10) summarized the then-present state of knowledge of the protein polysaccharides (now called proteoglycans; see reference 11) and HA. The biochemistry of the major polysaccharides (Fig. 2) was already well known. They consist of unbranching chains of repeating disaccharides; one unit is a hexosamine (usually *N*-acetyl glucosamine or *N*-acetyl galactosamine) which may be sulfated, and the second unit is hexuronate or D-galactose (10). The glycosidic bond between the hexuronate and hexosamine may be in the β-configuration (HA, KS, CS, Fig. 2) or α-configuration (heparin, Fig. 2). As a result of the work of Meyer and his colleagues (112), several enzymes were characterized which not only attack, but also distinguish among the structural configurations of these glycosidic bonds. Testicular hyaluronidase digests HA, chondroitin, and CS because it hydrolyzes the β1–4-linkage between galactosamine or glucosamine and glucuronic acid (see the diagram in reference 113). Streptococcal hyaluronidase seems only to attack the β1–4-linkage when the hexosamine is unsulfated, so it digests HA and chondroitin. Leech hyaluronidase is thought to hydrolyze the β1–3 between glucuronic acid and hexosamine when the hexosamine is *N*-acetylglucosamine (as opposed to *N*-acetylgalactosamine); it is specific for HA (Fig. 2).

These polysaccharidases have been of inestimable value to the cell and developmental biologist. Between 1968 and 1971, others have been discovered (14) including: chondroitinase AC (attacks HA, chondroitin, and CS); chondroitinase ABC (attacks HA, chondroitin, CS, and an epimer of CS, dermatan sulfate); heparinase (attacks HS and heparin); heparitinase (specific for HS); keratanase (specific for KS); and streptomyces hyaluronidase (specific for HA). In 1971, Toole and co-workers (114, 115) applied a number of these enzymes to analysis of GAG synthesis in embryos and thereby launched an important new horizon in developmental biology which will be described at the end of this chapter. Electron microscopists soon recognized the value of these enzymes for mapping the distribution of GAG in adult and embryonic tissues, particularly when used in conjunction with RR fixation, as we shall see now.

Because polysaccharides are poorly preserved by the commonly used fixatives for electron microscopy, they were not well visualized in tissues prior to the introduction of RR fixation (12), with the exception of cartilage. The PG granules in cartilage are fixed even by OsO_4 alone and were clearly described in cartilage by earlier workers who called them mucopolysaccharides (66). Matukas et al. (116) established their identity as GAG through their hyaluronidase susceptibility. PG granules (20–50 nm in diameter) similar in appearance to those in cartilage have been identified by RR fixation to be closely associated with collagen fibrils in adult and embryonic cornea, skin, reticular connective tissue, and even tendon (48). Small (5 nm diameter) filaments that are hyaluronidase-sensitive, but not neuraminidase-sensitive, connect the granules to each other and to the collagen fibrils (Fig. 9). RR is a polycation that presumably fixes GAG by electrostatic interaction with its multiple anion groups (12). Other compounds that interact with GAG may have similar potential as PG fixatives for electron microscopy (117).

Using RR fixation, Trelstad et al. (118) preserved small PG granules (10–20 nm in diameter) in the basal lamina (basement membrane) of embryonic cornea which are arranged in a regular pattern in the external and internal laminae rarae (Fig. 9). The PG granules form a monolayer on either side of the lamina densa, each granule located 60 nm from adjacent granules. The granules are digested by treatment of the tissue before RR fixation with chondroitinase (118) or testicular hyaluronidase (119), but not with leech hyaluronidase (118) and thus seem to contain chondroitin sulfate and/or chondroitin. The same type of granules are seen in embryonic lens capsule and neural tube basal lamina (119), but in embryonic salivary gland basal lamina, the RR-preserved CS-rich material does not seem to be granular and may contain as much as 50% hyaluronate (120). In the adult mammary gland, Gordon and Bernfield (121) found an RR-staining pattern like that in the salivary gland. The adult aorta (122), however, shows the same staining pattern observed in the cornea (Fig. 9). The different appearance observed by Bernfield and co-workers (120, 121)

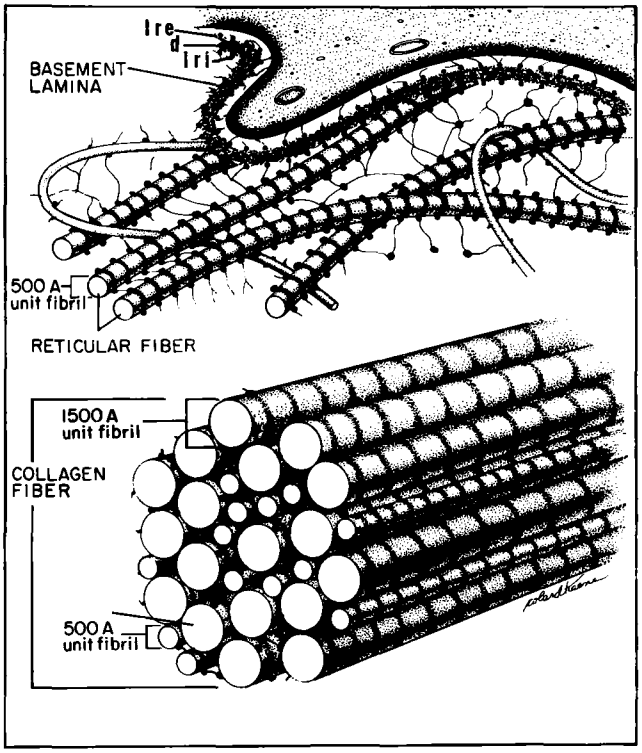

FIGURE 9 Diagram of the arrangement of PG granules revealed by RR staining of tissues viewed in the electron microscope. The lamina rara externa (lre) and lamina rara interna (lri) of the basement lamina contain small PG granules, whereas the lamina densa (d) is relatively free of PG. Larger PG granules, connected by fine filaments decorate unstriated fibrils and striated collagen fibrils. The reticular fiber consists of one or more small collagen fibrils (each 50 nm in diameter). The larger collagen fiber of connective tissue may contain collagen fibrils exhibiting a range of diameters (50–150 nm). From Hay et al. (48), courtesy of F. K. Schattauer Verlag.

may be due to differences in fixation (123).

Kanwar and Farquhar (124) described PG granules (10–20 nm diameter), spaced 60 nm apart like those in the cornea (118, 119), in the basal lamina of the adult kidney glomerulus. Located in the laminae rarae, PG is known to serve an important filtering role in the kidney (125). The renal PG granules are digested by heparitinase, but not by hyaluronidase (126). The results of the enzyme-digestion studies (126) are supported by the biochemical demonstration of heparan sulfate in isolated glomerular basement membrane (127). The biochemical studies also suggested that a small amount of hyaluronate is present in kidney basement membrane. It might have gone undetected in the histochemical study if its removal by Streptomyces hyaluronidase had not disrupted the granular appearance of the stained PG.

Remarkable progress has been made in the past decade in understanding the molecular organization of proteoglycans. In 1969, Sajdera and V. Hascall (128) introduced a nondisruptive method for extraction of cartilage proteoglycans using guanidinium chloride. It was found that a substance present at the top of the dissociative gradient was necessary for reaggregation of material on the bottom (129). Hardingham and Muir (130) in 1972 discovered the important role of HA in the aggregation, soon confirmed by V. Hascall and Heinegård (131) and others (129). In the molecular model of cartilage PG which rapidly emerged (13, 132), monomer PG is seen to be attached to a thread of HA with the help of link protein (Fig. 10).

The PG monomer consists of GAG chains attached to a core protein, which is approximately 300 nm long with a molecular weight of 250,000 (the core protein, thus, is almost as heavy as a tropocollagen molecule and is longer than four native collagen repeat units). An average of 80 CS chains (~20,000 mol wt each) are attached to the hydroxyl groups of serine residues in core protein by glycosidic bonds with the xylose residue at the end of each CS chain. In addition, ~100 KS and *O*-linked and *N*-linked oligosaccharides are attached by glycosidic bonds between *N*-acetylgalactosamine and the hydroxyls of serine and threonine in the core protein. The GAG chains with their arrays of negatively charged groups are stiff and they extend out from the core protein to occupy a large space (Fig. 10). One end of the core protein, the HA-binding region, has few or no attached GAG chains and resembles a glycoprotein. It contains an active site which binds to HA by a noncovalent interaction (see reference 13 for details) and is locked into place by a separate "link" protein (~50,000 mol wt).

Electron micrographs of extracted polymers (Fig. 10) and monomers spread on grids (133–136) confirmed and extended the biochemical predictions and led to immediate and widespread acceptance of the model. One of the intriguing questions to the electron microscopist studying sections of RR-fixed cartilage is how the structure of the isolated polymer relates to the PG granules one sees in the tissue. G. Hascall (137) has recently taken on this problem. She measured the dimensions of the structures seen in spreads and sections and has come to the rather convincing conclusion that the PG granules in cartilage are PG monomers, sectioned in different planes and condensed by dehydration (Fig. 10). The fine filaments connecting the PG granules, then, must correspond to the HA thread onto which the monomers are polymerized. The GAG would be expected to interact electrostatically with basic charges on collagen. In the expanded state, the monomers would fill all of the space around the collagen fibrils. In this way, the collagen fibrils define tissue shape and tensile strength and the interspersed PG aggregates provide a hydrated, viscous gel necessary for the compressibility of cartilage. Some of the functional and pathological implications of this model are discussed by V. Hascall and G. Hascall (13).

What can we say of the molecular structure of PG in tissues other than cartilage? Some tissues seem to resemble cartilage. In the aorta, monomeric PG aggregates with HA and link proteins (13). The collagen fibrils in the wall of the aorta are thicker than in cartilage and are lined with PG granules at regular intervals, whereas elsewhere in the matrix PG granules and narrow filaments (presumably HA) fill the spaces around the elastic fibers (122). The embryonic avian cornea has a similar appearance after RR fixation (48). In the more mature cornea, the stroma is highly condensed with little space around the collagen fibrils (88) and little or no HA (14, 138). Two classes of PG have been isolated from the adult corneal stroma, one of which contains CS and the other, KS (139). The CSPG seems to possess only one GAG (55,000 mol wt) and the core protein is short (80,000, mol wt). The KSPG contains one to three GAG chains and the total molecule is 80,000 in molecular weight (13). The small corneal PG monomers may be adapted to the refractive properties of the tissue, and it is possible that the PG pattern changes at the time of transition from embryonic to adult morphology. Heparan sulfate PG also occurs in many tissues, often if not always associated with the plasmalemma (140). It is a small PG (mol wt 80,000) and may be represented by the enzyme-resistant membrane plaques that

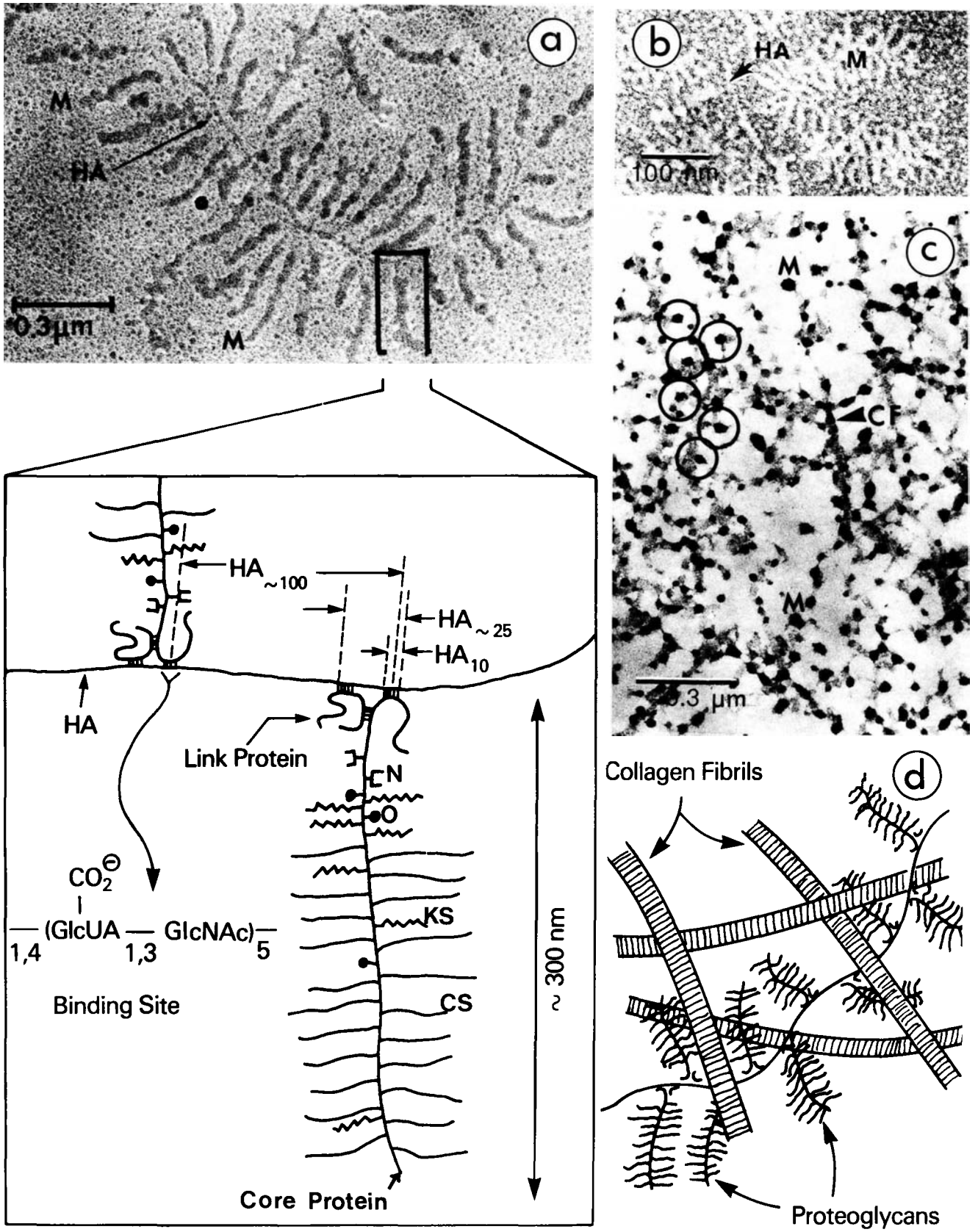

FIGURE 10 Structure of cartilage PG. (*a*) Electron micrograph of an isolated PG aggregate spread in a cytochrome *c* monolayer. The accompanying diagram shows a PG monomer (M in micrograph) and a portion of the hyaluronic acid (HA) thread as indicated in the rectangle. (*b*) Higher-power electron micrograph of part of a purified aggregate, revealing by negative staining the component chondroitin and KS side-chains of the monomer (M). (*c*) Electron micrograph of a section of cartilage matrix showing the nonstriated (or very finely striated) collagen fibrils (CF) and the characteristic PG granules (M). Each PG granule is a monomer, which if expanded would fill the space indicated by each circle. The relation of the PG aggregate to the collagen fibrils is diagrammed in *d*. (*a*) × 60,000 (bar = 300 nm); (*b*) × 130,000 (bar = 100 nm); (*c*) × 60,000 (bar = 300 nm). From Hascall and Hascall (13), courtesy of Plenum Press.

have been described in RR-fixed tissues (119, 122, 123).

In cartilage, the PG monomers do not seem to be aggregated into polymers when they leave the cell (141). Assembly may occur at some distance from the cell and, in contrast to collagen, some PG turnover does occur in adult cartilage (140). The synthesis of core protein occurs in the granular endoplasmic reticulum where addition of *O*-linked and *N*-linked oligosaccharides begins. Six different glycosyl-transferases and a sulfotransferase that may be membrane-bound are involved in CS synthesis. The initial step is transfer of xylose from uridine diphosphate xylose to the hydroxyl of serine in the core protein. Two galactose residues are added, then glucouronic acid and *N*-acetylgalactosamine, all involving different transferases. Repetitive glucuronic acid-*N*-acetyl galactosamines are then added by two final enzymes. Sulfation lags behind chain elongation (15, 142, 143).

The CS chains are attached to serine residues, and KS chains are probably added to *O*-linked oligosaccharides, as the core protein moves into the Golgi region (13). It has been known for some time that sulfation occurs in the Golgi region of chondrocytes (144). Recent in vitro experiments indicate that sulfate incorporation into PG from start to finish is very rapid (141). The Golgi vacuoles of chondrocytes contain various size PG granules and filaments (13) and they stain intensely with positively charged colloidal metals (145). Vertel and Dorfman (146) have recently used antibodies against core protein and type II collagen simultaneously to stain chondrocytes, using rhodamine and fluorescein labels for immunofluorescence (see 129 for history of the development of antibodies to core protein and their numerous uses). The same chondrocytes can be seen by immunohistochemistry to contain both core protein and collagen, sometimes in different regions of the cytoplasm and often concentrated in what appear to be vacuoles (146). Application of double-staining to the electron microscope in the future may tell us whether core protein (PG monomer) and collagen are packaged in the same or different secretory organelles.

Dorfman and co-workers have used a radioimmunoinhibition assay for core protein to follow inhibition of PG synthesis by BrdUrd in differentiating chondrocytes and to demonstrate that β-D-xylosides which inhibit PG synthesis do not inhibit core protein synthesis (129). Antibodies can also be used to show that cells in the cultures that are making type I collagen are not making CSPG; only the chondrocytes producing type II collagen produce CSPG (146). Presumably, the different proteoglycans of different tissues are products of different genes; it is also possible that diversity resulted from gene fusions (142). Dorfman (142) has recently reviewed the subject of PG synthesis in some detail.

Other Structural Proteins

The other structural proteins that occur in extracellular matrices fall into two groups: elastin and the glycoproteins. Elastin is an insoluble protein, with a soluble precursor, tropoelastin, which is free of neutral sugar, hexosamines, cystine, and tryptophan (52). Elastin is a peculiar protein, which Piez once speculated (147) might have evolved from collagen, because of its high glycine and hydroxyproline content (only two other proteins contain hydroxyproline; see reference 7). The main cross-links between the peptide chains of elastin are provided by two unique amino acids, desmosine and isodesmosine (147, 148). One of the discoveries in the past decade of particular interest to cell biologists is that of Ross and Bornstein (149) in 1969. They reported that the elastic fiber is often composed not only of the amorphous elastin described above, but also of another component, a microfibril (10 nm wide) differing from both elastin and collagen in amino acid composition (150) and belonging to the glycoprotein class. Ross and others (151–153) have also shown that isolated smooth muscle cells can synthesize elastin, thus removing this protein from the exclusive domain of the fibroblast. Antibodies to elastin have been obtained which promise to be quite useful to cell biologists (154).

The structural glycoproteins are proteins with one or more heterosaccharide chains containing hexosamine, galactose, fucose, and other sugars. Sialic acid is usually present, linked glycosidically in a terminal position of the heterosaccharide chain (52, 83). The glycoproteins are distinguished from the proteoglycans by their higher proportion of protein and the type of polysaccharide side-chains. Collagen is a glycoprotein, but is usually classified in a separate subgroup, because the side-chains are very short (galactose and glucosylgalactose bound to hydroxylysine) and no sialic acid is present (52).

Some measure of the rapid progress in this field can be seen by the fact that in comprehensive reviews of connective tissue as recent as 1975 (52), the names fibronectin, chondronectin, and laminin are not even mentioned. Of this group of "named" structural glycoproteins, fibronectin has received the greatest attention during the past half-decade. In 1973, Hynes (155) described a cell surface-associated protein sensitive to proteolytic digestion that was present on "normal" cell lines, but not on transformed cells, which he called "large, external transformation-sensitive" (LETS) protein (156), and Ruoslahti and co-workers (157, 158) described a "fibroblast surface antigen" that cross-reacted with serum. Vaheri, Ruoslahti, and co-workers (159) renamed this protein "fibronectin," to emphasize its association with fibroblasts and binding to fibrin during blood-clotting. Yamada and Weston (160) independently isolated a large "cell surface protein" with similar properties at about this same time, as did several other laboratories (160–162). By 1978 (163), the term fibronectin had been adopted by most investigators, because the protein turned out to occur within the extracellular matrix in fibrous form (Latin *fibra*, fiber) and to bind (Latin *nectere*, to tie) to a number of substances.

Two types of fibronectin are recognized. Cell surface or cellular fibronectin is synthesized by a variety of cells in culture, including fibroblasts, myoblasts, and certain epithelial cells (163), has a molecular weight of 220,000–240,000 daltons, and contains 5% carbohydrate. Plasma fibronectin, "cold insoluble globulin" (164), has a slightly smaller subunit molecular weight (200,000–220,000), but may be the same gene product rendered more soluble by posttranslational cleavage of terminal peptide. Cold insoluble globulin precipitates in the cold, if complexed to fibrin and fibrinogen, and thus is one of the components whose content varies in different batches of commercial fetal calf serum, a point of some importance to tissue culture methodology. Plasma fibronectin is a dimer of two disulfide-bonded polypeptides (subunits), whereas cellular fibronectin occurs as dimers and multimers. Most antisera to the two forms of fibronectin cross-react (163, 165, 166), but a monoclonal antibody has recently been described that distinguishes between the two (166a). Glycosylation is not essential to biological activity, but may help to protect the protein from proteolytic attack, to which it is very sensitive (166).

The multiple binding sites make it seem likely that fibronectin has a biological role in linking cells, collagen, GAG, and

fibrin (166). The collagen site, which binds to the same region of collagen that animal collagenase attacks (167, 168), seems to reside in a 40-K section of the molecule near its protease-sensitive, carboxyl terminal (Fig. 11). The cell-binding site is believed to occupy a 160-K segment between the collagen site and the amino terminal, which includes the 50-K heparin-binding site and possibly the HA-binding site (166). It has been suggested that a ganglioside-like molecule is the cell receptor for fibronectin (170). Fibronectin facilitates the attachment of certain cells to collagen in vitro, which might be due to the dual cell-binding, collagen-binding property; fibronectin might similarly help cells bind GAG (166, 171, 172).

Fibronectin has been shown to form extracellular fibrous networks in patterns which correlate with patterns of actin fibers within cells in vitro (173), and ultrastructural studies suggest that the two types of fibers are coaxial across the plasmalemma (174). Cytochalasin treatment disrupts the attachment of cells to fibronectin (175, 176). Thus, it is possible that extracellular fibronectin and intracellular actin are linked across the plasmalemma by a protein with a hydrophobic component, the putative receptor. Vinculin may be involved in the attachment (176a). Effects of fibronectin on cell adhesion and cell shape (177, 178), and on cell migration (179), might be mediated in part by modulation of the cytoskeleton (180).

Fibronectin is widely distributed. Immunohistochemical studies reveal that it makes an early appearance, together with laminin and collagen, in the mouse embryo at the morula and blastocyst stage (181). Fibronectin has been detected by immunofluorescence in basement membranes (182, 183) and in a variety of adult and embryonic connective tissues, excepting mature cartilage matrix and dense or mineralized connective tissue (182–185). In vitro, fibronectin forms extensive extracellular networks that may stain with collagen antibodies as well as with antifibronectin (186). Fibronectin is not present on epithelial-free surfaces (182, 183, 187, 188). At the electron microscope level, the immunoferritin technique demonstrates fibronectin in the internal and external laminae rarae of isolated glomerular basement membranes (189) and in extracellular fibrils (10 nm in diameter) and "amorphous" bodies secreted by cells in vitro (174, 190, 191). Embryonic tissues *in situ* have been studied with both these techniques at the electron microscope level (188). The interstitial bodies of Low, which

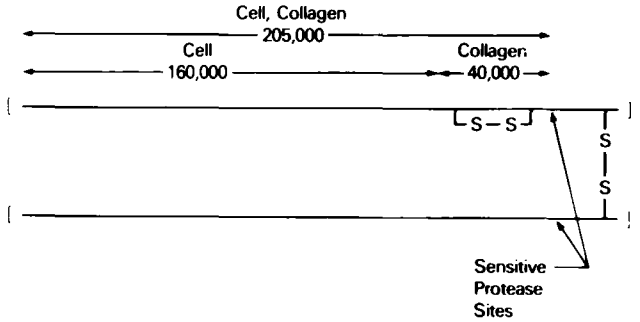

FIGURE 11 Model of the relationship of cell-binding and collagen-binding fragments of fibronectin. Protease cleavage separates the sites and abolishes the capacity to attach cells to collagen. The heparin-binding site is located near the middle of the cell-binding site. The dimer shown here consists of two unfolded subunits (each 220,000–240,000 daltons) linked by disulfide bonds at one end of the molecule. The carbohydrate (not shown) is of one class only, a mannose-containing oligosaccharide linked to asparagine residues of the protein. From Hahn and Yamada (169), courtesy of The MIT Press.

are associated with GAG (188), and the 10-nm fibrils of the embryonic matrix are well labeled by antifibronectin with the immunoferritin technique (Fig. 12). The immunoperoxidase technique reveals fibronectin in basement membranes (basal laminae) and possibly in cell membranes (188, 192), as well as in the cell surface-associated interstitial material and 10-nm fibrils (Fig. 12).

Laminin and chondronectin are more restricted in distribution. Laminin is a large glycoprotein that has been detected in basement membranes (basal laminae) by immunofluorescence (193, 194) and by immunoelectron microscopy (195, 196). Timpl and co-workers isolated laminin from the mouse tumor basement membranes in 1978 (193, 197). They report that it is composed of at least two polypeptide chains, one 220,000 in molecular weight, the other 440,000, joined by disulfide bonds. Laminin probably corresponds to one of the glycoproteins previously detected in basement membranes by Kefalides (28–30). It is relatively insoluble and difficult to characterize, but is clearly involved in epithelial attachment to collagen (198). Rotary shadowing and negative staining reveal that the macromolecule consists of a long arm and three short arms which contain terminal globular units (198a; Fig. 13).

Chondronectin (199, 200) is an adhesion factor for chondrocytes to collagenous substrates; it has not been fully characterized, but seems to be a relatively large protein (about 180,000 daltons). Fibronectin is present in the mesenchyme that gives rise to cartilage, but presumably is replaced by chondronectin in definitive cartilage matrix. Chondronectin may be to chondrocytes what fibronectin is to fibroblasts, at least as regards attachment of cells to collagen in vitro (201, 202).

We shall have more to say about the role of the structural proteins in cell differentiation in the concluding section. In the next decade, it is quite clear that many of the as yet "unnamed" glycoproteins of connective tissue (83) will be more fully characterized. The idea that cells are surrounded by a "glycocalyx" of both structural and functional significance has come a long way since Bennett (203) first introduced the term in 1963 to refer to the sugar-rich protein coat surrounding cells.

Embryonic Development

The functions of extracellular matrices (ECM) in the adult and embryo are numerous. They support and determine the shape of the body and are marvelously adapted to form rigid and semirigid structures, such as bone and cartilage, orthogonal collagenous gridworks, elastic tissues, loose connective tissues, and all the variations in between. By their charge and other characteristics, they filter the substances that reach cells and, in the case of the kidney glomerular basement membrane, they can affect what leaves the body as well. As we have seen in previous sections, cells are delicately tuned to make the proper collagens, proteoglycans, and other matrix proteins and, during differentiation, they may switch the type of matrix molecules they produce to meet the requirements of time and place. In closing, let us consider in a little more detail still another aspect of matrix cell biology, the feedback of information to cells by these same matrix molecules.

The idea that embryonic cells interact with the ECM produced by adjacent cells (and/or themselves) had its origin early in this century (204, 205). At the beginning of the period under review here, Grobstein (206) had discovered that ECM is instrumental in "tissue interaction," a term which he used synonomously with "embryonic induction" to refer to devel-

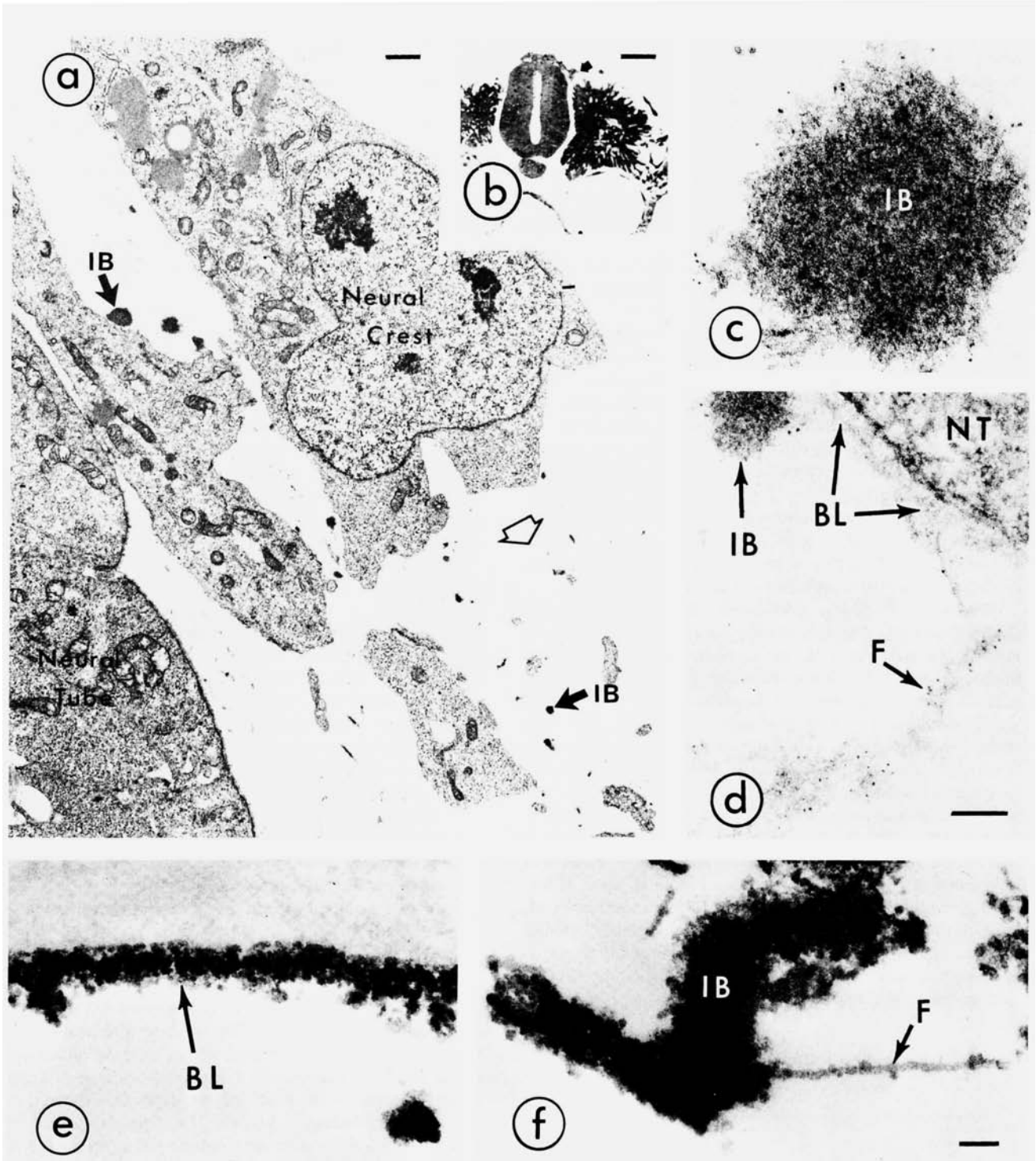

FIGURE 12 An immunohistochemical analysis of fibronectin distribution in the 2-day-old chick embryo. The neural crest cell shown in the electron micrograph (*a*) is located in the region indicated by the arrow in *b*. In the light micrograph (*b*), it can be seen that the dorsal ectoderm was removed to permit entry of the antibody. (*c*) Higher-power electron micrograph of an interstitial body (IB) similar to those depicted in a (IB); the small black dots are ferritin molecules (antifibronectin ferritin-IgG complex) indicating that fibronectin is present in the interstitial body. In *d*, ferritin-conjugates can be seen to localize fibronectin in small, nonstriated fibrils (F). The basement lamina (BL) is not well stained, presumably due to a problem in ferritin penetration. When the peroxidase-antiperoxidase technique is used to detect antibodies to fibronectin, the basement lamina is well stained (BL, *e*), as are interstitial bodies (IB, *f*) and the nonstriated (10-nm diameter) fibrils (F, *f*). (*a*) × 5500 (bar = 1 μm); (*b*) × 100 (bar = 50 μm); (*c, d*) × 100,000 (bar = 100 nm); (*e, f*) × 65,000 (bar = 100 nm). From Mayer et al. (188), courtesy of Academic Press, Inc.

opmentally significant interaction between two tissues of differing embryological origin. He found that during the interaction in vitro between mesenchyme and epithelium (e.g., salivary gland), or epithelium (e.g., neural tube) and mesenchyme, the "inducing" tissue secreted histochemically detectable extracellular matrix into a filter placed between the two tissues. In 1965, interest in the possibility that collagen might be one of the "inducing" molecules was stimulated by the report of

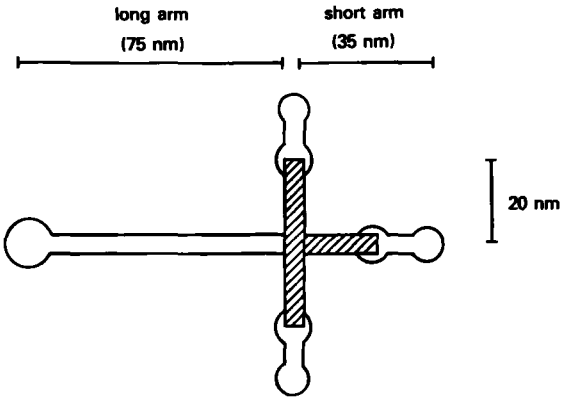

FIGURE 13 Structural model of laminin based on electron micrographs of isolated molecules viewed after rotary shadowing and negative staining. The two chains (220,000, 440,000 daltons) obtained by reduction of disulfide bonds lie within the long and short arms. Hatched area: disulfide-rich, protease-resistant fragment 1 (290,000 daltons). From Timpl and Martin (198a), courtesy of CRC Press.

Konisberg and Hauschka (207) that collagen promotes muscle differentiation in vitro. By the end of the 1960s, the idea that matrix components were "informational" during development was becoming widespread (88, 208, 209). In 1971, Dodson and Hay reported a direct effect of frozen-killed collagenous matrix on the ability of corneal epithelium to produce collagen (91) and, in 1972, Nevo and Dorfman described a stimulatory effect of purified PG and GAG on synthesis of GAG by chondrocytes (210).

The past decade has seen an increasing number of studies along these lines which cannot be reviewed adequately in the short space allowed here; the reader is referred to recent reviews for a full discussion of the morphogenetic roles of matrix molecules (14, 138, 180, 202, 209, 211–215). What is emerging, partly as a result of concurrent studies on the cytoskeleton (see other chapters in this volume) and on the role of cell shape in controlling metabolism (216, 217), is the idea that extracellular molecules, alone or in combination, affect the organization of the cytoplasm via putative receptors in the plasmalemma, and thereby influence the shape, mobility, and differentiation of the cell.

Consider the development of an epithelium. An epithelium is a group of cells that site on top of ECM; some evidence of the magnitude of the preprogramming of cell differentiation that occurs in embryogenesis is seen in the fact that true epithelial cells will not invade collagenous matrices (218). They develop this and other distinctive properties presumably during gastrulation, when the tissues are first formed (219). The requirement of corneal epithelium for underlying matrix (91) can be satisfied in vitro by several types of collagen; GAG also promotes differentiation in this system, and other matrix glycoproteins can have an effect on the cells (220–222). Direct contact of the epithelial with ECM is required for the stimulatory effect (221), which is measured by epithelial synthesis of corneal stroma (collagen and GAG). Even soluble matrix molecules can affect the shape of the cells and the organization of their basal surface and cytoskeleton (Fig. 14), and both soluble collagen and laminin seem to stimulate ECM synthesis (222).[1] The stimulatory effect on the cytoplasmic machinery may be mediated by an interaction between ECM molecules

[1] Sugrue, S. P., and E. D. Hay. Unpublished observations.

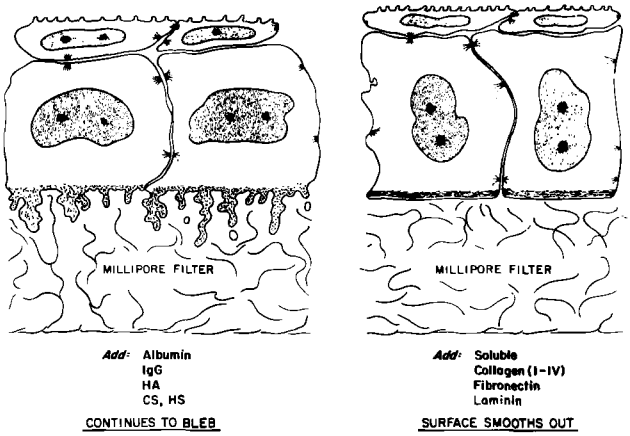

FIGURE 14 Diagram summarizing experiments testing the effect of soluble molecules on the organization of the basal corneal epithelial surface. The isolated epithelium placed on a Millipore filter has a disorganized basal cytoplasm and extends cell processes (blebs) into the pores of the filter. The epithelium continues to bleb in the presence of albumin, IgG, hyaluronic acid, chondroitin sulfate, or heparan sulfate. If, however, soluble collagen, fibronectin, or laminin is added to the culture medium, the epithelium withdraws the blebs and reorganizes its basal cytoskeleton within 2-6 h. The ECM molecules interact with the plasmalemma, even though a visible subepithelial ECM has not yet formed. From Sugrue and Hay (222), courtesy of The Rockefeller University Press.

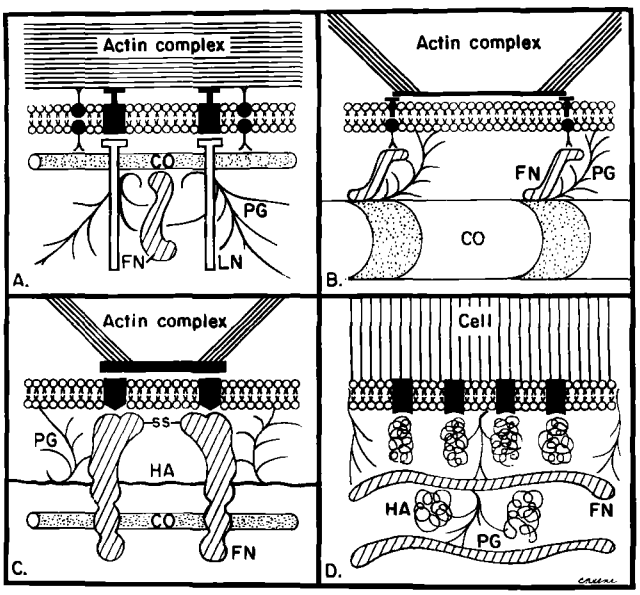

FIGURE 15 Diagrams of several models depicting the possible relation of ECM molecules to the cell surface. All models envision plasmalemmal receptors for one or more molecules. CO, collagen; FN, fibronectin; PG, proteoglycan; HA, hyaluronic acid. (A) Model of interaction of matrix molecules with the corneal epithelial basal surface to explain the data of Sugrue and Hay (222). (B) Model of interaction of ECM with mesenchymal cell surface based on data and diagrams of Singer (174) and Kleinman et al. (201, 245). (C) Model of interaction of fibronectin with fibroblast cell surface as visualized by Hynes (180). (D) Model of interaction of glycosaminoglycans with mesenchymal cell surface after Toole (215). From Hay (214), courtesy of Plenum Press.

and the cytoskeleton (Fig. 15A).

Mesenchymal cells, which are the inwandering cells that bud off the primitive germ layers to invade the embryonic ECM, are also affected by the ECM which they use as scaffoldings

for their migration. Lash and Kosher and their co-workers (223–225) have shown that embryonic somite (sclerotome) mesenchyme destined to become cartilage responds to PG and GAG in much the same way as do chondrocytes (210). Collagens also have a stimulatory effect on sclerotome differentiation (223, 225) and in the embryo these "inducing" matrix molecules derive from the adjacent neural tube and notochord (92, 123). There is evidence that type II collagen has more influence on presumptive chondrocytes than does type I, but myoblasts seem to respond to all collagens that have been tested (226). Solursh, Muir, and others (227–229) have studied the effects of ECM on mesenchymal cell differentiation; they report that HA diminishes GAG synthesis, whereas, as we have already seen (129, 142, 224), CS stimulates GAG synthesis by developing chondrocytes.

Toole and his co-workers, as we mentioned earlier (114, 115), introduced, in 1971, the enzyme degradation method as a way of characterizing embryonic GAG; this methodology has been in wide use since then. Toole et al. were able to show with this technique that, in a number of developing systems (cornea, limb, embryonic trunk), the migration phase of mesenchyme development is accompanied by domination of HA synthesis, whereas during differentiation (e.g., chondrogenesis), CS synthesis overrides HA and an enzyme, hyaluronidase, is produced which degrades existing HA (14, 114, 115). HA may also play an important role in endocardial (230) and neural crest migration (117, 231). It seems to characterize hydrated matrices and presumably promotes mesenchymal motility by an interaction with receptors in the plasmalemma (215; Fig. 15D).

Recent years have witnessed an enormous number of investigations on the effect of the glycoproteins, fibronectin, chondronectin, and laminin, on cell attachment in vitro, some of which have already been noted (177–181, 197–202). Martin and co-workers (197) report that laminin appears to be a specific attachment factor for epithelial cells to type IV collagen; it does not stimulate fibroblast attachment to collagen and promotes epithelial attachment only to type IV (not types I–III or V). Fibronectin does not promote adhesion of these epithelial cells to type IV (198). Chondronectin, not fibronectin, promotes attachment of cartilage cells to collagen (199). Fibronectin not only promotes attachment of fibroblasts and certain cell lines to plastic and/or collagen, but also seems to affect their migration (179). Kleinman et al. (201, 202) have recently reviewed this rapidly expanding field. Certain of these matrix glycoproteins have been implicated in the well-known phenomenon of platelet adhesion to collagen (202) and, together with collagen (232), seem even to possess chemotactic properties (202). Cell receptors have been suggested for fibronectin (Fig. 15 B, C) and there is good evidence that collagen can interact with the cell membrane via receptors (233, 234).

Even from this brief discussion, it is obvious that the effects of ECM on cells are varied and undoubtedly depend not only on the time and place, but also on the state of differentiation of the reacting cells. Some idea of the diverse nature of such interactions is seen in gland development, where collagen seems to act in some cases to inhibit degradation of GAG (235), rather than to promote its synthesis. Bernfield and co-workers have suggested (120, 236) that, in the salivary gland, degradation of ECM, presumably by mesenchymal cells, promotes epithelial branching and thus influences the morphogenesis of the gland. Nerve myelination is influenced by collagen (237). Basement membranes retain the memory of their previous association with neuromuscular junctions (238). Elastin biosynthesis is promoted by ECM, possibly by the elastin component (239, 240). Materials associated with collagenous bone matrix can induce nonchondrogenic cells to become cartilage (103, 212, 241). Endothelial cells may inhibit smooth muscle growth by secreting heparin-like molecules (242). Mammary epithelial differentiation is promoted by growing the cells on floating collagen gels (243, 244).

In the next era of research on the role of ECM in embryonic development, these systems and effects will be catalogued and classified more fully. The interactions among the ECM molecules and their effects on the cell surface and cytoskeleton will be clarified and, as more cell biologists enter the field, the exact mechanisms of action of ECM on cells will be discovered. It is important to remember when isolating cells from either adult or embryonic tissues that the ECM and the cell surface form a continuum, in the sense that the adjacent ECM is a part of the cell and the cell, of the ECM. For a full understanding of cell biology, then, we need to know the compositions and sources of the extracellular matrices, the manner in which they are produced and secreted by cells, and the mechanisms by which these extracellular molecules interact with cells to affect their growth and metabolism.

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V. Energy Sources

Mitochondria: A Historical Review

LARS ERNSTER and GOTTFRIED SCHATZ

Known for over a century, mitochondria have become during the last three decades an important subject of research within several disciplines of experimental biology. For the cytologist, they represented the ideal test objects for applying electron microscopy to the exploration of cellular ultrastructure and for the elaboration of tissue-fractionation techniques with the aim of isolating cytoplasmic organelles. For the biochemist, the identification of mitochondria as the site of cell respiration and respiration-linked phosphorylation implied a decisive step towards the resolution and reconstitution of these processes at a molecular level and the elucidation of their relationship to cellular membranes. For the physiologist, mitochondria afforded the first opportunity for an experimental approach to structure-function relationships, in particular those involved in active transport, vectorial metabolism, and metabolic control mechanisms on a subcellular level. And for the molecular biologist, the discovery of mitochondrial DNA and protein synthesis and the study of mitochondrial biogenesis opened up a new chapter of eukaryotic gene expression.

The purpose of this review is to give a brief account of these developments by selecting some of the highlights of the long and eventful history of mitochondrial research. Detailed historical accounts are found in numerous monographs (1–7) and review articles (8–12) covering various aspects of the field.

The Beginnings

CYTOLOGICAL OBSERVATIONS: The earliest records on intracellular structures that probably represent mitochondria go back to the 1840s (13–19), only a few years after the discovery of the cell nucleus (20). However, Altmann (21) in 1890 was the first to recognize the ubiquitous occurrence of these structures (Table I). He called them "bioblasts" and concluded that they were "elementary organisms" living inside cells and carrying out vital functions. Altmann would have been greatly satisfied by knowing that his idea of the symbiotic origin of mitochondria would be revived several decades later, based on similarities between mitochondria and bacteria (22). The name mitochondrion was introduced in 1898 by Benda (23), and originates from the Greek "mitos" (thread) and "chondros" (granule), referring to the appearance of these structures during spermatogenesis.

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In 1900, Michaelis (24) found that the redox dye Janus Green B serves as a specific supravital stain of mitochondria. As pointed out by Palade (25) in 1964, this feature became the "official portrait" of mitochondria until 1952, when the first high-resolution electron micrographs of mitochondria were published (26). It is remarkable, in view of Michaelis's active interest in biological redox processes, that he did not relate this finding to a possible role of mitochondria in cellular oxidations. In fact, it took 50 years until Lazarow and Cooperstein (27) demonstrated that the specific staining of mitochondria by Janus Green B is due to their capacity to reoxidize the reduced dye by way of cytochrome oxidase.

Plant mitochondria were first described in 1904 by Meves (28). Four years later, Regaud (29) concluded that mitochondria contain protein and lipid. Both Meves (28) and Regaud (30) suggested a role of mitochondria as "bearers of genes." In 1912, Kingsbury (31) arrived at the foresighted conclusion that mitochondria serve as "a structural expression of the reducing substances concerned in cellular respiration." However, these proposals, like many others put forward during the following 20 years (32–40), were based almost exclusively on morphological observations, without direct chemical evidence. As Cowdry (41) pointed out in 1924, "... it is quite obvious that the investigation of mitochondria will never achieve the usefulness which it deserves as an instrument for advance in biology and medicine until we know much more of their chemical constitution as the only accurate basis for interpretation of our findings. In other words, we must wait upon the slow development of direct, quantitative cellular chemistry."

The first decisive step towards this goal was taken when, in 1934, Bensley and Hoerr (42) described the isolation of a fraction containing globular or rod-shaped structures from guinea-pig liver after homogenization in a physiological salt solution and subsequent centrifugation at 2,000 rpm. Although these granules did not stain with Janus Green B, they most probabaly consisted, at least partly, of mitochondria. This method offered the first opportunity for biochemical analysis of an isolated cytoplasmic fraction, and opened the way to the identification of mitochondria as the site of cell respiration.

EARLY STUDIES ON CELL RESPIRATION AND OXIDATIVE PHOSPHORYLATION: From the early 1910s, beginning with the studies of Battelli and Stern (43) on cell-free preparations of dye-reducing dehydrogenases, it has been recognized that biological oxidations are intimately associated with insoluble cellular structures. In 1913, Warburg (44) reported that in extracts of guinea-pig liver, respiration is linked to particles. He called these particles "grana," and suggested

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TABLE I

Some Key Discoveries on Mitochondrial Structure and Function

Year	Discovery	Author(s)	Reference(s)
1890	Description of "bioblasts," a cytoplasmic structure of ubiquitous occurrence, resembling bacteria and functioning as "elementary organisms"	Altmann	21
1912–1922	Recognition of the particulate nature of cell respiration	Battelli and Stern	43
		Warburg	44
		Wieland	46
1925	The cytochrome system is associated with cellular structures	Keilin	47
1934	First attempts to isolate mitochondria by cell fractionation	Bensley and Hoerr	42
1940–1946	First correlated morphological and biochemical studies on isolated mitochondria	Claude	64, 65, 67
1946	Demonstration of the localization of succinoxidase and cytochrome oxidase in mitochondria	Hogeboom et al.	69
1948	Isolation of morphologically well-preserved mitochondria	Hogeboom et al.	72
1948–1951	Mitochondria contain the enzymes of the citric acid cycle, fatty acid oxidation, and oxidative phosphorylation	Kennedy and Lehninger	74
		Schneider and Potter	75
		Green	76
		Lehninger	77
1950–1955	The enzymic complement of mitochondria as revealed by tissue-fractionation studies	Schneider and Hogeboom	73
		Hogeboom and Schneider	105
		de Duve et al.	89, 104
1951–1952	Demonstration of respiratory control, latency of ATPase, and uncoupling effect of dinitrophenol with isolated mitochondria	Lipmann et al.	82, 88
		Rabinovitz et al.	83
		Lardy and Wellman	84, 87
		Kielley and Kielley	86
1952–1953	Early studies on mitochondrial swelling and contraction	Slater and Cleland	138
		Raaflaub	139
1952–1953	First high-resolution electron micrographs of mitochondria	Palade	26, 120
		Sjöstrand	121, 122
1953	"Chemical" hypothesis of oxidative phosphorylation	Slater	179
1952–1955	Localization of coupling sites of the respiratory chain	Lardy and Wellman	84, 87
		Lehninger	78
1952–1956	Introduction of rapid and sensitive physical methods for the study of mitochondrial electron transport. Kinetics and metabolic states of the respiratory chain.	Chance	165, 171
		Chance and Williams	169, 170
1953–1956	Partial reactions of oxidative phosphorylation (P_i-H_2O and P_i-ATP exchange)	Cohn	180
		Boyer et al.	181
		Swanson	182
1953–1957	Demonstration of the membranous localization of the respiratory chain.	Cleland and Slater	52
		Watson and Siekevitz	376
		Siekevitz and Watson	377
1956–1960	Introduction of the use of beef-heart mitochondria for the study of the respiratory chain and oxidative phosphorylation. Demonstration of the participation of ubiquinone, nonheme iron, and metalloflavoproteins as redox carriers of the respiratory chain. Isolation and characterization of electron-transport complexes.	Crane et al.	301, 304
		Singer et al.	302
		Beinert and Sands	309
		Hatefi et al.	310, 312, 315
		Ziegler and Doeg	311
		Kuboyama et al.	318
1957–1961	Demonstration of the reversal of oxidative phosphorylation	Chance and Hollunger	184, 189
		Klingenberg et al.	111, 185–187
		Löw et al.	196, 197
1958–1962	Reconstitution of the respiratory chain	Keilin and King	300
		Hatefi et al.	316
1960	Isolation of mitochondrial ATPase and demonstration of its action as coupling factor (F_1)	Pullman et al.	336
		Penefsky et al.	337
1961	Chemiosmotic hypothesis of oxidative phosphorylation	Mitchell	405
1961–1963	Demonstration of energy coupling in the respiratory chain without the participation of the phosphorylating system	Azzone and Ernster	193
		Ernster	194, 206–208
		Klingenberg and v. Häfen	195

that their role is to enhance the activity of the iron-containing "respiratory enzyme" (*Atmungsferment*) (45). Similarly, Wieland (46), who extended Battelli and Stern's (43) early observations to a generalized concept of cellular dehydrogenases, recognized the particulate nature of these enzymes. Despite diverging views concerning the chemical nature of cell respiration—involving a transfer of oxygen according to Warburg (45), and a transfer of hydrogen according to Wieland (46)—

TABLE I—*Continued*

Year	Discovery	Author(s)	Reference(s)
1961–1963	Energy-linked uptake of Ca^{2+} and other divalent cations	Vasington and Murphy	225
		DeLuca and Engstrom	226
		Brierley et al.	227
		Chappell et al.	228
		Lehninger et al.	229, 232
		Saris	230
		Chance	233
1962	Discovery of projecting subunits on the mitochondrial membrane	Fernández-Morán	322
1963–1966	Energy-linked transhydrogenase and its use as a tool for the study of mitochondrial energy transduction	Danielson and Ernster	215
		Lee and Ernster	216, 219
		Lee et al.	218
1964	Discovery of the action of valinomycin as K^+ ionophore	Moore and Pressman	241
1965	Conformational hypothesis of oxidative phosphorylation	Boyer	404
1966	Development of the chemiosmotic hypothesis as a general mechanism of oxidative and photosynthetic phosphorylation	Mitchell	406
1966	Discovery of mitochondrial anion translocators	Chappell and Haarhoff	155
1966–1969	Separation and characterization of the inner and outer mitochondrial membranes	Lévy et al.	379
		Schnaitman et al.	380, 381
		Parsons et al.	382
		Sottocasa et al.	81
		Ernster and Kuylenstierna	389
1966–1969	Evidence for chemiosmotic coupling in native membranes	Jagendorf and Uribe	416
		Mitchell and Moyle	413, 415
1966–1976	Isolation and characterization of coupling factors. Resolution and reconstitution of the ATPase complex	Kagawa and Racker	343
		Thayer and Hinkle	351
		Capaldi	352
		MacLennan and Tzagoloff	349
		Racker et al.	347, 348
		Lam et al.	350
1971–1975	Reconstitution of oxidative phosphorylation and related reactions in artificial phospholipid vesicles	Kagawa and Racker	428
		Racker and Kandrach	432
		Ragan and Racker	430
		Hinkle et al.	425
		Skulachev	419
		Leung and Hinkle	426
		Ragan and Hinkle	427
		Rydström et al.	429
		Racker and Stoeckenius	433
1973–1979	Evidence for electron transport-linked proton pumps	Azzone and Massari	235
		Brand et al.	439, 440
		Papa et al.	444
		Guerrieri and Nelson	445
		Höjeberg and Rydström	221
		Wikström	441
		Wikström and Krab	442

they both agreed that the role of the particulate cellular structure may be to enlarge the catalytic surface. Warburg (45) referred to the "charcoal model," and Wieland (46) to the "platinum model," in attempting to explain how this may be achieved.

In 1925, Keilin (47) described the cytochromes, a discovery that led the way to the definition of the respiratory chain as a sequence of catalysts comprising the dehydrogenases on one end and *Atmungsferment* on the other. This resolved the Warburg-Wieland controversy. To achieve this, however, another, equally controversial problem had to be settled, namely, that of the relationship between Keilin's cytochromes and Warburg's *Atmungsferment.* This was not done until 1939, when Keilin and Hartree (48) established the identity between *Atmungsferment* and cytochrome a_3. Keilin's studies were carried out first with the living wax moth and later, in collaboration with Hartree, with a particulate preparation from mammalian heart muscle. This preparation, which catalyzed the aerobic oxidation of succinate and NADH, was subsequently studied in great detail by Slater (49, 50), especially regarding the catalyst responsible for the interaction of the dehydrogenases and the cytochrome system ("BAL-sensitive factor"). It also became in many laboratories the starting material for the isolation and characterization of various respiratory-chain catalysts.

Keilin and Hartree (51) early recognized the need for a cellular structure for cytochrome activity, pointing out that "it is quite possible that the paramount conditions for existence of this pigment are found in some properties connected with the physico-chemical structure of the cell." In contrast to the charcoal and platinum models, they suggested that the cellular structure may be necessary, not for the activity of the individual

catalysts, but rather for determining their mutual accessibility and thereby the rates of reaction between different members of the respiratory chain. Such a function, according to Keilin and Hartree (51), could be achieved by "unspecific colloidal surfaces." Interestingly, the possible role of phospholipids was not considered in these early studies, and it was not until 1953 that the membranous nature of the Keilin and Hartree heart-muscle preparation and its mitochondrial origin were recognized by Cleland and Slater (52).

During the second half of the 1930s, considerable progress was made in elucidating the reaction pathways and energetics of aerobic metabolism. In 1937 Krebs (53) formulated the citric acid cycle, and Kalckar (54) presented his first observations leading to the demonstration of aerobic phosphorylation, using a particulate system derived from kidney homogenates. Earlier, Engelhardt (55) had obtained similar indications with intact pigeon erythrocytes. Extending these observations, Belitser and Tsybakova (56) in 1939 deduced from experiments with minced muscle that at least two molecules of ATP are formed per atom of oxygen consumed. These results indicated that phosphorylation probably occurs coupled to the respiratory chain. In 1941, Lipmann (57) developed the concept of "phosphate-bond energy" as a general form of energy conservation in cellular metabolism.

In the following years several laboratories reported studies with "washed tissue particles" in which various qualitative and quantitative aspects of the aerobic oxidation of citric acid-cycle metabolites and accompanying ATP synthesis were investigated. A paper of special importance was published in 1943 in Ochoa (58), in which it was concluded that the aerobic oxidation of pyruvate probably gives rise to 3 moles of organically bound phosphate for each atom of oxygen consumed (P/O ratio-3). During these years also the first evidence was presented of the capacity of tissue particles to carry out fatty acid oxidation (59). In 1948–1949 , using a particulate fraction from rat liver and β-hydroxybutyrate or NADH as substrate, Friedkin and Lehninger (60, 61) provided conclusive evidence for the occurrence of respiratory chain-linked phosphorylation. At about the same time, Green and associates (62, 63), in a series of papers, described a particulate system from rabbit kidney which was given the name "cyclophorase" and was shown to catalyze the aerobic oxidation of citric acid cycle metabolites and accompanying phosphorylation. This system displayed certain "organized" properties not observed with earlier-studied particulate systems; for example, it contained a complement of endogenous NAD^+, which was lost upon mechanical damage of the particles. All of these important developments took place before the relationship of these particles to mitochondria was known. The establishment of this relationship had to await the availability of reliable methods for tissue fractionation.

Structure-Function Relationships

ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF MITOCHONDRIA: From the late 1930s, Claude (64) was engaged in a detailed study of the conditions for cell fractionation that was based on the original procedure of Bensley and Hoerr (42). Claude's contributions came to be of fundamental importance for the separation and the morphological and biochemical characterization of cell organelles. He introduced the tissue fractionation technique based on differential centrifugation (65), and worked out basic criteria for the identification and the chemical and enzymic characterization of the fractions obtained. These criteria included examination of the size, shape, and, whenever possible, the fine structure of the particles recovered in the various fractions, as well as the protein content of each fraction in relation to that of the total homogenate. Most importantly, Claude (66) pointed out that the assessment of the localization of an enzyme or another chemical constituent in a given organelle must be based on quantitative criteria, such as the total recovery of the constituent and its relative concentration in the organelle in question. He was also first to stress the importance of using an isotonic solution as the homogenizing medium, in order to prevent osmotic changes in the organelle structures. Claude's (67) fractionation procedure yielded four fractions: a heavy fraction, consisting of nuclei and cell debris; an intermediate, "large-particle" fraction, containing mitochondria; a light fraction, consisting of "submicroscopic" particles that Claude called "microsomes" (later identified by Palade and Siekevitz [68] as consisting mainly of fragments of the endoplasmic reticulum); and a soluble fraction, including the cell sap.

Through the the use of the above procedure, Hogeboom, Claude, and Hotchkiss (69) concluded in 1946 that succinoxidase and cytochrome oxidase in rat liver are localized exclusively in the mitochondria. Although the mitochondrial fraction obtained in these studies differed from mitochondria *in situ* by being round rather than elongate and not being stained by Janus Green B, the size and homogenous appearance of the particles were taken as sufficient evidence to identify them with mitochondria. Significantly, the oxidase activities were highly concentrated in this fraction. Succinoxidase activity earlier had been found (70, 71) in the large-particle fraction isolated by the original procedure of Bensley and Hoerr (43), but in those studies considerable activity was recovered in the small-particle fraction as well.

In 1948, Hogeboom, Schneider, and Palade (72) modified Claude's procedure by using a hypertonic (0.88 M) sucrose solution as the homogenizing medium. This improved the quality of the isolated mitochondria, which now remained elongate and stainable with Janus Green B. In addition, the use of sucrose instead of a salt solution eliminated aggregation of the particles, improving the purity of the fractions. Succinoxidase activity again was localized exclusively in the mitochondria. Later this procedure was further modified (73) by employing isotonic (0.25 M) rather than hypertonic sucrose as the fractionation medium. This modification facilitated the sedimentation of the cell fractions and also eliminated the inhibitory effect of high concentrations of sucrose on certain enzymes. This procedure became the routine method for preparing mitochondria.

The stage was now set for a direct biochemical approach to the elucidation of mitochondrial function. In 1949, Kennedy and Lehninger (74) demonstrated the aerobic oxidation of citric acid cycle metabolites and of fatty acids as well as the accompanying formation of ATP from inorganic phosphate and ADP with rat liver mitochondria prepared in 0.88 M sucrose. Other cell fractions were devoid of these activities, which was to be expected in view of the earlier conclusion by Hogeboom, Claude, and Hotchkiss (69) that cytochrome oxidase is located exclusively in the mitochondria. Similar results were independently reported by Schneider and Potter (75). In 1951, Green (76) concluded that the cyclophorase system consists of mitochondria.

Using isolated mitochondria, Lehninger (77, 78) also confirmed and extended the results with washed particles, which he and Friedkin had obtained (60, 61), that demonstrated the occurrence of phosphorylation coupled to the aerobic oxidation

of externally added NADH. Respiration was accompanied by a phosphorylation with an estimated P/O ratio approaching 3, which appeared to be in agreement with earlier proposals concerning the existence of three sites of phosphorylation in the respiratory chain. At the same time, however, these experiments indicated that mitochondria are impermeable to added NADH and that they possess an "external," nonphosphorylating pathway of NADH oxidation that can be demonstrated in the presence of added cytochrome *c*. This pathway was later found to differ from that involved in the oxidation of intramitochondrial NADH in being insensitive to antimycin (78), amytal (79), and rotenone (80), and to be associated with the outer mitochondrial membrane (81).

An important advance that was made possible by the isolation of structurally well-preserved mitochondria was the discovery of certain organized features of the mitochondrial enzyme system, not seen in earlier work with washed particles. In 1951–1952, several laboratories (82–84) demonstrated "respiratory control" with isolated mitochondria, an effect consisting of a control of the respiratory rate by the availability of inorganic phosphate and the phosphate acceptor ADP. It was proposed that this phenomenon, which required a certain degree of structural intactness, was a reflection of the capacity of the organism as a whole to adjust its respiration according to the actual need for energy. Respiratory control became an important parameter for the study of mitochondrial energy transduction at both the biochemical and the physiological and pathological levels (85) (see further p. 233s). Another organized feature of intact mitochondria, discovered in 1951 by Kielley and Kielley (86), was the "latency" of the ATPase activity, which was stimulated by agents that damage the mitochondrial structure. An enhanced ATPase activity and abolition of respiratory control was also found (84, 87) to occur with the known uncoupler of oxidative phosphorylation, 2,4-dinitrophenol (88). These observations indicated that the coupling of respiration to phosphorylation requires a structural feature of the mitochondrion in addition to a set of functional enzymes. However, the understanding of the nature of this structural feature had to await better knowledge of mitochondrial ultrastructure and, in particular, of the role of membranes in energy transduction.

ENZYME-DISTRIBUTION STUDIES: Simultaneously with the above developments, several laboratories were actively engaged in studies of enzyme distribution among the various cell fractions as prepared by differential centrifugation (1, 9, 89, 90). These studies led to the recognition of mitochondria as the site of several enzymes in addition to those involved in the citric acid cycle, fatty acid oxidation, respiration, and phosphorylation. Among these enzymes were adenylate kinase (86, 91, 92), glutamate dehydrogenase (93, 94), transaminases (95), pyruvate carboxylase (96), nucleoside diphosphokinase (97), and nicotinamide nucleotide transhydrogenase (98), as well as enzymes involved in the substrate-level phosphorylation linked to the oxidation of α-ketoglutarate (93), the synthesis of porphyrine and heme (99), citrulline (100,101), and phospholipids (102). Mitochondria were also found to contain a part of the cellular hexokinase (103).

An important result of these studies was the discovery that several enzymes in mitochondria were present also in the microsomal fraction or the cell sap. The question arose whether these enzymes really had a dual localization or whether their presence of two cell fractions was due to incomplete separation of the various cell components. In most cases this question could be settled by carrying out a careful quantitative analysis of the various cell fractions, using a refined fractionation technique and a set of "marker enzymes" for the different organelles as devised by de Duve and associates (104). These studies also led to the discovery of a new cell organelle, the lysosome, with a sedimentation rate intermediate between those of mitochondria and microsomes, and characterized by a complement of hydrolytic enzymes.

A much-debated case of an enzyme with dual localization was the $NADP^+$-linked isocitrate dehydrogenase. It was pointed out (105) that since only slightly more than 10% of this enzyme in a liver homogenate is recovered in the mitochondrial fraction, with the remainder in the cell sap, the enzyme may not be a true mitochondrial constituent. On this basis, the part played by mitochondria in the citric acid cycle as a whole was questioned (106). The problem was settled, however, by showing, first, that the small portion of the enzyme found in the mitochondrial fraction fails to react with externally added $NADP^+$ and is thus truly mitochondrial (107); and, second, that the citric acid cycle proceeds via an NAD^+-linked isocitrate dehydrogenase (108), which is exclusively mitochondrial (109). A bimodal distribution between mitochondria and cell sap was later found to be characteristic of several enzymes, including malate dehydrogenase and glutamate-oxalacetate transaminase, and to be related to the transfer of reducing equivalents between extra- and intramitochondrial nicotinamide nucleotides (110). The distribution of the nicotinamide nucleotides between the cell sap and mitochondria was first determined by Bücher and Klingenberg in 1957 (111).

Data concerning the gross chemical composition of mitochondria also began to appear in the early 1950s. In 1951, Schneider and Hogeboom (112) estimated that rat liver mitochondria account for about 35% of the total tissue protein. Earlier estimates had indicated that 70–75% of the mitochondrial dry weight consists of protein and the remainder mainly of phospholipid (65). The lipid composition of mitochondria was determined in 1958 by Marinetti et al. (113), who also carried out a quantitative analysis of various phospholipids. That cardiolipin is localized almost exclusively in mitochondria was demonstrated in 1968 by Getz et al. (114).

Isolated, intact mitochondria were found to contain adenine nucleotides (115) as well as a variety of inorganic ions including phosphate, Na^+, K^+, and Mg^{2+} (116). Mitochondria were also shown to take up and accumulate Ca^{2+} (117) and Mn^{2+} (118). These early observations foreshadowed the occurrence of active ion-transport across the mitochondrial membrane.

MITOCHONDRIAL STRUCTURE AND ITS VARIATIONS: That mitochondria are surrounded by a membrane was suggested on the basis of early observations with the light microscope (18). The first electron micrographs of mitochondria, published by Claude and Fullam (119) in 1945, confirmed this conclusion. However, detailed studies of the mitochondrial ultrastructure became possible only after the development of thin-sectioning techniques in the early 1950s. The first high-resolution electron micrographs were published in 1952–1953 by Palade (26, 120) and Sjöstrand (121, 122), who used osmium-fixed thin sections of various animal tissues (Fig. 1). Palade (26) found that the mitochondrion is surrounded by a membrane, which is folded to form ridges inside the mitochondrion; he named these ridges *cristae mitochondriales*. Sjöstrand's micrographs revealed a double limiting membrane surrounding the mitochondrion, and a number of double membranes inside the mitochondrion forming divisions, *septae*, of the inner chamber. The existence of a double limiting membrane was soon confirmed by Palade (120), who concluded

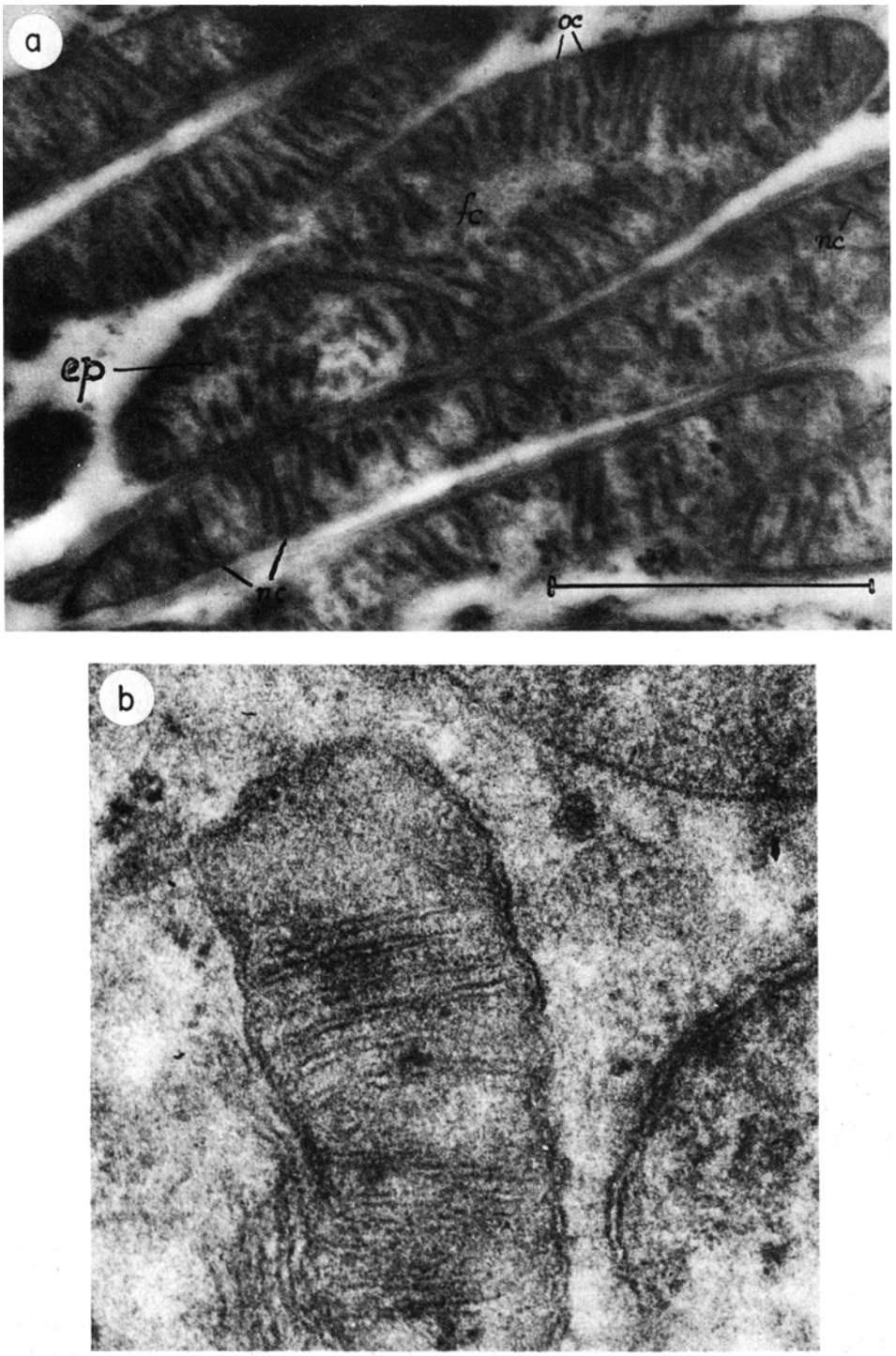

FIGURE 1 Electron micrographs of kidney mitochondria. (*a*) From Palade, 1953 (120); ×45,700. (*b*) From Sjöstrand, 1953 (121), × 120,000.

that the cristae are infoldings of the inner membrane. According to Palade's (123) definition, "Two spaces or chambers are outlined by the mitochondrial membranes, an outer chamber contained between the two membranes, and an inner chamber bounded by the inner membrane. The inner chamber is penetrated and, in most cases, incompletely partitioned by laminated structures which are anchored with their bases in the inner membrane and terminated in a free margin after projecting more or less deeply inside the mitochondrion." This definition of the mitochondrial structure has become widely accepted over the years. The space inside the inner membrane is usually referred to as the *matrix*, and the space between the inner and outer membranes as the *intermembrane space* (124).

An explanation of why the cristae seen in electron micrographs of thin sections often lack connection with the inner limiting membrane—an apparent inconsistency with Palade's (123) model—was offered by Whittaker (125), who proposed that this connection may consist of a relatively narrow orifice. That the primary function of the cristae is connected with an increase of the internal surface, rather than a compartmentation

of the inner chamber, is consistent with the early observation that in certain tissues, e.g., adrenal cortex (126), the disc-shaped cristae are replaced by tubular structures, which protrude as fingerlike invaginations from the inner membrane. Tubular cristae are also common in protozoans (127, 128) and have been suggested to represent a phylogenetically basic type of intramitochondrial structure (129). Great variations in the conformation of cristae have been found among different tissues and organisms (4, 5, 9), but the functional implications of these variations are still poorly understood. Irrespective of shape, however, a high respiratory activity seems to be correlated with an abundance of cristae (120). Examples of tissues with mitochondria rich in cristae are insect flight muscle (130), mammalian cardiac muscle (120), and brown adipose tissue (131).

Striking variations are also found in the number, size, shape, and intracellular localization of the mitochondria (4, 5, 9). These variations are clearly related to the specific functions of the tissue. A classic example of specialized mitochondria, described as early as 1871 by Bütschli (16), is the mitochondrial syncytium in the midpiece of sperms (*Nebenkern*). A "syncytial reticulum" of skeletal muscle mitochondria, which extends in the plane of the I band of the fibers, was observed by Palade 20 years ago (132), and demonstrated recently by three-dimensional reconstruction of electron micrographs carried out by Skulachev and associates (133). A slab-like orientation of mitochondria is also found in insect flight muscle, as Smith described (130). The regularly oriented mitochondria in the distal convoluted tubules, demonstrated by Sjöstrand and Rhodin (134) in 1953, are another striking example of specialized mitochondrial topography.

Mitochondria are highly dynamic structures. As early as 1914–1915, Lewis and Lewis (135) described extensive changes in the position and shape of mitochondria in animal tissue cultures. These observations were later extended by several investigators who used the phase-contrast microscope in combination with time-lapse cinematography. These studies, which were pioneered by Frédéric and Chèvremont (136), revealed striking movements of the mitochondria in various phases of cell activity, e.g., during mitosis, as well as in response to varied physiological, pathological, and experimental conditions. Despite many observations during the past 25 years (4, 5, 9, 137), however, the mechanism and physiological significance of these movements remain largely unexplained. Similarly, many changes that occur in mitochondrial structure after the administration of various drugs or toxic substances to experimental animals have been described (8), but in most cases no causal relationship between these changes and the pharmacological or toxic effects has so far been established.

Shape and volume changes of mitochondria *in vitro*, usually referred to as mitochondrial "swelling" and "contraction," occupy a large chapter in mitochondrial research. Swelling of mitochondria was probably first observed in 1888 by Kölliker (18), who described volume changes of structures isolated from skeletal muscle; these structures were studied in great detail by Retzius (19), who named them sarcosomes. In 1946, Claude (65) demonstrated that mitochondria suspended in a hypotonic medium swell; this was revealed by a decrease in light scattering. From the early 1950s, several laboratories were actively engaged in studies of this phenomenon and its relation to mitochondrial function. It was found that swelling can also occur in an isotonic medium and is promoted by Ca^{2+} (138, 139), inorganic phosphate (140), short-chain fatty acids (141), thyroxine (142), bilirubin (143), and other agents (144, 145); in some instances, the swelling was dependent on energy in the form of an oxidizable substrate and was inhibited by respiratory inhibitors, anaerobiosis, or by uncouplers (8). During the swelling process, the mitochondria gradually lost their ability to concentrate various ions (116, 146) and nucleotides (115) and to carry out oxidative phosphorylation (115, 147–149); and, simultaneously, they acquired certain hydrolytic activities (86, 140). In most cases, the swelling was prevented by Mg^{2+}, Mn^{2+}, and ATP (147–151), and, as first shown by Raaflaub (138), ATP also was able to reverse the swelling. This effect of ATP was promoted by Mg^{2+} and Mn^{2+}, and was accompanied by a restoration of oxidative phosphorylation and related structure-dependent functions (147–150). The ATP effect in reversing swelling has been compared with muscle contraction (138) and it has been suggested that mitochondria contain a contractile protein similar to actomyosin. Some studies describing such a protein were reported in the early 1960s (152) but were apparently not further pursued. In recent years it has been established that the mitochondrial swelling and contraction concern primarily the inner membrane (153) and are at least partly related to the movements of water across this membrane that accompany the uptake and release of ions by way of specific translocators (154, 155). In fact, the swelling phenomenon has served as an important tool in discovering and characterizing some of these translocators (155) (see the section on "*Ion Translocation*"). It has also played a significant role in the development of methods for the separation of the outer and inner mitochondrial membranes (124), as will be discussed below.

Besides these large-amplitude volume changes, mitochondria also exhibit a low-amplitude swelling-contraction cycle, which was discovered and studied extensively by Packer (156). This cycle follows the metabolic state of the mitochondria, and is probably related to changes in the prevailing protonmotive force. Also apparently related to the metabolic state of the mitochondria are the so-called "orthodox" and "condensed" conformational states, which were described in 1966 by Hackenbrock (157). These changes are restricted to the conformation of the inner membrane—probably resulting from drastic volume changes of the matrix—whereas the outer membrane is unaltered. Interestingly, the "condensed" mitochondria reveal the existence of multiple points of attachment between the outer and inner membranes. Metabolism-dependent conformational changes of mitochondrial cristae have also been observed by Penniston et al. (158).

The Energy-Transduction System of Mitochondria

Since the early 1950s, an important part of mitochondrial research has been concerned with the mechanism of electron transport and oxidative phosphorylation. It became clear that the mitochondrion was the long sought particulate structure necessary for these processes. However, as Lehninger (2) pointed out, "It was a part of the biochemical *Zeitgeist* that particles were a nuisance and stood in the way of purification of the respiratory enzymes," and this was even more true for membranes. Thus, even when it was proved and generally accepted that the catalysts of the respiratory chain and the phosphorylating system are associated with the inner mitochondrial membrane, it took a long time to begin to understand the role of the membrane in the function of these catalysts. Does the membrane serve as Keilin and Hartree's (51) "colloidal surface," regulating the mutual accessibility of the catalysts?

Do these catalysts form an assembly or do they interact by collisions in the plane of the membrane? Does the membrane serve as a hydrophobic environment protecting labile intermediates? Does it serve as a permeability barrier, and, if so, a barrier to what? Are the catalysts oriented across the membrane in a specific way, and, if so, why? These were some of the questions that governed mitochondrial research from the early 1950s. The general experimental approach included work with intact mitochondria as well as attempts to resolve and reconstitute the components of the respiratory and phosphorylating enzyme system.

OXIDATIVE PHOSPHORYLATION AND ITS PARTIAL REACTIONS. THE "CHEMICAL" HYPOTHESIS: As mentioned above, isolated, well-preserved mitochondria were found to exhibit such organized features as a high degree of respiratory control and a latency of ATPase activity. These findings stimulated interest in assessing the maximal number and the location of phosphorylations in the respiratory chain. One approach was based on determining the P/O (or P/2e$^-$) ratios obtained with mitochondria that were respiring in the presence of various substrates or utilizing artificial electron donors (e.g., ascorbate + cytochrome *c* [61] or TMPD [159], or menadiol [160]) or acceptors (e.g., ferricyanide [161] or coenzyme Q_1 [162]) in combination with appropriate electron-transport inhibitors. The most commonly used inhibitors were cyanide for cytochrome oxidase (163), antimycin for the cytochrome *b-c* segment of the respiratory chain (164, 165), and amytal (79) or rotenone (80, 166) for the electron flow between NADH and cytochrome *b*. Suitable electron-transfer "shunts" were also used to bypass the sites of action of amytal (167) and rotenone (80) and of antimycin (168). To ensure maximal yields of phosphorylation, in most cases the system was supplemented with an efficient "ATP trap" in the form of hexokinase and glucose.

A powerful method for the study of oxidative phosphorylation, which was introduced by Chance and Williams (169, 170), was based on the use of a dual-wavelength spectrophotometer in combination with an oxygen electrode. The redox states of various respiratory-chain components were determined spectrophotometrically, simultaneously with the polarographic measurement of oxygen uptake. Such determinations were carried out in various metabolic states of the mitochondria, e.g., during respiration in the presence of substrate and phosphate, with and without ADP ("State 3" and "State 4") (Fig. 2). The transition between the two states was accompanied by characteristic redox shifts of certain electron-transport carriers, some becoming more reduced, others more oxidized. From the location of these "crossover" points, the sites of phosphorylation in the respiratory chain were determined. At the same time, from the increment in oxygen uptake due to the addition of a given amount of ADP, the ADP/O (P/O) ratio was calculated. This method of Chance and Williams (169, 170) made possible the first quantitative study of the concentrations and kinetics of electron-transport catalysts not only in intact mitochondria but also in other integrated biological systems, including intact cells and tissues (171, 172).

The above lines of investigation constituted the experimental basis for the widely accepted view that the respiratory chain contains three sites of phosphorylation—located between NADH and cytochrome *b*, cytochrome *b* and cytochrome *c*, and cytochrome *c* and oxygen, respectively—each giving rise to the synthesis of one molecule of ATP from ADP and inorganic phosphate per two electrons transferred. The actual

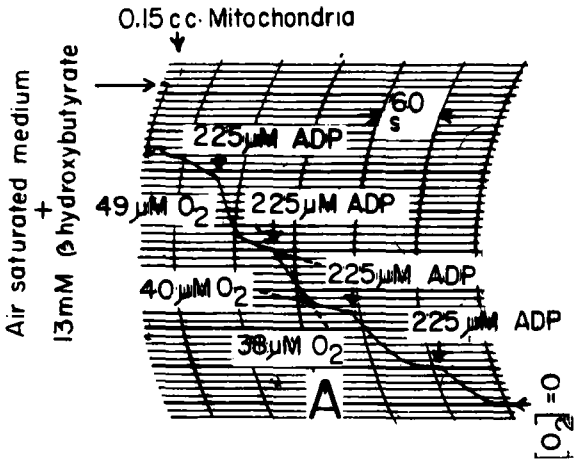

FIGURE 2 Respiratory control: a polarographic recording of the effect of ADP upon the respiration of a suspension of rat liver mitochondria. From Chance and Williams, 1955 (169).

mechanism of these phosphorylations, however, remained unsettled.

In 1946, Lipmann (173) suggested that phosphorylations in the respiratory chain follow a mechanism similar to that of the phosphorylation in glycolysis, involving a phosphorylated derivative of the oxidized electron donor:

$$AH_2 + B + P_i \rightleftharpoons A \sim P + BH_2; \quad (1)$$

$$A \sim P + ADP \rightleftharpoons A + ATP; \quad (2)$$

$$\text{Net: } AH_2 + B + ADP + P_i \rightleftharpoons A + BH_2 + ATP, \quad (3)$$

where *A* and *B* are redox carriers. In 1952 Krimsky and Racker (174) demonstrated that the glycolytic phosphorylation proceeds via a thiol ester prior to the formation of the phosphorylated intermediate. One year earlier, Kaufman (175) and Sanadi and Littlefield (176) had shown that the phosphorylation coupled to the oxidation of α-ketoglutarate (discovered by Hunter and Hixon [93] in 1949) also involves a thiol ester, succinyl ~CoA, and in the same year, Lynen and Reichert (177) demonstrated the occurrence of acetyl~CoA as a product of pyruvate oxidation, after the discovery of coenzyme A by Lipmann (178). Prompted by these developments, several investigators considered the possibility that respiratory chain-linked phosphorylations also may proceed by way of nonphosphorylated high-energy intermediates (1, 78, 179). Slater (179) in 1953 was the first to formulate such a mechanism in general terms:

$$AH_2 + B + C \rightleftharpoons A\sim C + BH_2; \quad (4)$$

$$A\sim C + ADP + P_i \rightleftharpoons A + C + ATP; \quad (5)$$

$$\text{Net: } AH_2 + B + ADP + P_i \rightleftharpoons A + BH_2 + ATP, \quad (3)$$

where *C* is a hypothetic ligand. Slater's mechanism, which is often referred to as the "chemical" hypothesis of oxidative phosphorylation, constituted a widely accepted framework for designing and interpreting experiments in this field during the following 15 years.

Slater's (179) mechanism appeared to account for a number of findings relating to respiratory chain-linked phosphorylation. For example, the phenomenon of respiratory control

could be explained by assuming that, in the absence of P_i and/or ADP, $A\sim C$ accumulates and this leads to an inhibition of electron transport via A. The proposed mechanism also accounted for the effect of uncouplers in abolishing respiratory control and oxidative phosphorylation and stimulating ATPase activity, by postulating that uncouplers cause a cleavage of A~C into A and C. Similarly, structural damage would induce a splitting of $A\sim C$, thus explaining why mitochondrial membrane fragments such as the Keilin-Hartree preparation can respire in the absence of P_i (179).

An important development was the discovery that mitochondria catalyze a number of exchange reactions which represent partial reactions of respiratory chain-linked phosphorylation. One reaction, an oxygen exchange between inorganic phosphate and water, was discovered in 1953 by Cohn (180). Another, described by Boyer et al. (181) and by Swanson (182), involved an exchange of phosphate between inorganic phosphate and ATP. Both reactions were sensitive to uncouplers and proceeded at rates that were higher than the net rate of oxidative phosphorylation. These findings could be explained in terms of Slater's mechanism (179) by assuming that the conversion of $A\sim C$ + ADP + P_i into A + C + ATP (reaction 5) is readily reversible and proceeds via a phosphorylated high-energy intermediate ($C\sim P$), the formation of which involves the splitting of a P-O bond of inorganic phosphate. The occurrence of such an intermediate was also supported by the demonstration of an ADP-ATP exchange by Wadkins and Lehninger (183).

A further extension of the chemical hypothesis was proposed by Chance and Williams (166), who postulated the occurrence of two types of nonphosphorylated high-energy intermediate, one that contained a redox carrier and was individual for each coupling site of the respiratory chain, and a second that contained no redox carrier and was common for the three coupling sites. They also introduced the symbol I instead of Slater's C, (175) to indicate that this ligand inhibits respiration when bound to an electron carrier. Moreover, Chance and Williams (166) concluded that I binds to the reduced, rather than to the oxidized, redox carrier.

One of the most important tenets of Slater's hypothesis (179) and its subsequent extensions was that the energy liberated during electron transport via the respiratory chain can be conserved without the participation of the phosphorylating system. Experimental proof for this concept was obtained during the late 1950s and early 1960s through a series of discoveries, primarily the reversal of respiratory chain-linked phosphorylation, and the phosphorylation-inhibitor oligomycin, which led to the demonstration of energy transfer directly between the coupling sites of the respiratory chain.

ENERGY-TRANSFER BETWEEN COUPLING SITES OF THE RESPIRATORY CHAIN: In 1957, Chance and Hollunger (184) briefly reported that intact mitochondria catalyze an energy-dependent reduction of endogenous NAD^+ by succinate. Similar findings were reported shortly thereafter by Bücher and Klingenberg (111), who used α-glycerophosphate as the reducing substrate. Both laboratories subsequently extended these observations and interpreted them as evidence for a reversal of electron transport through the first energy-coupling site of the respiratory chain (185–189). Initially this interpretation was received with skepticism (190–192), but several laboratories carried out further studies with both intact mitochondria (by use of succinate-linked acetoacetate reduction as the test system [193–195]) and submitochondrial particles (196, 197) that soon eliminated the objections raised. Continued work, mainly in Chance's (198) and Klingenberg's (195, 199) laboratories, has led to the general concept that electron transport through all three coupling sites of the respiratory chain is reversible and that the reversibility is a reflection of a dynamic equilibrium between the respiratory and phosphorylating systems.

Oligomycin, an antibiotic introduced by Lardy and associates (200) in 1958 as an inhibitor of respiratory chain-linked phosphorylation, has proved to be a most valuable tool for the study of mitochondrial energy transduction. It was shown to inhibit the P_i + ADP-induced stimulation of respiration of tightly-coupled mitochondria (201, 202), but not uncoupled respiration. It was also shown to inhibit the P_i-ATP (200) and P_i-H_2O (202) exchange reactions as well as the ATPase activity induced by uncouplers (202), but not that induced by arsenate (203, 204). These effects were consistent with the interpretation that oligomycin is a specific inhibitor of the phosphorylating system of the respiratory chain—a conclusion that was later confirmed with the isolated mitochondrial ATP synthetase (205).

In 1960, Ernster reported (206) that oligomycin does not inhibit the succinate-linked NAD^+ reduction when the aerobic oxidation of succinate is the source of energy. In fact, oligomycin was able to restore the reaction when it was inhibited by P_i + ADP (206) or by arsenate (207). Furthermore, the reaction was uninhibited in P_i-depleted mitochondria (208). These findings, which are summarized schematically in Fig. 3, demonstrated conclusively that energy can be conserved and utilized by the respiratory chain without the participation of the phosphorylating system (209). Evidence to support this conclusion was reported soon from other laboratories (195, 210–212; see also review 213).

OTHER ENERGY-LINKED FUNCTIONS OF THE RESPIRATORY CHAIN: Another important development that began in the early 1960s was the demonstration of reactions that are capable of utilizing energy derived from the respiratory chain without the participation of the phosphorylating sys-

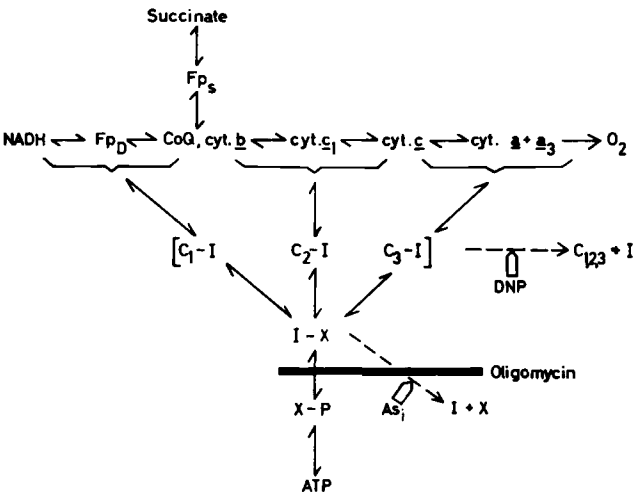

FIGURE 3 Schematic representation of the respiratory chain and the oxidative phosphorylation system. C_1, C_2, C_3 denote electron carriers at the coupling sites of the respiratory chain; I and X denote hypothetic energy-transfer carriers. Open bars indicate probable sites of uncouplind by 2,4-dinitrophenol (DNP) and by arsenate (As_i). From Ernster, 1965 (148).

tem, such as the energy-linked transhydrogenase, various ion translocators, and thermogenesis, as well as some pathological conditions resulting in impaired respiratory control.

Energy-linked Transhydrogenase

In their studies of the energy-linked reduction of endogenous nicotinamide nucleotide in intact mitochondria, Klingenberg and Slenczka (185) observed that both NAD^+ and $NADP^+$ were reduced, the latter to a higher degree than the former. They interpreted this phenomenon as an energy-linked shift of equilibrium of the mitochondrial nicotinamide nucleotide transhydrogenase reaction. Similar findings were reported by Estabrook and Nissley (214).

In 1963 Danielson and Ernster (215) demonstrated an energy-linked transhydrogenase reaction catalyzed by submitochondrial particles. The reaction consisted of an energy-dependent enhancement of the reduction of $NADP^+$ by NADH and resulted, as later shown by Lee and Ernster (216), in about a 500-fold shift of equilibrium of the transhydrogenase reaction towards the formation of NADPH and NAD^+. The energy for the reaction could be supplied either by substrate oxidation through the respiratory chain or by ATP hydrolysis; in the former case the reaction was insensitive to oligomycin, whereas in the latter it was oligomycin-sensitive. In both cases, the reaction was sensitive to uncouplers. From these results it was concluded (215) that the transhydrogenase reaction is functionally linked to the respiratory chain-linked ATP-synthesizing system in such a way that it can utilize energy captured by the energy-conservation mechanisms of the respiratory chain without the participation of the phosphorylating system. In more general terms, this conclusion implied that energy derived from the respiratory chain can be utilized for purposes other than ATP synthesis. An actual competition between the energy-linked transhydrogenase and oxidative phosphorylation was subsequently demonstrated by Lee and Ernster (217). Due to its ability to utilize energy directly from the respiratory chain, the transhydrogenase reaction became a useful tool for studying energy coupling in nonphosphorylating electron-transport systems (218, 219).

The transhydrogenase has subsequently been studied in great detail with respect to its kinetics and reaction mechanism (220), but it is only recently that the enzyme has been purified (221, 222) and the energy-linked reaction has been reconstituted (223).

Ion Translocation

As already mentioned, in the early 1950s several laboratories found that isolated mitochondria take up Ca^{2+} from the suspending medium, and that this causes swelling and uncoupling of oxidative phosphorylation. In 1955 Chance (224) observed that repeated additions of small amounts of Ca^{2+} to respiring mitochondria in the presence of phosphate caused transient enhancements of the rate of oxygen uptake, similar to those found with ADP. In 1962–1963 several laboratories (225–230) independently demonstrated an energy-dependent accumulation of Ca^{2+} and other divalent cations by respiring mitochondria in the presence of phosphate. As first shown by Saris (230), the Ca^{2+} uptake was accompanied by a release of protons. The Ca^{2+} accumulation resulted in a deposition of calcium phosphate as hydroxyapatite-like, electron-dense granules in the matrix (229, 231). The respiration-driven Ca^{2+} uptake was uncoupler-sensitive but insensitive to oligomycin, and was thus another example of a process capable of deriving energy from the respiratory chain without the involvement of the phosphorylating system. Initially it was believed that phosphate rather than Ca^{2+} was the actively accumulating species (232), but subsequent work in Chance's laboratory revealed that Ca^{2+} uptake occurred also in the presence of other penetrating anions (233) and to some extent even in the absence of added anions (234). The energetic stoichiometry of the anion-linked, massive accumulation of Ca^{2+} was estimated at two atoms of Ca^{2+} taken up per pair of electrons traversing each coupling-site of the respiratory chain (233–235). This stoichiometry and, in general, the mitochondrial transport of Ca^{2+} has been a very active field of research in the last 15 years (235–237). It is now established that the carrier functions as an electrogenic uniporter, mediating an active influx of Ca^{2+} across the inner mitochondrial membrane at the expense of an efflux of protons driven by electron transport or ATP hydrolysis. The carrier also mediates the transport of Mn^{2+}, Sr^{2+}, and Ba^{2+} but not Mg^{2+}. Crompton et al. (238) recently discovered a second Ca^{2+} carrier in mitochondria, which mediates the efflux of Ca^{2+} against an influx of Na^+, and which is found in the mitochondria of heart and other excitable organs. The occurrence of a mitochondrial glycoprotein which might be involved in Ca^{2+} transport has been described by Sottocasa and co-workers (239, 240).

In 1964, Moore and Pressman (241) made the important discovery that the antibiotic valinomycin, earlier described as an uncoupler of oxidative phosphorylation, required K^+ for its uncoupling effect. Closer examination of this phenomenon led to the recognition of valinomycin as a K^+ carrier, which facilitated the energy-linked uptake of K^+ by the mitochondria at the expense of respiratory energy (242). This discovery opened up the new field of ionophores, and had important implications not only for bioenergetics and membrane biology but also for bio-organic chemistry (243) and medicine (242).

By using the swelling of mitochondria as the test, Chappell and Haarhoff (155) discovered in 1967 that mitochondria in the presence of a permeant cation (e.g., NH_4^+, which penetrates the membrane as NH_3) take up various anions including phosphate, malate, and citrate. The uptake of malate was dependent on the simultaneous presence of phosphate, and the uptake of citrate required the presence of both phosphate and malate. From the steric requirements of the anion uptake, it appeared that the transport was mediated by a set of carriers, each specific for a certain anion or combination of anions. These observations set the stage in various laboratories for the demonstration and characterization of a series of translocators that are present in the mitochondrial inner membrane and responsible for the transport of various metabolites into and out of the mitochondrial matrix (Table II) (244–280; cf. reviews 237, 281). Among the metabolic functions of these translocators is to bring pyruvate (the product of glycolysis) and long-chain fatty acids (as the carnitine esters [267]) into the mitochondria, to facilitate amino acid catabolism and urea synthesis, to mediate the transfer of the reducing equivalents between extra- and intramitochondrial nicotinamide nucleotides via various shuttle mechanisms (110), and to provide for the import of phosphate and ADP and the export of ATP in connection with oxidative phosphorylation. The glutamate, aspartate and ADP,ATP translocators are electrogenic, and are responsible for the maintenance of the known disequilibrium that exists in the intact cell between the extra- and intramitochondrial $[NADH]/[NAD^+]$ (111) and $[ATP]/([ADP]\cdot[P_i])$ (282) poten-

tials. The ADP,ATP translocator, which is inhibited by atractylate (275) and bongkrekic acid (276), has recently been purified (277, 278) and its mode of action has been extensively studied in both native and reconstituted membranes (280).

Thermogenesis

Brown fat, discovered in 1551 by the Swiss naturalist Gessner (283), is the organ responsible for nonshivering thermogenesis (284). Heat production by brown-fat mitochondria is another example of a process that derives energy directly from the respiratory chain. For some time, it was believed that the norepinephrine-induced stimulation of respiration and heat production originated from a re-esterification of fatty acids (set free by a hormone-activated lipase), and resulted in a continuous splitting and resynthesis of ATP (285). Subsequent work revealed, however, that brown-fat mitochondria are relatively poor in ATP synthetase (286), and it became clear that the increased heat production is due to the presence of an endogenous "uncoupler," the effect of which is inhibited by certain nucleotides (287). Prompted by these observations, Heaton et al. (288) have demonstrated the occurrence in brown-fat mitochondria of a protein that acts as an unspecific anion channel, and the function of which is blocked by extramitochondrial purine nucleotides (289). Recently, Lin et al. (290) have purified this protein and found that it has some structural resemblance to the ADP,ATP translocator. How norepinephrine regulates the function of this protein is not known, but its effect seems to involve an adenyl cyclase-controlled lipase as well as the mitochondrial Ca^{2+}-Na^{+} exchange carrier (291).

TABLE II

Mitochondrial Metabolite Translocators

Metabolites	Species translocated (in ⇌ out)	Selected references
Dicarboxylates	$malate^{2-} \rightleftharpoons phosphate^{2-}$	155, 244
Tricarboxylates	$citrate^{3-} + H^{+} \rightleftharpoons malate^{2-}$	155, 245-248
α-Ketoglutarate	α-$ketoglutarate^{2-} \rightleftharpoons malate^{2-}$	244, 249-251
Phosphate	$phosphate^{-} \rightleftharpoons OH^{-}$	155, 252-256
Pyruvate	$pyruvate^{-} \rightleftharpoons OH^{-}$	257-259
Glutamate	$glutamate^{-} \rightleftharpoons OH^{-}$	260, 261
Glutamine	$glutamine \rightleftharpoons glutamate^{-} + H^{+}$	262, 263
Ornithine	$ornithine^{+} \rightleftharpoons H^{+}$	264, 265
Neutral amino acids	neutral amino acids	266
Acyl carnitine, carnitine	acyl carnitine ⇌ carnitine	267-269
Glutamate, aspartate	$glutamate^{-} + H^{+} \rightleftharpoons aspartate^{-}$	260, 270, 271
ADP, ATP	$ADP^{3-} \rightleftharpoons ATP^{4-}$	272-280

PATHOLOGICAL CHANGES IN MITOCHONDRIAL RESPIRATORY CONTROL: The discovery of mitochondrial respiratory control stimulated interest in factors and conditions that may control the coupling of respiration to phosphorylation in vivo. In the early 1950s, several laboratories (see reviews 85, 209) proposed that thyroid hormones may exert their physiological effect by uncoupling or loose-coupling respiration from phosphorylation. (The term "loose-coupling" was defined as a state of the mitochondria in which respiratory control is virtually abolished while phosphorylation still can take place in the presence of phosphate and phosphate acceptor.) Early evidence seemed to support this concept by showing that thyroid hormones added to mitochondria in vitro or administered in vivo caused loose-coupling or uncoupling of oxidative phosphorylation and a swelling of the mitochondrial structure (cf. 85, 209). Closer examination of these effects revealed (292, 293), however, that they are observed only at toxic levels of the hormone and thus probably unrelated to the physiological action of thyroid hormones. At the same time, evidence was obtained (292, 293) which indicated that the physiological effect of thyroid hormones is on the synthesis of mitochondrial proteins. This conclusion has received considerable support in recent years.

In 1959, Ernster, Ikkos, and Luft (294), in the course of investigations of skeletal-muscle mitochondria from thyrotoxic patients, discovered a defect of mitochondrial respiratory control in a case of severe hypermetabolism of nonthyroid origin. The mitochondria showed a high degree of "loose-coupling"; they almost completely lacked of respiratory control by P_i and ADP, but could still make ATP from P_i and ADP (Fig. 4). The respiration was insensitive to oligomycin (295, 296). There were also some striking structural changes of the skeletal-muscle mitochondria as well as a large increase in their number and

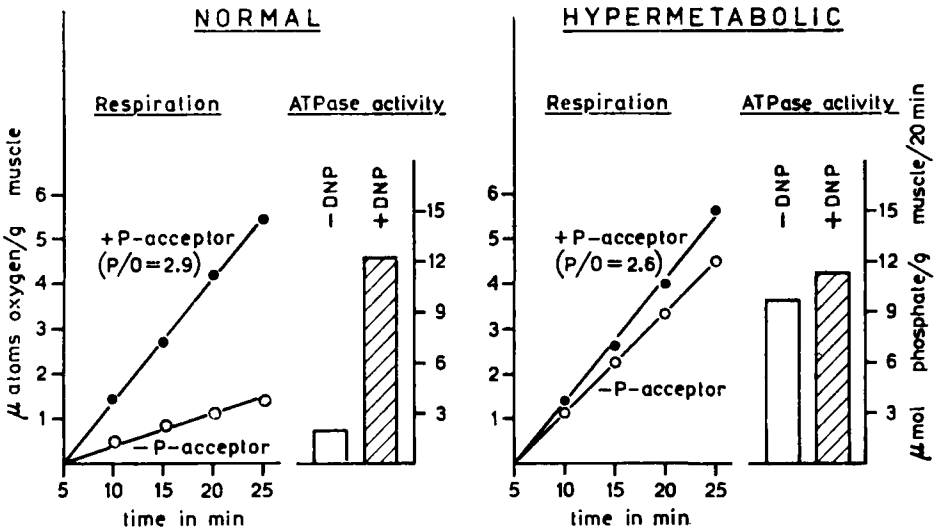

FIGURE 4 "Loose-coupling" of muscle mitochondria from a patient with severe hypermetabolism of nonthyroid origin. From Ernster, Ikkos, and Luft, 1959 (294).

size (295). A second case of this disease, the etiological origin of which is not known, was reported in 1976 by Di Mauro et al. (297).

A comprehensive review of mitochondrial disorders has recently been published by Carafoli and Roman (137).

RESOLUTION AND RECONSTITUTION OF THE ENERGY-TRANSDUCING SYSTEM OF MITOCHONDRIA. MITOCHONDRIAL ENZYME TOPOLOGY: Parallel to the above developments, important progress was made during the 1950s and 1960s towards the resolution and reconstitution of the mitochondrial electron-transport and phosphorylating systems. During the latter half of the 1960s, several laboratories also initiated attempts to separate and characterize the inner and outer mitochondrial membranes and to determine the localization and topology of various mitochondrial catalysts and other chemical constituents.

Resolution and Reconstitution of the Respiratory Chain

A first reconstitution of the respiratory chain was achieved by Keilin (298) in 1930. The oxidation of cysteine by an oxidase preparation was restored by cytochrome *c*, and all the properties of the reconstituted system were those of "a true respiratory system of the cell." The use of cytochrome *c*-deficient heart-muscle preparations led in 1940 to the demonstration of the role of cytochrome *c* in the succinoxidase system (299). In 1958, Keilin and King (300) reconstituted succinoxidase by combining an alkali-treated Keilin-Hartree preparation with solubilized succinate dehydrogenase.

A comprehensive study of large-scale preparations of beef heart mitochondria was begun in the mid-1950s in Green's laboratory (301). Mitochondria from beef heart proved to possess a remarkably high degree of stability; they were capable of withstanding a preparation procedure involving disruption of the tissue by relatively harsh mechanical means, and subsequent storage of the mitochondria in the frozen state for long periods of time. These preparations thus became the material of choice for future studies that aimed at a resolution and reconstitution of the respiratory chain and the phosphorylating system.

In 1956, Singer et al. (302) purified succinate dehydrogenase from beef heart mitochondria and demonstrated that the enzyme is a flavoprotein. The same conclusion was reached simultaneously by Wang et al. (303). A year later Crane and associates (304) reported evidence that ubiquinone is a redox carrier of the respiratory chain between the NADH- and succinate dehydrogenases and the cytochrome system. For some time, the participation of ubiquinone in the respiratory chain was questioned on kinetic grounds (305), but finally was established by reconstitution experiments (306, 307) and by reevaluation of the kinetic data (308), which took into account the fact that the molar amount of ubiquinone in mitochondria is in large molar excess over other respiratory-chain components. In 1960, Beinert and Sands (309) discovered a new type of iron-containing redox catalysts that consisted of nonheme iron proteins. These catalysts, which were later found to contain iron-sulfur centers as their redox groups, were shown to be components of both succinate and NADH dehydrogenase.

In the early 1960s, several groups in Green's laboratory were engaged in the separation and characterization of particulate protein complexes that catalyze partial reactions of the respiratory chain. Four such complexes were isolated: NADH-ubiquinone reductase (complex I), containing FMN and nonheme iron (310); succinate-ubiquinone reductase (complex II), containing FAD and nonheme iron (311): ubiquinol-cytochrome *c* reductase (complex III), which contains cytochromes *b* and c_1, some bound ubiquinone (312), and, as later shown by Rieske et al. (313), a nonheme iron protein (recently identified with Slater's BAL-sensitive factor [314]); and cytochrome *c* oxidase (complex IV), containing cytochrome *a* ($+a_3$) and copper (315). In 1962, Hatefi et al. (316) succeeded in reconstituting NADH oxidase and succinoxidase by combining complexes I, III, and IV and complexes II, III, and IV, respectively, in the presence of cytochrome *c*. In both cases, the reconstitution required high concentrations of the complexes and resulted in a particulate preparation which did not dissociate upon subsequent dilution.

These results gave rise to the concept (317) that the components of the respiratory chain exist in mitochondria as a fixed assembly ("elementary particles"). Indeed, it was found that cytochrome *c* can form stable complexes with complex III and complex IV (318) and that mitochondria contain the cytochromes in near-stoichiometric amounts (319). On the other hand, the flavin-containing complexes, and especially complex I, were found to occur in smaller amounts than the cytochromes, and it was suggested that ubiquinone functions as a mobile redox carrier between these complexes and the cytochrome system (308). In recent years it has become evident that this relationship is far more complex than had been anticipated. It is now known (see reviews 320, 321) that both complex I and complex II contain multiple iron-sulfur centers, and that some of these interact with protein-bound ubiquinone in a highly complex fashion, the functional significance of which is not yet fully understood.

In 1962, Fernández-Morán (322) discovered in negatively stained specimens of mitochondria the occurrence of regularly spaced, globular projections on the inner surface of the inner membrane. Initially these projections were believed to correspond to the "elementary particles" (323) and to contain, besides the enzymes of the respiratory chain, a large amount (>50%) of inert "structural protein" (324). However, later work in Racker's and Chance's laboratories (325) led to the conclusion that the globules consist of the coupling factor F_1, which is the catalytic unit of the ATP-synthesizing system of the respiratory chain (see below). The "structural protein" was found to consist mainly of denatured F_1 (326). Whether the projections exist as such in the native mitochondrion or are preparation artifacts has been much debated over the years. In any case, they are found with great regularity in negatively stained mitochondrial preparations and have served as an important landmark for the identification and orientation of the inner membrane. For example, it was on this basis, in addition to biochemical evidence, that the inverted orientation of the membrane of submitochondrial particles prepared by sonication was first proposed (219).

Resolution and Reconstitution of Oxidative Phosphorylation

The first submitochondrial preparation capable of carrying out oxidative phosphorylation was described by Cooper, Devlin, and Lehninger (327) in 1955. By treating rat liver mitochondria with digitonin, they isolated a particulate fraction which catalyzed the oxidation of β-hydroxybutarate and succinate with P/O ratios of 2.4 and 1.5, respectively. The prepa-

rations contained endogenous NAD^+, and external NADH was oxidized by an external type of cytochrome *c* reductase, similar to that found in intact mitochondria. The size of the particles was estimated to be 1/2,000 of that of mitochondria. Interestingly, these particles, in contrast to preparations obtained by sonication, apparently had the same orientation of the membrane as exists in intact mitochondria.

Submitochondrial particles prepared by sonication and capable of oxidative phosphorylation were first described by Kielley and Bronk (328) through the use of mitochondria from rat liver. In Green's laboratory, it was found that electron transport particles from beef heart mitochondria, when prepared in the presence of media containing Mg^{2+} and ATP (329), or Mg^{2+}, Mn^{2+}, and succinate (330), retained the ability to carry out phosphorylation with relatively high efficiency if either succinate or NADH was used as substrate. These "heavy" electron transport particles (ETP_H), and especially those prepared in the presence of Mg^{2+} and ATP, proved to be valuable for future studies of oxidative phosphorylation and related energy-linked functions. It was, for example, with these particles that a reversal of oxidative phosphorylation was first demonstrated in a submitochondrial system (196); such studies eliminated most of the objections to the conclusions about the mechanism of this process earlier based on experiments with intact mitochondria. With this system it was also first demonstrated (331) that oxidative phosphorylation in submitochondrial particles is not specific for adenine nucleotides—in contrast to intact mitochondria—a phenomenon that was later explained through the discovery of the adenine nucleotide-specific mitochondrial ADP, ATP translocator. Another result of principal importance obtained with phosphorylating sonic particles was the demonstration that energy-linked transhydrogenase driven by the respiratory chain was insensitive to oligomycin whereas that driven by ATP hydrolysis was oligomycin-sensitive (215, 218).

In the late 1950s work was also initiated in Green's laboratory in order to resolve and reconstitute the phosphorylating enzyme system associated with ETP_H. In 1958, Linnane (332) reported that disruption of beef heart mitochondria by sonication in the presence of EDTA resulted in submitochondrial particles ("modified ETP_H") that required a soluble protein fraction for maximal phosphorylation. This factor was purified 8- to 15-fold by Linnane and Titchener (333), who showed that it restored phosphorylation coupled to the oxidation of both succinate and NADH.

After this initial success, Green and associates (334) embarked on a comprehensive study of the individual coupling sites of the respiratory chain. The interpretation of the results became difficult, however, because some of the data, notably those relating to coupling site 3, could not be verified. In the meantime, Racker and associates had begun their work on mitochondrial coupling factors which came to be of fundamental importance for the resolution and reconstitution of the enzyme system involved in electron transport-linked phosphorylation.

Extending an observation reported briefly in 1958 (335), Racker and associates described in 1960 the isolation of an ATPase from beef heart submitochondrial particles (336, 337). The soluble enzyme was cold-labile, which may explain why it escaped detection earlier. The enzyme had the important property of being able to restore oxidative phosphorylation to nonphosphorylating submitochondrial particles. This ability led to the denotion of the ATPase as "coupling factor 1" (F_1). When F_1 was rebound to the membrane, its ATPase activity again became cold-stable. Furthermore the bound enzyme was sensitive to the phosphorylation-inhibitor oligomycin, whereas the soluble enzyme was oligomycin-insensitive. These findings constituted strong evidence for the notion that the ATPase functions as the terminal enzyme in electron transport-linked ATP synthesis. The binding of F_1 to the membrane of F_1-depleted submitochondrial particles was accompanied by a reappearance of the projecting subunits (325) (Fig. 5).

A much-discussed feature of F_1, observed at an early stage, was that its ability to restore phosphorylation in nonphosphorylating submitochondrial particles actually consisted of a combination of two effects: a catalytic and a "structural" effect (338). It was observed (339) that in certain instances phosphorylation can be restored by catalytically inactive (e.g., chemically modified) F_1. This phenomenon was explained when Lee and Ernster (340) demonstrated that low concentrations of oligomycin can replace F_1 in restoring phosphorylation in certain types of nonphosphorylating particles. These particles contained residual F_1, which was catalytically competent, and the added F_1 served to plug an energy leak—as we now know, a proton leak—that had arisen because of the partial removal of F_1 and caused a dissipation of the energy generated by electron transport. It was this "structural" effect of F_1 that was duplicated by low concentrations of oligomycin (high oligomycin concentrations caused an inhibition of the residual F_1 and thereby an inhibition of phosphorylation). Oligomycin also induced a respiratory control in these particles (219, 341, 342). When particles were completely depleted of F_1, oligomycin was no longer able to replace F_1 in restoring phosphorylation (343).

An important development in the mid-1960s was the demonstration by Kagawa and Racker (205) that the phosphorylation inhibitor oligomycin does not act on F_1 itself but on a segment of the ATPase complex that is imbedded in the membrane. This segment has been denoted F_O (where o refers to oligomycin). F_O consists of several hydrophobic proteins. One of these has been identified by its covalent binding to *N,N'*-dicyclohexylcarbodiimide (DCCD), which has been shown by Beechey and associates (344) to act as a phosphorylation inhibitor similar to oligomycin. This DCCD-binding protein had been purified from several sources, including mitochondria, chloroplasts, and bacteria; the amino-acid sequences of these proteins show an exceedingly high degree of homology (345).

The discovery of F_1 was followed by extensive efforts in several laboratories to identify and characterize additional coupling factors involved in the mitochondrial ATP-synthesizing system (see review 346). Among the many factors described, two have proved to exhibit well-defined functions: coupling factor F_{c2} (347) (or F_6 [348]), involved in the binding of F_1 to F_O, and OSCP (oligomycin sensitivity-conferring protein) (349), responsible for the conferral of oligomycin sensitivity. Interestingly, both factors have been found thus far only in mitochondria and do not seem to occur in chloroplasts or bacteria. Another coupling factor of continued interest is factor B, which was described by Sanadi and associates (350) and is required for the P_i-ATP exchange reaction that is catalyzed by membrane-bound ATPase (cf. ref. 346).

The whole, oligomycin-sensitive, ATPase complex was first isolated by Kagawa and Racker (205) in 1966, and its action as a reversible proton pump was demonstrated (351) a few years later (see further below). Reconstitution of the ATPase complex

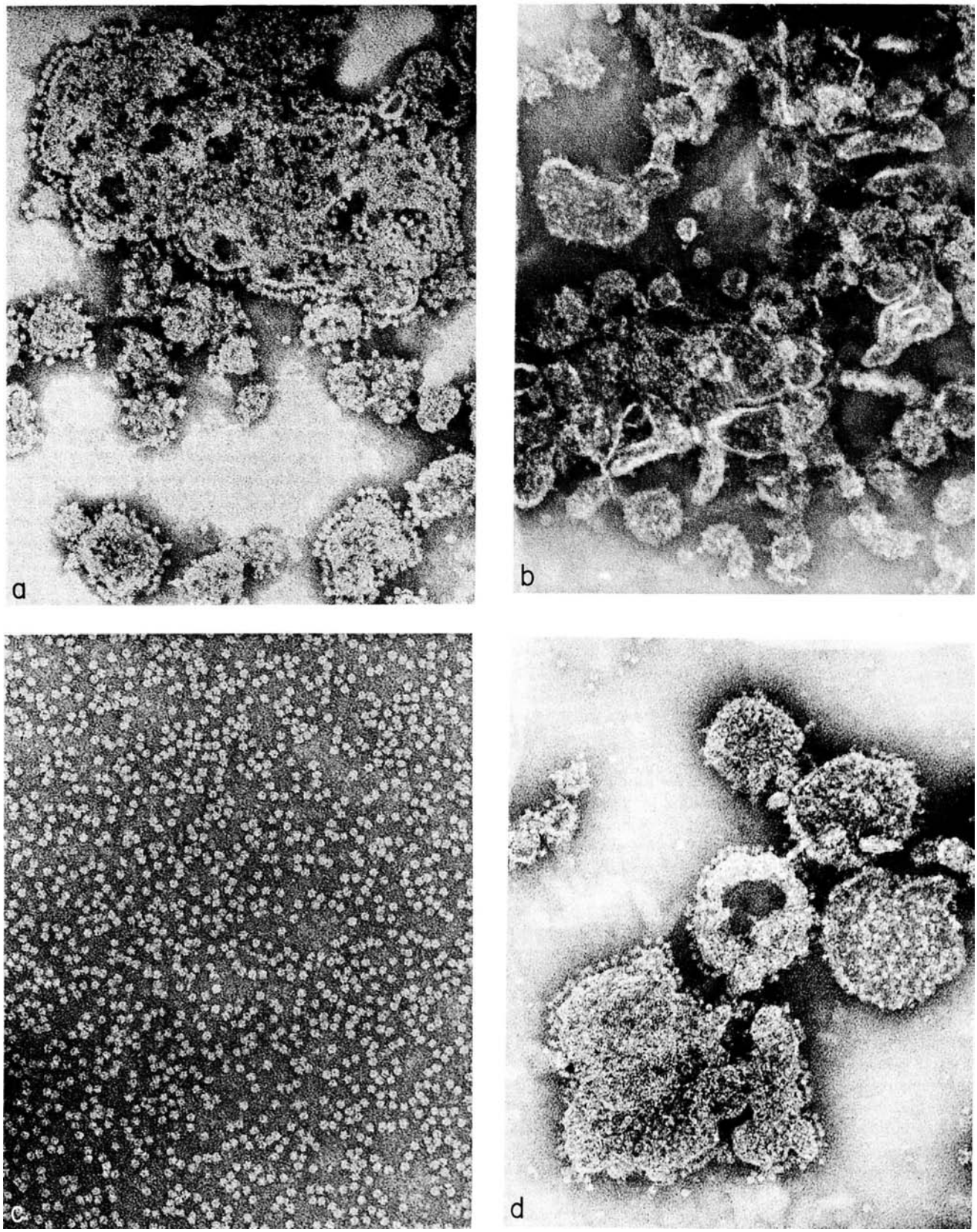

FIGURE 5 Resolution and reconstitution of the projecting subunits of submitochondrial particles from beef heart, containing coupling factor F_1. (*a*) Native particles. (*b*) Particles after removal of F_1. (*c*) Coupling factor F_1. (*d*) Reconstituted particles. For details, see Racker et al., 1965 (325).

from isolated F_O and F_1 was first described by Capaldi (352) and its quantitative aspects were investigated by Glaser et al. (353, 354).

Isolated mitochondrial F_1, together with similar proteins from chloroplasts and bacteria, has been the subject of extensive studies (cf. review 346). As first shown by Senior and

Brooks (355, 356), F_1 consists of five subunits (α–ϵ), with molecular weights of 53,000, 50,000, 30,000, 17,000, and 7,500 daltons. Initial estimates, based on Coomassie blue staining of SDS gels, indicated a subunit stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ (355, 357), but the validity of these estimates was later questioned on the basis of sulfhydryl-group titrations (358). Recent data, however, seem to support the originally proposed stoichiometry (359, 360). The β-subunit is generally believed to contain the catalytic center of F_1 (cf. ref. 346). The enzyme contains firmly bound adenine nucleotides (361), the role of which is thought to be partly catalytic and partly regulatory. Extensive studies of the reaction mechanism of F_1 have been carried out by means of various exchange reactions as well as by the use of various activators and inhibitors. Among the latter aurovertin (which forms a fluorescent complex with F_1 [326–364]), the covalent inhibitor nitrosobenzofurazan chloride (365), and tris-bathophenanthroline-ferrous chelate (the effect of which is relieved by uncouplers [366, 367]) have been shown to bind to the β-subunit of the enzyme.

A naturally occurring inhibitor of mitochondrial F_1-ATPase was described in 1963 by Pullman and Monroy (368). It consists of a protein of about 10,000 daltons. It has been proposed (369) that the inhibitor acts as a unidirectional regulator of ATP synthesis, inhibiting the backflow of energy from ATP to the respiratory chain. The binding and release of the inhibitor are regulated by the energy state of the mitochondria (370–373). Recent evidence suggests that the inhibitor acts on both the forward and reverse reactions catalyzed by F_1 (374) and that its function may be to regulate ATP synthesis in relation to other energy-linked functions of the respiratory chain, e.g., Ca^{2+} uptake (375).

Isolation and Characterization of the Outer and Inner Membranes. Intramitochondrial Localization of Enzymes

Although the existence of two mitochondrial membranes had been established in the early 1950s, it was not until the second half of the 1960s that methods for their separation were developed. Perhaps the main reason for this long interval was that interest in mitochondrial research was focused primarily on studies of the respiratory chain and oxidative phosphorylation. The association of respiration with the cristae was indicated by early morphological evidence (26), and, thus, when it was later found that the Keilin and Hartree heart-muscle preparation (52) or a deoxycholate-solubilized succinoxidase from liver mitochondria (376, 377) consisted of membranes, it was plausible to assume that these membranes were derived from the inner mitochondrial membrane. Support for the association of the respiratory chain with the inner mitochondrial membrane was also obtained by histochemical techniques (378).

In the mid-1960s several laboratories independently reported the successful separation of mitochondrial inner and outer membranes (see review 124). Lévy et al. (379) and Schnaitman et al. (380) used low concentrations of digitonin to detach the outer membrane of liver mitochondria, after which they separated the outer and inner membrane fractions by differential centrifugation. The rationale for this method is that the outer membrane is rich in cholesterol, which binds digitonin. Later Schnaitman and Greenawalt (381) refined this method to allow the preparation of "mitoplasts," i.e., mitochondria without an outer membrane and with a relatively intact inner membrane and matrix. Another procedure, worked out by Parsons et al. (382) and Sottocasa et al. (81), took advantage of the selective shrinking of the inner membrane following exposure of liver mitochondria to a swelling-contraction cycle. The distended outer membrane ruptures either spontaneously or, more efficiently, after gentle sonication, and can be separated from the inner-membrane fraction by density-gradient centrifugation.

Striking differences between the two mitochondrial membranes were found in regards to osmotic behavior and permeability. As already mentioned, the inner membrane readily unfolds and refolds in response to changes in osmotic pressure. In contrast, the outer membrane shows no reversible response to such changes, and the distention and rupture during mitochondrial swelling may be interpreted as a passive process following the unfolding of the inner membrane. Extensive studies (383), based on measurements of the space occupied by various substances present in the medium in relation to the total water space of the mitochondria, and correlated with morphological observations, have led to the conclusion that the inner membrane is practically impermeable to most substances except uncharged molecules of a molecular weight not greater than 100–150. The majority of charged molecules of physiological significance pass through the inner membrane by way of specific translocators associated with this membrane (see Table II). In contrast, the outer membrane seemed to be permeable to a wide range of substances, both charged and uncharged, with molecular weights up to about 5,000. Parsons et al. (382) observed in negatively-stained specimens of isolated outer membranes the occurrence of pore-like structures. Recently, two laboratories (384, 385) reported the isolation from the mitochondral outer membrane, of a protein with a molecular weight of about 30,000 and the ability to make phospholipid bilayers permeable to a variety of substances. A similar "pore protein" ("porin") had been described previously in the outer membrane of Gram-negative bacteria (386).

Chemically the most striking quantitative difference between the mitochondrial inner and outer membranes is in the relative contents of protein and lipid; the outer membrane contains, on a protein basis, two to three times more phospholipid than the inner membrane (382). Like energy-transducing membranes in general, the inner membrane is particularly rich in proteins deeply embedded in the membrane, a circumstance strikingly revealed by freeze-fracture techniques (387) (Fig. 6). A conspicuous qualitative difference in phospholipid composition is the virtually exclusive localization of cardiolipin in the inner membrane (382). Cholesterol is found predominantly in the outer membrane (388).

The availability of procedures for the separation of the two mitochondrial membranes opened the way to a determination of the intramitochondrial distribution of enzymes. Such data soon became available regarding almost all enzymes known to be present in mitochondria (124, 389). The outer membrane was found to contain a diversity of enzymes including a rotenone-insensitive NADH-cytochrome *c* reductase (81) (similar to, but not identical with, the microsomal NADH-cytochrome b_5 reductase-cytochrome b_5 system), monoamine oxidase (380, 381), as well as various enzymes involved in phospholipid metabolism (390). The intermembrane space was identified as the location of adenylate kinase (124, 381, 389, 391), nucleoside mono- and diphosphokinases (381, 389), sulfite oxidase (392) and yeast cytochrome *c* peroxidase (393). The inner membrane proved, as expected, to be the site of the catalysts of the respiratory chain and respiratory chain-linked phosphorylation. Also found to be associated with the inner membrane were the nicotinamide nucleotide transhydrogenase

FIGURE 6 Freeze-etch preparation of glutaraldehyde-fixed mitochondrion showing the two membrane faces exposed by fracture. From Wrigglesworth and Packer, 1970 (387).

(389), β-hydroxybutyrate dehydrogenase (381, 390), fatty acyl carnitine transferase (394), and two enzymes involved in heme synthesis (ferrochelatase [395, 396] and δ-aminolevulinic acid synthetase [396]). Enzymes localized in the matrix include those involved in the citric acid cycle (except succinate dehydrogenase) and related processes such as substrate-level phosphorylation, pyruvate and phosphopyruvate carboxylation, glutamate oxidation, transamination, citrullin synthesis, and fatty acid oxidation (see review 124). The localization of some of these enzymes in the matrix was questioned by Green and associates (cf. 124, 397 and refs. therein), who claimed that the enzymes of the citric acid cycle and fatty acid oxidation are associated with the outer membrane. However, this controversy was successfully settled through a joint effort between two of the laboratories involved (398).

Enzyme Topology of the Inner Membrane

In the 1960s information also began to be available concerning the enzyme topology of the inner mitochondrial membrane. As membrane proteins in general, the enzymes associated with the inner membrane of mitochondria proved to possess a well-defined orientation in relation to the plane of the membrane—a transverse topology, or "sidedness," that was of special interest in connection with the chemiosmotic hypothesis (see below).

A first striking illustration of this sidedness was the demonstration that the coupling factor F_1 resides in the projections on the inside surface of the inner mitochondrial membrane. More general criteria used for the assessment of the transverse topology of various enzymes included accessibility to proteases, antibodies, and other macromolecules as well as nonpenetrant substrates, effectors, and protein reagents (see review 399). The inverse relationship in membrane orientation between intact mitochondria (or mitoplasts) and sonic submitochondrial particles was also exploited. The results could be verified further by reconstitution experiments using isolated components. The available information (see reviews 308, 399) may be summarized as follows (Fig. 7).

The flavin prosthetic groups of NADH- and succinate-ubiquinone reductase face the matrix (M) side of the membrane. Ubiquinone and the cytochrome *b* moiety of ubiquinol-cytochrome *c* reductase are probably inaccessible to either side of the membrane, whereas cytochrome c_1 faces the cytosolic (C)

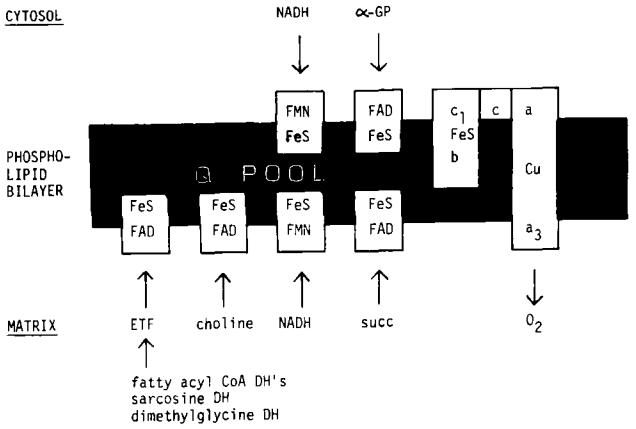

FIGURE 7 Possible topology of various FeS-flavoproteins, the Q pool and the cytochrome system in the inner mitochondrial membrane. Abbreviations: α-GP, α-glycerophosphate; ETF, electron-transferring flavoprotein; succ, succinate; DH, dehydrogenase. From Ernster, 1977 (538).

side. Cytochrome *c* is located on the C side of the membrane, which is its site of interaction with both cytochrome c_1 and cytochrome *a*. Cytochrome *c* oxidase is thought to span the membrane, with its O_2-reactive site (cytochrome a_3) oriented toward the M side. Also facing the M side of the membrane are the catalytic sites of nicotinamide nucleotide transhydrogenase and ATPase as well as β-hydroxybutyrate dehydrogenase, choline dehydrogenase, and "ETF dehydrogenase," the enzyme serving as the catalytic link between the electron-transferring flavoprotein (ETF) and the cytochrome system. The flavoenzymes glycerol phosphate dehydrogenase and an "external" NADH dehydrogenase found in yeast and plants react with their substrates on the C side of the membrane. It should be pointed out that all these localizations of the various enzymes concern their catalytic sites and that the enzyme as a whole spans the membrane in many cases. This is the case with the proton-translocating energy-transducing units (complexes I, III, IV, ATPase, transhydrogenase) and probably with several other enzymes as well.

SEARCH FOR CHEMICAL HIGH-ENERGY INTERMEDIATES. ALTERNATIVE HYPOTHESIS: Despite the vast amount of information that accumulated during the 1950s and early 1960s concerning the pathways and components of mitochondrial energy transduction, relatively little progress was made towards elucidating the mechanism of electron transport-linked phosphorylation. Although most data were consistent with the predictions of a "chemical" mechanism, which, as formulated by Slater (179) and others, involved "high-energy intermediates" as energy transducers between electron transport and ATP synthesis, there was no success in isolating or identifying such intermediates, in spite of efforts in many laboratories. There is hardly any component of the respiratory chain that has not been proposed as part of such an intermediate (400). An involvement of Ca^{2+} (234) and of K^+ (+ valinomycin) (241) in high-energy intermediates has also been considered in connection with the active uptake of these cations by respiring mitochondria. None of the proposed or, in some cases, allegedly demonstrated intermediates could be verified experimentally. A promising exception was a protein-bound phosphohistidine, described in Boyer's laboratory (401), which, however, turned out to be an intermediate of the succinyl thiokinase reaction (402). As could be expected, the search for chemical "high-energy" intermediates gradually decreased in intensity from the mid-1960s (although new proposals still appear occasionally [403]).

In 1965, Boyer (404) proposed that the energy-yielding oxidoreduction steps in the respiratory chain might give rise to "energized" conformational states of the catalysts involved. These conformational states of the redox catalysts would induce the formation of a covalent high-energy bond in an adjacent ATPase molecule, with the result that the latter now would be able to synthesize ATP from ADP and P_i. While this mechanism provided an alternative explanation of how energy may be conserved by electron-transport catalysts or ATP-synthesizing enzymes, it did not readily account for the mechanism by which energy may be transferred *between* such enzymes. By the middle of the 1960s there was evidence that in mitochondria energy can be transferred between a relatively large number of energy-transducing units, including the three coupling-sites of the respiratory chain, the ATP-synthesizing system, the nicotinamide nucleotide transhydrogenase, and various ion translocators. It appeared unlikely that all these enzymes and translocators would transfer energy by direct molecular collision within the membrane.

A solution to this problem was opened in 1961 when Mitchell (405) advanced his chemiosmotic hypothesis, which he subsequently developed to represent a general mechanism for energy-coupling in respiratory and photosynthetic phosphorylation (406) (Fig. 8). According to this hypothesis energy conservation in the oxido-reduction chain proceeds by way of a proton gradient across the coupling membrane; the gradient is brought about through the action of alternating hydrogen and electron carriers, which form loops across the membrane in such a way that hydrogen ions are taken up on one side of the membrane and given off on the other side. It was further proposed that the same membrane contains a proton-translocating reversible ATPase, which can utilize the proton gradient generated by the oxido-reduction chain for the synthesis of ATP from ADP and P_i. The same year, Williams (407) proposed a mechanism which also involves a flux of protons generated by the respiratory chain as the driving force for ATP synthesis. However, instead of establishing a gradient *across* the membrane, as in the case of chemiosmotic hypothesis, this proton flux was assumed to give rise to a proton activity in the lipid phase *within* the membrane, localized so that it can bring about a dehydration around the active center of the ATPase and thereby promote the condensation of ADP and P_i to ATP (408). The chemiosmotic mechanism elicited especially great interest, since it provided a rational explanation for the role of the membrane in oxidative and photosynthetic phosphorylation and constituted a common link between these processes and active transport.

Historically, the concept of vectorial transport of protons originated from the suggestion by Lundegårdh (409) in 1945 that if oxido-reduction through the cytochrome system were anisotropically organized across the membrane, protons would be produced on one side and consumed on the other. In their studies of acid secretion by gastric mucosa, Davies and Ogston (410) concluded in 1950 that proton secretion may involve the parallel operation of two mechanisms: (1) a redox-linked proton pump; and (2) a proton pump driven by the hydrolysis of a labile phosphate compound (e.g., creatine phosphate). They pointed out (410) that "this is an interesting possibility, because, each mechanism being reversible, mechanism 1 could drive mechanism 2 backwards, leading to phosphorylation of crea-

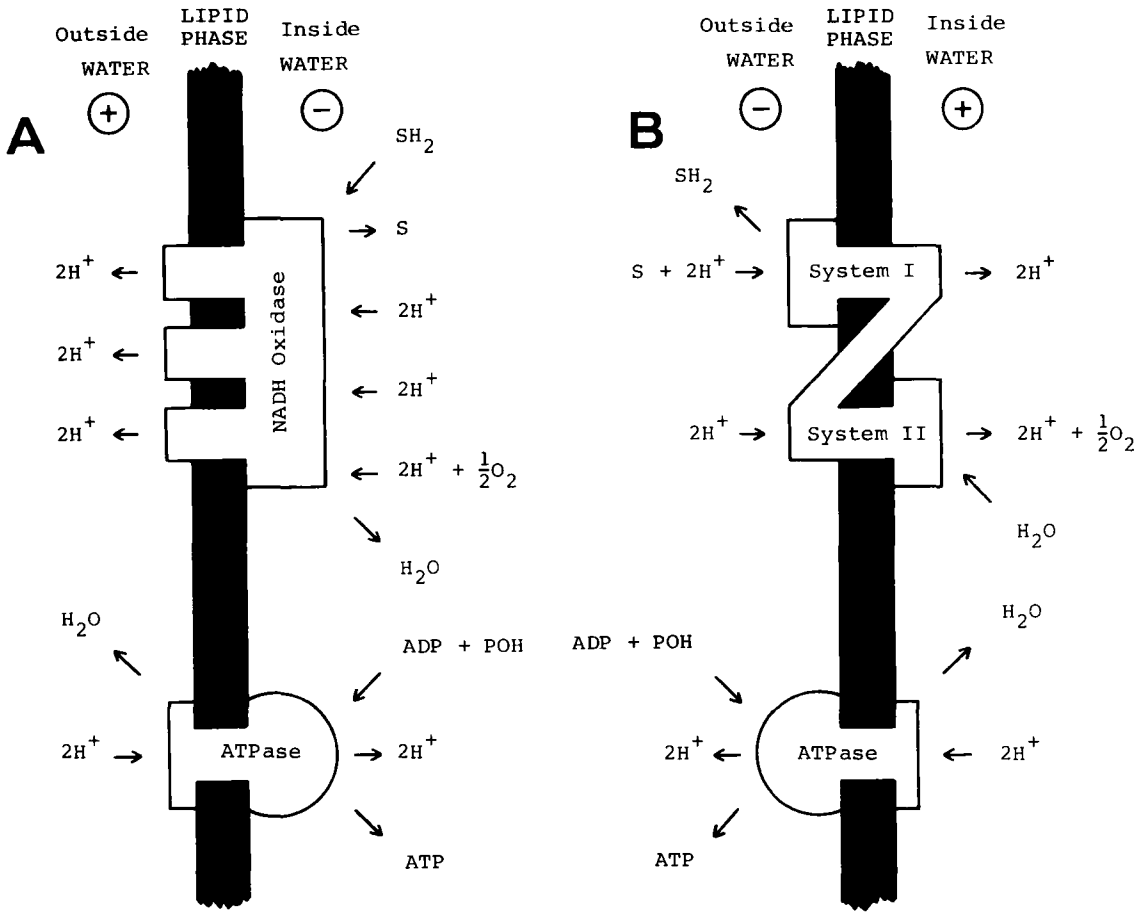

FIGURE 8 Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. From Mitchell, 1970 (539).

tine and this might explain how oxidation in the cytochrome system at high E_h' could bring about phosphorylation." However, they discarded this possibility in view of Friedkin and Lehninger's (60) demonstration of phosphorylation linked to the oxidation of NADH in a liver-particulate preparation, pointing out (410) that "it is doubtful whether such a degree of organization as would be required by our mechanism could have survived in such a preparation."

Mitchell's interest in chemiosmotic mechanisms arose in the early 1950s from studies of bacterial membrane transport. He noted that some of these processes resembled enzyme-catalyzed reactions and, furthermore, that bacterial membranes contained both electron-transferring and phosphorylating enzymes. These observations gave rise to the idea that certain transport processes across membranes might be catalyzed by vectorial group-translocating enzymes that may be related to oxidative phosphorylation. In a lecture published in 1954, Mitchell (411) stated: "... in complex biochemical systems, such as those carrying out oxidative phosphorylation, the osmotic and enzymic specificities appear to be equally important and may be practically synonymous."

The official debut of the chemiosmotic hypothesis took place in a short paper in *Nature* in July, 1961 (405); one of the reasons given for the proposal of the hypothesis was "to acknowledge the elusive character of the $C \sim I$ intermediates by admitting that they may not exist." Shortly afterwards, Mitchell (412) reported evidence that uncouplers of oxidative phosphorylation act as proton conductors through the membranes of mitochondria and bacteria. However, the real breakthrough of the hypothesis did not begin until 1966 when Mitchell (406) published a book entitled: *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation.* In this book he outlined his hypothesis in detail, specifying its basic postulates and defining the "protonmotive force" as the sum of an electric and a chemical component. At the same time, experimental evidence for these concepts began to appear (413–416). As a result, work was initiated in many laboratories in order to evaluate Mitchell's hypothesis, and constituted the principal task in bioenergetics for the next decade.

THE CHEMIOSMOTIC DECADE: The chemiosmotic hypothesis was based upon four postulates (406): (*a*) a proton-translocating ATPase; (*b*) a proton-translocating oxido-reduction chain; (*c*) exchange-diffusion systems, coupling proton translocation to that of anions and cations and permitting entry and exit of essential metabolites; and (*d*) an ion-impermeable membrane, in which systems 1, 2, and 3 reside. The protonmotive force, Δp (or $\Delta\tilde{\mu}_{H^+}$, as designated by many investigators), was defined (406, 414) as the sum of the electric potential ($\Delta\psi$) and the pH difference (ΔpH) across the membrane, according to the equation

$$\Delta p (\text{or } \Delta\tilde{\mu}_{H^+}) = \Delta\psi - Z\Delta pH,$$

where Z stands for $-2.303RT/F$ (-59 at 25° when Δp [or $\Delta\tilde{\mu}_{H^+}$] and $\Delta\psi$ are expressed in mV and ΔpH in pH units).

Early evidence reported by Mitchell and Moyle (413, 415) showed that the addition of oxygen to anaerobic mitochondria in the presence of an NAD^+-linked substrate resulted in an ejection of protons with a stoichiometry of $6H^+/O$ (Fig. 9 *top*), and that the proportion of the electrical and chemical compo-

nents of the protonmotive force varied with the experimental conditions (Fig. 9 *lower panel*), all in accordance with the predictions of the chemiosmotic hypothesis. An early piece of evidence that acquired great attention was the "acid-bath" experiment of Jagendorf and Uribe (416), in which it was shown that a proton gradient imposed across the thylakoid membrane of chloroplasts gave rise to ATP synthesis.

Further progress in this area was greatly dependent upon technical developments that made possible the demonstration and quantitation of the protonmotive force and its electric and chemical components (see reviews 417, 418). The availability of sensitive pH and ion-specific electrodes, in combination with suitable ionophores, and of various optical probes was of great significance. Another important development was the introduction by Skulachev, Liberman, and their associates (419) of a series of synthetic organic anions and cations, among them several boron compounds, which proved very useful for determining $\Delta\tilde{\mu}_{H^+}$. A number of elegant physicochemical techniques that facilitated the measurement of transient phases of the electrochemical events, especially those accompanying light-induced electron transport and phosphorylation, were developed in Witt's laboratory (420). Another crucial technical development, initiated in Racker's laboratory (421, 422) and pursued and further developed by Skulachev's group (423, 424), was the elaboration of methods to incorporate energy transducing systems—electron transport catalysts, ATPases, and ion translocators—into artificial phospholipid vesicles.

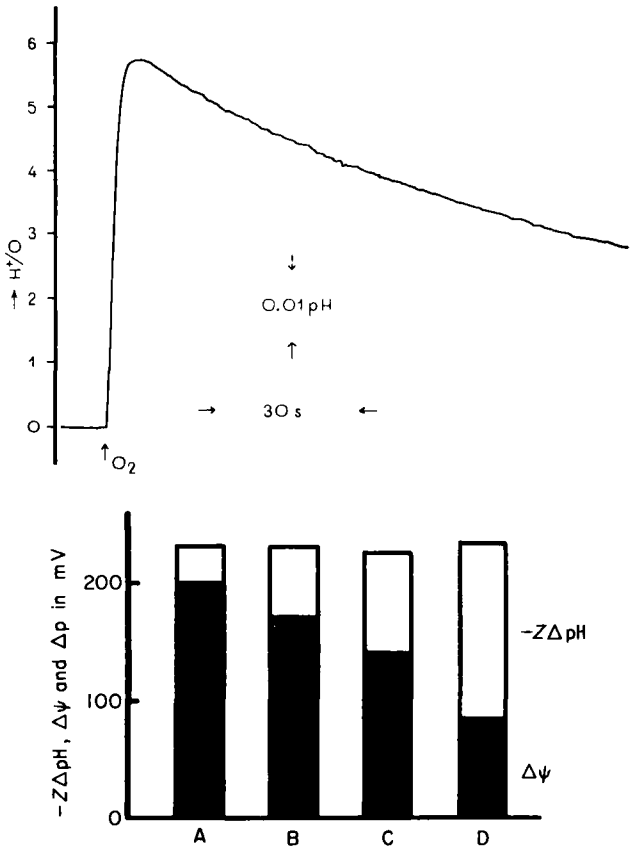

FIGURE 9 Demonstration and measurement of protonmotive force in respiring mitochondria. (*Top*) Proton pulse driven by β-hydroxybutyrate oxidation. From Mitchell and Moyle, 1967 (413). (*Bottom*) Electrical (Δψ) and chemical (−ZΔ pH) components of the protonmotive force (Δp) in mitochondria respiring under different conditions. From Mitchell and Moyle, 1969 (415).

Due to these techniques, and to a large amount of experimental data collected in many laboratories, the four basic postulates of Mitchell's chemiosmotic hypothesis were essentially proven by the middle of the 1970s. It was established that mitochondria and submitochondrial particles can form a transmembrane proton gradient concomitant to electron transport or ATP hydrolysis, and that the generation of a proton gradient by the former can be utilized for ATP synthesis by the latter. It was also shown that the mitochondrial inner membrane contains a substrate-specific exchange diffusion carrier system (cf. Table II), and that the membrane itself has a low permeability to protons and other ions. Similar results were obtained with chloroplasts and bacteria. Incorporation of the isolated respiratory-chain complexes representing the three coupling sites of the chain (425–427), of ATPase (428), and of transhydrogenase (221, 429) into liposomes was shown to result in an ability of these catalysts to bring about transmembrane proton gradients. Furthermore, when one of the three electron-transport complexes and ATPase were incorporated into the same liposome, the system was capable of exhibiting oxidative phosphorylation (430–432). Recently, ATP-driven transhydrogenase was also reconstituted (223). In a spectacular reconstitution experiment performed in 1974, Racker and Stoeckenius (433) incorporated bacteriorhodopsin and mitochondrial ATPase in the same liposome and demonstrated light-induced phosphorylation.

Over the years, the chemiosmotic hypothesis has been the subject of much debate. Some of the criticism (408, 434, 435) addressed the basic principles of the hypothesis and gave rise to lively rebuttal (436). Other criticism (e.g., 437) concerned details of Mitchell's mechanism and often led to modifications of the hypothesis. This debate is still going on (see below). An overview of the field (see e.g., 438), however, shows that the basic idea of the chemiosmotic hypothesis:

$$\text{electron transport} \rightarrow \Delta\tilde{\mu}_{H^+} \rightarrow \text{ATP synthesis}$$

was widely accepted by the middle of the 1970s as the "central dogma" of membrane bioenergetics.

PRESENT STATE OF THE ART: For the last five years, research in mitochondrial energy transduction (and in membrane bioenergetics in general) has been centered mainly on three questions (cf. 438): (*a*) the mechanism of proton translocation by various energy-producing units; (*b*) the possible role of conformational changes of proteins as the primary events in energy transduction; and (*c*) the possible involvement of localized (intramembrane) electrochemical mechanisms in energy transfer between energy-transducing units.

A controversial issue relating to the mechanism of proton translocation that has been actively pursued concerned the number of protons that are translocated across the mitochondrial inner membrane for each pair of electrons transferred through the individual coupling sites of the respiratory chain. According to Mitchell (406) this number is 2, in accordance with the concept of redox loops. Estimates from other laboratories (235, 439, 440) indicate higher values, probably 3 or 4, which suggests the occurrence of proton pumps. Evidence for a proton pump linked to cytochrome *c* oxidase has been reported by Wikström (441) and others (see review 442) but is questioned by Mitchell (443). The possibility that cytochrome *b* functions as a proton pump has also been considered (444, 445) with reference to Chance's concept of "membrane Bohr effect" (446). Alternatively, the generally observed $H^+/2e^-$ stoichiometry of 4 of the ubiquinol-cytochrome *c* reductase

may be explained in terms of Mitchell's "Q-cycle" (447). There is strong evidence for a proton pump linked to transhydrogenase (221), in agreement with earlier predictions (409, 448). It is also generally agreed that the ATPase functions as a proton pump, but the H^+/ATP ratio is not yet settled (cf. 438). The possibility that different energy-coupling sites may have different proton stoichiometries has led to a reevaluation of the concept of integral $P/2e^-$ and P/O ratios; various current proposals are listed in Table III (449).

Another controversial issue is the mechanism of interaction between the proton-translocating (F_O) and catalytic (F_1) moieties of ATPase. According to Mitchell (443), protons conducted by F_O interact directly with the active site of F_1 in the course of the catalytic event. Boyer (450) and Slater (451) have proposed an indirect mechanism, according to which proton conduction via F_O alters the conformation of F_1 so as to release bound ATP; the synthesis of the latter from bound ADP and P_i would take place at the expense of energy stored in the "energized" conformational state of the enzyme. Later Boyer and associates (452) extended this hypothesis to involve catalytic cooperativity between identical subunits of the enzyme, where the binding of ADP and P_i to one subunit is accompanied by a release of ATP from another subunit. This "alternate-site" or "binding-change" mechanism has received significant experimental support in recent years (453). In general, there is growing evidence (cf. 438) that both the ATPase and various energy-transducing electron-transport catalysts are capable of undergoing energy-linked conformational changes and that proton gradients may serve not only as energetic links but also as effectors of these catalysts.

Several laboratories are pursuing the important problem of whether energy-transducing catalysts located in the same membrane interact merely by way of a bulk proton gradient or whether there may be more localized interactions, e.g., electrochemical activities along or within the membrane. Indications for the latter type of interaction have been obtained by using the ATP-driven transhydrogenase of mitochondria as the test system (371, 372, 454) as well as with various membrane probes (455–458). Evidence for a localized energy transfer has also been obtained with photosynthetic systems (459–463).

The final answers to these questions will have to await more detailed knowledge of the chemical structure of the individual energy-transducing catalysts. A great deal of information has accumulated during the last few years concerning the subunit composition and topology of both the ATPase complex (cf. 346) and various electron-transfer complexes (464–467). For the latter, information of great interest has been obtained from X-ray studies of two-dimensional membrane crystals followed by image reconstruction (Fig. 10); such information is now available concerning the shape and membrane topology of

TABLE III

Proposed H^+/site, $P/2e^-$ and P/O Ratios

	H^+/site					$P/2e^-$			P/O
Author(s), year (ref.)	Site 1	Site 2	Site 3	ATP synth.*	ATP transp.‡	Site 1	Site 2	Site 3	Sites 1-3
Mitchell, 1966 (406)	2	2	2	2	0	1	1	1	3
Brand et al., 1976 (439)	4	4	4	3	1	1	1	1	3
Wikström and Krab, 1978 (442)	3	2	4	2	1	1	0.67	1.33	3
Brand et al., 1978 (440)	2	2	4	2	1	0.67	0.67	1.33	2.67
Hinkle and Yu, 1979 (449)	2	2	2	2	1	0.67	0.67	0.67	2

* H^+/site used for ATP synthesis.

‡ H^+/site used for ATP transport via the ADP, ATP translocator.

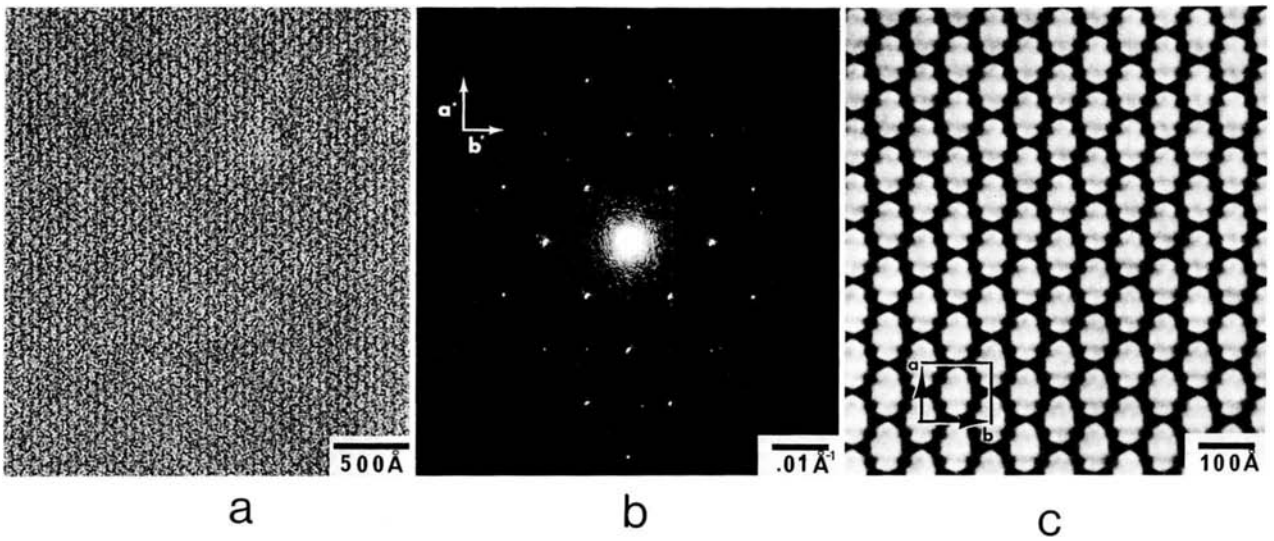

FIGURE 10 Structure of negatively stained crystalline cytochrome *c* oxidase. (*a*) Electron micrograph of crystalline cytochrome *c* oxidase stained with 1% uranyl acetate. (*b*) Optical diffraction pattern of (*a*). Reciprocal space lattice vectors (a^* and b^*) are indicated. (*c*) Optically filtered image of (*a*). Reconstruction is based on the information at or around the reciprocal lattice points of the optical diffraction pattern (*b*). One unit cell is outlined and lattice vectors (*a* and *b*) are indicated. From Frey, Chan, and Schatz, 1978 (470).

cytochrome *c* oxidase (468–470) and ubiquinol-cytochrome *c* reductase (467). The primary structures of several subunits of cytochrome *c* oxidase (466) and of the DCCD-binding protein of the ATPase complex (345) have recently been determined. Progress in this area has been greatly accelerated by the advent of DNA-sequencing techniques and by the rapid advance of the field of mitochondrial genetics.

In an introduction to a symposium on the Biochemistry of Mitochondria 15 years ago, Slater (471) ended his lecture with the following words: "I suspect, however, that it will be a long time before we understand the mechanism of oxidative phosphorylation. It is an open bet whether it will be the enzymologist, the membranologist or the protein chemist who will give the answer." Today it appears that the answer will come from all three, in a relay, and that the bâton has just passed from the membranologist to the protein chemist.

Biogenesis

GENETIC EVIDENCE FOR SEMIAUTONOMOUS REPLICATION: Research on mitochondrial biogenesis is one of the youngest areas of "mitochondriology" (Table IV). This is not surprising since the problem of how mitochondria are formed could only be attacked after the morphology, composition, and function of the organelles had become reasonably well known. About 30 years ago it was widely held that mitochondria arise *de novo* by transformation of other cellular structures. For example, experiments by Harvey with sea urchin eggs (472) and by Zollinger with mouse kidney tubules (473) seemed to indicate that these cells could regenerate their mitochondria after all preexisting mitochondria had been removed or destroyed. Also, several light- and electron micrographs that were made of animal- and plant cells in the

TABLE IV

Some Key Discoveries on Mitochondrial Biogenesis

Year	Discovery	Author(s)	Reference(s)
1950–1952	Mitochondrial formation in yeast and *Neurospora crassa* is controlled by "non-Mendelian" genetic factors	Ephrussi	479
		Mitchell and Mitchell	480
1958	Mitochondria synthesize protein	McLean et al.	482
1963–1964	Mitochondria contain DNA	Nass and Nass	493
		Schatz et al.	494
		Luck and Reich	495
1964	Mitochondria synthesize RNA	Wintersberger and Tuppy	498
1966	Mitochondrial biogenesis in vivo is blocked by some antibiotics	Clark-Walker and Linnane	484
1966	The extrachromosomal "petite" mutation in yeast alters mitochondrial DNA	Mounolou et al.	501
1966	Iso-1-cytochrome *c* is coded by a nuclear gene	Sherman et al.	492
1967	Mitochondria contain ribosomes	Küntzel and Noll	500
1967–1968	Mitochondrially inherited drug-resistant yeast mutants	Linnane et al.	505
		Thomas and Wilkie	506
1969–1970	Yeast mitochondria are not formed *de novo*	Plattner et al.	478
1968–1970	Methods for mapping mitochondrial genes	Thomas and Wilkie	508
		Gingold et al.	509
		Coen et al.	510
1972–1973	Identification of mitochondrially synthesized polypeptides	Tzagoloff and Meagher	489
		Weiss	490
		Mason and Schatz	488
1974–1975	Mitochondrially-inherited yeast mutants with specific phenotypic lesions (mit^- mutants)	Flury et al.	512
		Tzagoloff et al.	513
1975–1976	Genetic map of yeast mitochondrial DNA	Molloy et al.	535
		Schweyen et al.	515
1976	Physical map of mitochondrial DNA from various species	Sriprakash et al.	536
		Sanders et al.	520
		Morimoto et al.	520
		Bernard and Küntzel	520
		Attardi et al.	520
		Terpstra et al.	520
		Dawid et al.	520
1977	Mitochondrially-translated polypeptides are coded by mitochondrial DNA	Douglas and Butow	534
		Sebald et al.	516
		Cabral et al.	517
		Claisse et al.	518
		Mahler et al.	519
1978	Some mitochondrial genes are "interrupted"	Bos et al.	524
		Slonimski et al.	521
		Haid et al.	522
		Mahler et al.	519
1979	Identification of larger precursors to cytoplasmically synthesized mitochondrial proteins	Maccecchini et al.	531
		Neupert et al.	533
1979–1980	Nucleotide sequences of mitochondrial genes. The mitochondrial genetic rode has some unique features	Hensgens et al.	525
		Macino and Tzagoloff	526
		Fox	528
		Barrell et al.	529

1950s and early 1960s appeared to show formation of mitochondria from other cellular membranes, in particular the nuclear outer membrane (e.g., 474). *De novo* formation of mitochondria was still debated as recently as 11 years ago: two laboratories claimed that anaerobically-grown yeast cells (which lack respiratory enzymes) were devoid of mitochondria, yet could reform them upon oxygenation (475, 476). *De novo* formation of mitochondria ceased to be taken seriously only in the late 1960s when it was shown that, during anaerobic growth, the mitochondria of yeast are not lost but are merely dedifferentiated into respiration-deficient promitochondria which are difficult to detect by conventional electron microscopy (477, 478). In retrospect, it is not easy to understand how the *de novo* formation of mitochondria could have been considered seriously after the discovery of mitochondrial DNA. However, initially, the genetic system of mitochondria was often compared to that of bacteriophages, which suggested the possibility that a "master copy" of mitochondrial DNA could be transiently incorporated into nuclear DNA under "mitochondria-free" growth conditions.

Modern research on mitochondrial biogenesis was started in the early 1950s by Ephrussi (479) and Mitchell and Mitchell (480), who found that the properties of certain respiration-deficient *Saccharomyces cerevisiae*- and *Neurospora crassa* mutants were not inherited according to Mendelian laws (Table IV). Whereas earlier studies, particularly with higher plants, had suggested that hereditary determinants may reside outside the nucleus (481), the properites of these new microbial mutants suggested that "extrachromosomal genetic factors" might reside in, or even be identical with, mitochondrial organelles. This set the stage for a merger between genetics and mitochondrial physiology, but this merger was slow in coming. In our opinion, a major hurdle was the reluctance of most "mitochondriologists" to accept the fact that yeast and *Neurospora* cells possessed *bona fide* mitochondria. Interest in these mutants thus remained confined to a few specialized laboratories and research on mitochondrial formation was unnecessarily delayed.

MITOCHONDRIAL PROTEIN SYNTHESIS: It is particularly fortunate that the next development occurred with rat liver mitochondria which, at that time, were the best characterized mitochondrial species. When McLean et al. (482) reported in 1958 that isolated rat liver mitochondria could incorporate labeled amino acids into protein, their paper immediately attracted widespread attention and raised hopes that a thorough study of this process would quickly provide insight into the mechanism of mitochondrial formation. However, disappointment was not far behind. Amino acid incorporation by isolated mitochondria proved to be very slow and critically dependent on a seemingly endless list of trivial parameters. Even more frustrating was the search for labeled protein product(s): none of the matrix enzymes or cytochromes appeared to be labeled by isolated mitochondria. Instead, most of the label was found in a highly insoluble, ill-defined protein fraction which, for a while at least, was viewed as a "structural protein" for the mitochondrial inner membrane (483). In the early 1960s, many workers in the field had begun to doubt that mitochondria could synthesize any functional polypeptide. Finally, Clark-Walker and Linnane broke the impasse; they discovered in 1966 (484) that the formation of respiring mitochondria in intact yeast cells could be blocked by chloramphenicol—precisely the antibiotic that had been shown several years before to specifically inhibit protein synthesis by isolated mitochondria but not by microsomes (485). This convinced most workers that mitochondrial protein synthesis was a biological reality and that it was necessary for the biogenesis of respiring mitochondria. Because yeast and animal cells grown in the presence of chloramphenicol proved to be deficient in cytochrome *c* oxidase and cytochrome *b* (12), it seemed likely that these mitochondrial components were made at least partly within mitochondria. Direct proof came from several technical advances. In 1969, two laboratories independently showed that the mitochondrially-made proteins could be specifically labeled *in vivo* by pulse-labeling cells in which the extramitochondrial ribosomes had been blocked with cycloheximide (486, 487). The labeling rates of these in vivo systems were one or two orders of magnitude higher than those attainable with isolated mitochondria. Another crucial step was the decision to isolate labeled products by immunoprecipitation rather than by conventional fractionation and to analyze them by the newly developed technique of SDS-polyacrylamide gel electrophoresis. By 1973 it had become clear that mitochondria synthesize three (out of the seven) subunits of cytochrome *c* oxidase (488), at least two (out of the approximately ten) subunits of the oligomycin-sensitive ATPase complex (489), and one (out of the six to nine) subunits of the cytochrome bc_1 complex (490, 491). The main conclusion from these studies is still valid today: mitochondria do not synthesize complete enzymes, but only some subunits of oligomeric enzymes; the remaining subunits of these enzymes are manufactured outside the mitochondria under the direction of nuclear genes. The involvement of nuclear genes in mitochondrial formation had already been implicated in 1950 by the discovery of respiration-deficient yeast mutants which, unlike the mutants mentioned above, exhibited classical Mendelian inheritance (491). In 1966, Sherman et al. (492) had analyzed one of these "Mendelian" mutants in detail and had furnished the first rigorous evidence that a mitochondrial protein (iso-1-cytochrome *c*) is coded by a typical nuclear gene. The identification of mitochondrially-made subunits of cytochrome *c* oxidase, the cytochrome bc_1 complex, and the ATPase complex now opened the way for studying the interplay between the mitochondrial and the nuclear-cytoplasmic genetic systems at the level of relatively simple and well-defined enzyme complexes.

THE MITOCHONDRIAL GENETIC SYSTEM. Where are the genes coding for the mitochondrially-translated polypeptides? Now we have to backtrack by almost a decade and recount the discovery of mitochondrial DNA. Late in 1963, Nass and Nass (493) startled the biological community with electron micrographs which indicated that the mitochondria of chick embryo cells contained threadlike structures that could be digested by DNAse, but not by RNAse. A few months later, DNA was detected and quantitated in highly purified yeast mitochondria by biochemical procedures (494). Then DNA was extracted from *Neurospora* mitochondria and shown to differ from the bulk of *Neurospora* DNA by its buoyant density in CsCl gradients (495). From the very beginning, it was expected that this "mitochondrial DNA" would turn out to be the "extrachromosomal factor" that, more than a decade before, had been reported to control mitochondrial formation in lower eukaryotes. Just as the discovery of mitochondrial protein synthesis had prompted many classical "mitochondriologists" to take a closer look at mitochondrial formation, so the discovery of mitochondrial DNA brought to the field an ever increasing number of molecular biologists. Now things started to happen fast. In 1966, van Bruggen et al. (496) and Nass (497)

reported that mitochondrial DNA from many higher eukaryotes is a circular double-stranded molecule of which the size may differ between different species. In 1965, Wintersberger and Tuppy (498) isolated RNA from yeast mitochondria and separated it into two ribosomal-type RNA species and a 4S fraction with the functional properties of tRNA. They also showed that isolated yeast mitochondria could synthesize RNA from nucleoside triphosphates by a process that was partly sensitive to actinomycin. Three years later, DNA-RNA hybridization experiments by Wintersberger and Viehhauser (499) indicated that mitochondrial RNA was homologous to, and thus probably transcribed from mitochondrial DNA. In 1967, Küntzel and Noll (500) isolated ribosomes from *Neurospora* mitochondria and demonstrated that these ribosomes were similar in many respects to bacterial ribosomes. Thus, although the evidence available in 1968 was still incomplete, it left little doubt that mitochondria contain a typical genetic system capable of replicating, transcribing, and translating genetic information.

But how could one prove that this genetic information was in any way related to mitochondrial biogenesis? The first clear indication for this was a report by Mounolou et al. (501) that the overall base composition of mitochondrial DNA was altered in some of the "extrachromosomally-inherited" respiration-deficient yeast mutants that had been discovered in the early 1950s. (Because of their small colony size on certain media, these mutants are generally referred to as "petite" mutants). Subsequent analysis of these abnormal "petite" mitochondrial DNAs suggested that they had suffered massive deletions and that the extent and the nature of these deletions differed between different petite mutants (502). In 1970, two laboratories reported that some petite mutants are totally devoid of mitochondrial DNA (503, 504). However, because all petite mutants had lost the same mitochondrial proteins and neither reverted nor complemented each other, they were unsuited for unraveling the genetic content of mitochondrial DNA. Clearly, more restricted mitochondrial mutations were needed that could be identified by their specific phenotype and mapped by recombinational analysis or other reliable genetic methods.

Such mutants were first isolated by Linnane et al. (505) and Thomas and Wilkie (506) in 1968 and from then on, yeast has remained the organism of choice for studying mitochondrial biogenesis. The newly discovered mutants differed from wild-type yeast cells in that their mitochondrial protein synthetic system had become resistant to chloramphenicol or erythromycin. Later, mutants resistant to other "antimitochondrial" antibiotics such as oligomycin, paromomycin, or antimycin were discovered (507). These mutants showed "non-Mendelian" inheritance, had specific and easily measurable phenotypes, could be readily obtained by appropriate selection and, most important, were amenable to some of the classical genetic tests. Soon procedures for studying recombination between mitochondrial mutations were worked out (508–510) and, in 1969, the field of "mitochondrial genetics" was formally inaugurated at a meeting in Canberra (511). The first circular genetic map of yeast mitochondrial genome was published in 1975 (535). However, additional mitochondrial markers were badly needed. It was thus a major breakthrough when, in 1974-1975, many mitochondrially inherited yeast mutants became available that were not simply resistant to a particular antibiotic, but had suffered specific lesions in their oxidative phosphorylation system (512, 513). These "mit$^-$-mutants" initiated a particularly fruitful and exciting period in research on mitochondrial biogenesis for they led to the discovery of several new loci on the mitochondrial genome (514, 515). Because these mutants had lost individual mitochondrial components, they made possible the identification of the structural genes for mitochondrially translated polypeptides on mitochondrial DNA (516–519), which settled the decade-old question of whether the mRNAs for these proteins are imported into the mitochondria from the nucleus (Fig. 11). In 1976, several laboratories used the new tool of restriction enzyme analysis to work out the physical map of mitochondrial DNA from several species including yeast (520). As a consequence of these parallel developments, the structure of several mitochondrial genes is now being studied by genetic (521–523), electron microscope (524), or DNA sequencing methods (525–529), with many surprising results (537). For example, mitochondrial genes in yeast are usually separated from each other by long AT-rich sequences and, in some instance, contain intervening sequences. In contrast, human mitochondrial genes appear to lack intervening sequences and are so closely packed on the genome that the terminal codon of one gene can be immedi-

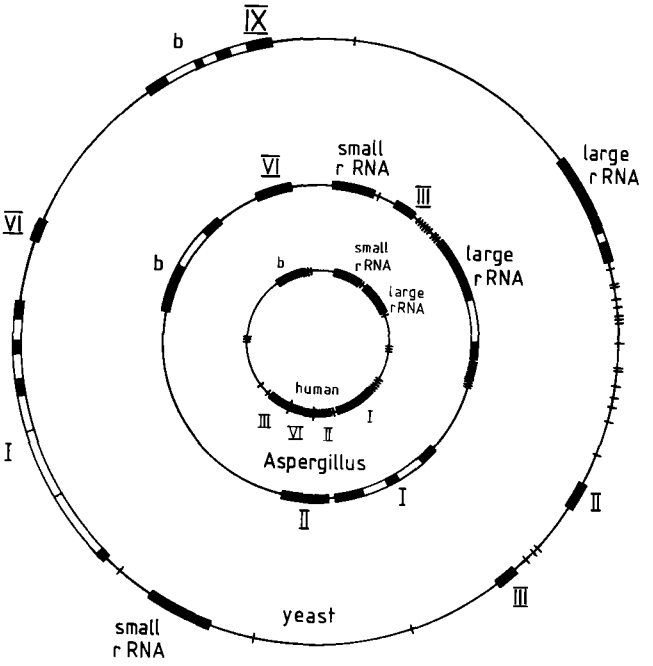

FIGURE 11 Genetic and physical maps of mitochondrial DNAs from yeast (*Saccharomyces cerevisiae*), *Aspergillus nidulans*, and humans. The different sizes of the rings are roughly proportional to the molecular weights of these mitochondrial DNAs. The blocks on the rings signify genes for proteins or ribosomal RNAs, the short thin lines pointing towards the center signify genes for transfer RNAs. Shaded areas of the blocks stand for exons, open areas for introns. The exact number of transfer RNA genes on each of the three mitochondrial DNAs is still uncertain, as in the evidence for some of the indicated introns. (*b*) Cytochrome *b*. (I, II, and III) Subunits I, II and III of cytochrome *c* oxidase. (VI, IX) Subunits VI and IX of the ATPase complex (subunit IX is often referred to as the dicyclohexylcarbodiimide-binding protein). Yeast mitochondrial DNA contains an additional gene between the subunit IX gene and the large rRNA gene; this additional gene controls the expression of a polypeptide associated with the small mitochondrial ribosomal subunit. Because it is not clear whether this gene codes for any protein (537), it has been omitted from this simplified map. Adapted from refs. 537, 540, and 541.

TABLE V

Some Unique Features of the Mitochondrial Genetic Code

Codon	"Normal" codon assignment	Codon assignment in mitochondria from		
		yeast	*Neurospora*	humans
UGA	stop	trp	trp	trp
CUN	leu	thr	leu	leu
AUA	ile	?	?	met
AGA or AGG	arg	arg	?	stop

See ref. 537 for details.

ately succeeded by the first codon of the following gene. Finally, the genetic code in mitochondria differs in several respects from that which, up to now, has been taken to be "universal" (Table V). More exciting findings are emerging almost by the month, but now our narrative deals no longer with history but the present.

All this new information has shattered the view that the mitochondrial genetic system is "bacteria-like." This view was quite popular in the past two decades because ribosomes, initiation factors, and RNA polymerase of mitochondria from most species are sensitive to inhibitors, which also are specific for the analogous components from bacteria (12). Although this fact is certainly in harmony with the hypothesis that mitochondria evolved from free-living prokaryotes (22), it must now be weighed against equally striking "eukaryotic" features of the mitochondrial genetic system such as "split" genes and mRNA-splicing events. Indeed, present information suggests that the mitochondrial genetic system should be considered as unique.

What is the future of the field? We suspect that the mitochondrial genetic system will soon have yielded its most important secrets and that research will shift to the interaction between the mitochondrial and the nucleo-cytoplasmic genetic systems. Almost certainly, new recombinant DNA technologies and our recent ability to transform eukaryotic cells with defined pieces of DNA (530) will help us to decipher the language through which the nucleus and the mitochondria "talk" to each other. On another front, evidence has accumulated that nuclearly-coded proteins are imported into mitochondria via post-translational, energy-dependent steps that frequently involve proteolytic processing of larger precursors (531–533). In order to unravel these processes, we will finally be forced to take a closer look at the mitochondrial outer membrane which, for much too long, has remained a stepchild of mitochondrial research. Possible interactions between mitochondria and the cytoskeleton are another promising hunting ground for future researchers. Clearly, the merger between mitochondrial research and cell biology has just begun.

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Chloroplasts

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Joseph Priestley discovered photosynthesis, which he described as "a method of restoring air which has been injured by the burning of candles." That was in 1771. In 1779, the remarkable Dutch physician, J. Ingen-housz, found that only the green parts of plants could carry on photosynthesis.

Studies of photosynthesis expanded in the 19th century. In 1846 Nageli followed the division of chloroplasts in *Nitella syncarpa*. In the first half of the century, von Mohl's students examined the starch grains contained in the chlorophyll bodies of angiosperms; Schimper and Meyer, in 1883, continued the attention to the chlorophyll bodies. In 1888 Haberlandt connected chloroplasts directly with oxygen production by showing that oxygen-seeking motile bacteria were attracted to illuminated chloroplasts of *Funaria hygrometricia* Hedw which had been liberated by cutting a leaf under sucrose solution on a microscope slide.

This chapter begins by outlining how the study of photosynthesis was revolutionized in the early 1930s, and how the process has come to be largely understood. The rate of progress toward our current level of understanding accelerated rapidly after the discovery of ^{14}C, and received repeated boosts as our knowledge of chemistry, physics, and biochemistry grew. For two centuries, the study of photosynthesis has been strongly influenced by and tightly linked to new discoveries in the physical sciences. Starting in the 1950s, the tools of cell biology began to be used to explore the structure of chloroplasts; as a result, although many questions remain today, the molecular aspects of photosynthesis and the organization of the chloroplast photosynthetic membrane are somewhat reconciled.

The other development with which this chapter deals is our knowledge of the molecular biology of plastids. At the beginning of the 20th century, just a few years after the rediscovery of Mendel's works, the detection by Correns and Bauer of pecularities in the transmission of genetic factors affecting chloroplasts caused botanists to think not only of the genome—referring to the collection of nuclear genes—but also of the plastome, implying a collection of plastid genes. One of the remarkable achievements of molecular biology has been to materialize chloroplast and other genes and, therefore, to make it possible to study them physically. However, even though our knowledge of the chloroplast genome, i.e., the plastome, is rudimentary, it seems far advanced to those who easily remember the very recent quest for DNA, RNA, and the other elements of life in chloroplasts.

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The Photosynthetic Apparatus

The era of modern research into photosynthesis and its relation to the structure of chloroplasts opened in the decade 1930–1940, but it was long before, in the 1860s, that the summary formula for photosynthesis,

$$6\ CO_2 + 6\ H_2O \rightarrow C_6H_{12}O_6 + 6\ O_2,$$

became established. The first product of carbon fixation in this formulation was written as a six-carbon sugar, but, in fact, the identity of the "first product" remained uncertain for almost a century. The mechanistic theory that prevailed until the 1930s held that $C_6H_{12}O_6$ was produced from six molecules of formaldehyde. Some investigators were satisfied that they understood this condensation and believed that the formaldehyde was produced by light acting on a complex of water, carbon dioxide, and chlorophyll. Blackman showed in 1905 that there are reactions in photosynthesis that do not require light, i.e., dark reactions (referred to in the literature also as Blackman reactions), as well as light-requiring steps. These subsets of photosynthetic reactions were taken into account and included in the von Baeyer–Wilstatter-Stoll formaldehyde theory given in elementary plant physiology text books as late as the 1940s.

In the early 1930s van Niel (1), influenced by the concept that many biological processes involve electron or hydrogen transfer, produced a generalized scheme for photosynthesis:

$$CO_2 + H_2A \xrightarrow{\text{light}} [HCOH] + 2A + H_2O.$$

[HCOH] represented one-sixth of $C_6H_{12}O$. *A* represented sulphur in certain photosynthetic bacteria but represented oxygen in blue-green algae and eukaryotic green plants.

The recognition that this overall reaction could be further subdivided has provided the basis for experiments in photosynthesis to the present. For the higher green plants that produce oxygen in photosynthesis, the subdivision takes the following form:

(a) The path of energy and electrons is:

$$HOH \xrightarrow[\text{chlorophyll}]{\text{light}} [H] + [OH].$$

Water is split using the energy derived from light by chlorophyll.

(b) Oxygen is produced by the reaction summarized as: $4\ [OH] \rightarrow O_2 + 2H_2O$. Steps *a* plus *b* together lead to the conclusion that oxygen is produced by and as a result of oxidation of the electron donor, water.

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(c) The path of carbon is summarized as: $CO_2 + 4\,[H] \rightarrow [HCOH] + H_2O$.

Initially, the impact of van Niel's work was muted. Robert Hill (2) believes that it was because van Niel was a student of chemosynthetic and photosynthetic bacteria (rather than of green plants) that the announcement of his "generalized scheme for photosynthesis ... which was based on his accurate quantitative experiments with bacteria ... [produced] ... no immediate stir in the domain of plant physiology." Hill, who himself was not a physiologist of green plants, goes on to state "thus I published my contribution on the green plant in 1937 and 1939 with no reference to van Niel's work; I had found that chloroplasts from higher plants would produce oxygen in light by the reduction of substances other than CO_2 ... It was shown that the oxygen produced in light corresponded with the reduction of a hydrogen or electron acceptor. One of the reactions catalyzed in light was the reduction of ferric potassium oxalate to the ferrous state. It seemed that the oxygen must have come from the water, thus confirming van Niel ..." In Hill's experiment, the ferric ions substituted for the natural electron acceptor and permitted the splitting of water and the liberation of oxygen. The ability to analyze partial reactions of photosynthesis has facilitated, indeed permitted, correlation of physical components of the membranes with the biochemistry of photosynthesis. Hill's experiment was the first in which a partial reaction could be followed.

In 1932 Emerson and Arnold (3) reported experiments designed to determine "how much chlorophyll must be present for the reduction of one molecule of carbon dioxide." Put another way, they asked: Does each chlorophyll molecule work independently in photosynthesis? They found that about 300 chlorophyll molecules can be considered to be operating together per photon absorbed.

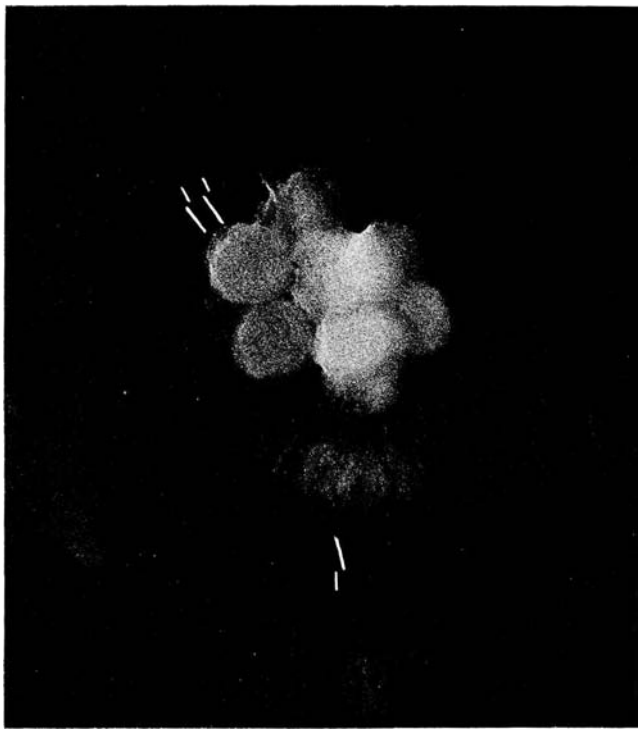


FIGURE 1 An electron micrograph of spinach chloroplasts dried on an electron micrograph grid and shadowed with gold. Stacks of very electron-dense grana (double arrows) are seen in the upper part of the photograph. In the lower part of the micrograph, one of the grana stacks (single arrow) is spread out revealing the component thylakoids. From an electron micrograph taken in December 1946 and kindly provided by Professor Keith Porter (7).

Their experiments were interpreted as follows (3, 4): 300 chlorophyll molecules form a "photosynthetic unit." Each unit is comprised of a pool of light-harvesting chlorophyll molecules and one reaction center. Once a quantum is absorbed by any chlorophyll molecule in the unit, the excitation energy migrates rapidly throughout the population of chlorophyll molecules in that unit until the energy is released (as fluorescence, for example) or, if and when the chlorophyll molecule(s) at the reaction center become excited, the excitation energy is converted into some form usable for oxidizing water and reducing carbon. These experiments and their interpretation gave, for the first time, some indication of the possible organization of the photosynthetic apparatus. The photosynthetic unit could be statistical or it could be a physical entity, but the idea that there might be some regular structure—some periodicity—in the photosynthetic apparatus was powerful.

Another important advance of this period was Granick's discovery (5), published in 1938, of a method for partially purifying tomato or tobacco chloroplasts by grinding leaf tissue in 0.5 M sucrose or glucose followed by differential centrifugation. Such plastids produced oxygen for several minutes. Granick's isolation procedure is the basis for purification of chloroplasts and mitochondria to this day.

The method for the partial purification of chloroplasts opened the way for chemical analytical and structural studies. The first electron micrographs of plastids were published in 1940. Kausche and Ruska (6) simply dried down chloroplasts on electron microscope grids and examined them in the microscope. The edges of somewhat electron-transparent disks could be seen in these primitive pictures. Using the light microscope, Meyer had observed, in 1883, that plastids of angiosperms contain dark dots, which he called grana, within the more transparent stroma. In electron micrographs of dried membranes from spinach chloroplasts (Fig. 1), published by Granick and Porter in 1947 (7), Meyer's grana were seen to be stacks of disks.

Starting out on the True Path of Carbon: 1940s and Early 1950s

The identity of the first sugar formed in photosynthesis had been the object of a search dating to almost the middle of the 19th century. The award of the Nobel Prize in chemistry to Melvin Calvin in 1961 recognized a solution of this problem. Radioactive carbon (^{14}C) was discovered by Ruben and Kamen in 1940 and was used in a series of elegant classical tracer experiments in photosynthesis. The early part of this work, from 1940 to 1955, is described succinctly, but in detail, by Kamen (8). The full Calvin (or Calvin-Benson) cycle has been discussed by Bassham and Calvin (9), and at least outlines of the cycle appear in virtually every current biochemistry textbook.

Among the crucial steps are (*a*) the combining of carbon dioxide with the five-carbon sugar ribulose bisphosphate by the enzyme ribulose bisphosphate carboxylase (RuBPcase) to produce two molecules of phosphoglyceric acid and (*b*) the reduction of this acid to the three-carbon sugar phosphoglyceraldehyde. The resemblance between the photosynthetic reduction of phosphoglyceric acid to phosphoglyceraldehyde and the reverse reaction known in glycolysis was not lost on the early investigators. The glycolytic reaction yields a molecule of ATP (from ADP plus phosphate) and a molecule of NADH (from NAD^+ plus two electrons and one proton). This sug-

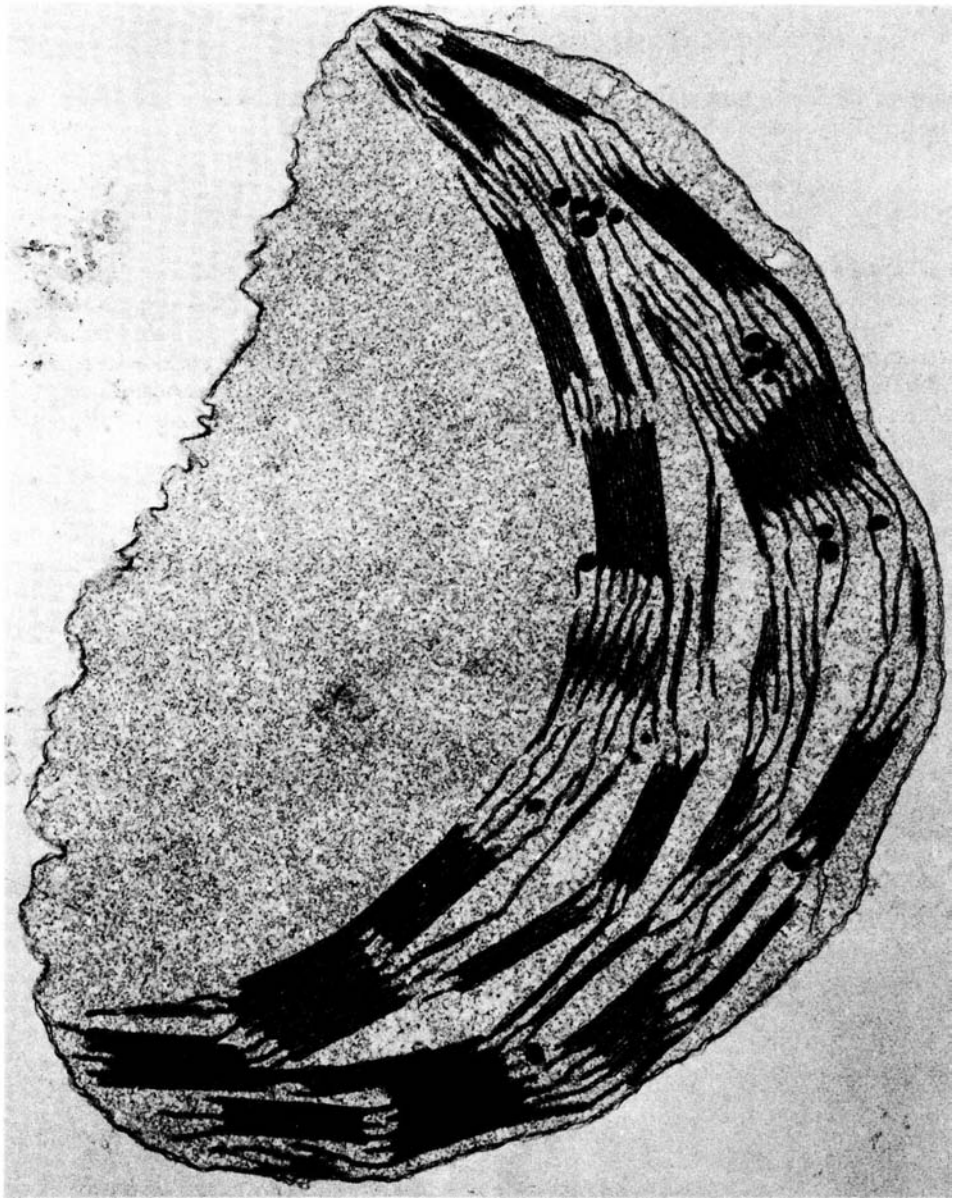


FIGURE 2 An electron micrograph of a thin section of a spinach leaf chloroplast. × 40,000. (Kindly provided by K. R. Miller).

gested that, in the reverse reaction, ATP might be produced photosynthetically and be expended along the path of carbon fixation, and that some reduced pyridine nucleotide might also be produced photosynthetically and be used to drive the reduction of phosphoglyceric acid. Although further details of the paths of carbon emerged to reveal an enormously complex web of metabolic reactions—interacting cycles for regeneration of the carbon dioxide acceptor in photosynthesis, substrates for polysaccharide formation, etc.—the search was also on to see whether chloroplasts could produce ATP and some sort of reduced pyridine nucleotide photosynthetically.

The fine Structure of Chloroplasts—the First Approaches

While the paths of carbon driven by photosynthesis were being further delineated and discoveries in the enzymology of carbon metabolism were emerging, great advances were being made in the preparation of samples for electron microscopy. In 1953 Finean, Sjöstrand, and Steinmann (10) published an electron micrograph of a thin section of a chloroplast of *Aspidistra elatior*.

A thin section of a chloroplast from a spinach leaf is shown in Fig. 2. It is surrounded by an outer double membrane. The most conspicuous internal features are the grana. They are made up of groups of flattened vesicles each designated a "thylakoid" (saclike). The network is complex, with vesicles extending in the stroma, i.e., the unstructured space outside of thylakoids, from granum to granum. Thylakoid membranes are about 70–80 Å in thickness and the spaces shown between them are about the same size. The larger dark dots in the stroma are granules which take up osmium and are lipid in nature. The granal structure seen in Fig. 1 is fairly characteristic of plastids in vascular plants although in maize, as in some other grasses, certain tissues contain agranal plastids. Agranal plastids are common in algae although a few thylakoids may be appressed for the entire length of a plastid.

The main contributions of electron microscope studies of thin sections of chloroplasts to the understanding of chloroplast structure in the 1950s and 1960s were:

(a) Chloroplasts are limited by double membranes with a space of about 70–100 Å between the two layers (in some cases there seem to be more than two layers).

(b) Each disk seen in a dried plastid preparation of grana is a vesicle limited by a membrane of 70–80 Å.

(c) The inner lamellar system of plastids is a complex of appressed and interconnected flattened vesicles. Adjacent grana seen in chloroplasts of vascular plants are connected to one another by longer flattened vesicles which extend so far as to be parts of two or more stacks.

(d) Plants in major taxonomic groups (e.g., algal subgroups, angiosperms) differ in the arrangement of thylakoids. Grana stacks are characteristic, but not universal among angiosperms. Algal plastids are commonly agranal but groups of vesicles may be appressed, sometimes for the entire length of the plastid.

(e) Plastids are sedimented during centrifugation in 0.5 sucrose for a few minutes at 400–1000 *g*, but when plastids are broken osmotically or mechanically, a green pellet is recovered only after centrifugation at higher speeds and forces. Thin sections show that the pellets contain the lamellar system including grana. The nonmembranous stroma elements are in the supernatant fluid.

Although advances in understanding the relationships between photosynthesis and plastid structure were limited somewhat by electron microscope technology, the major impediment in this period was the lack of knowledge of energy transduction and electron transport processes in photosynthesis. The only partial reaction that could be studied was still the Hill reaction.

Biophysics and More Biochemistry

Starting in the early 1950s biochemical and biophysical research into photosynthesis brought important and interesting data and ideas to those concerned with relating thylakoid lamellar structure to function. One of the major concepts underlying the photosynthetic unit idea is the transfer of energy among pigment molecules in the light harvesting pool and the final transfer of this energy to a reaction center.

The greatest influence on this field was undoubtedly L. M. N. Duysens's Ph.D. thesis published in 1952 (11). Duysens, following some earlier similar experiments of Dutton and Manning (12) and Wassink and Kersten (13), compared the fluorescence emitted by chlorophyll *a* in an organism illuminated at a wave length at which other pigments did not absorb (i.e., in the red region of the spectrum) with the fluorescence of chlorphyll *a* in cells illuminated at a wave length where the major absorber was another pigment (e.g., the carotenoid fucoxanthol in brown algae, phycobiliproteins in red or blue-green algae, or other "accessory" pigments). Highly efficient energy transfer from accessory pigment to chlorophyll *a* was observed. Duysens considered mechanisms by which such transfer could occur and concluded that excitation energy is most probably transferred through inductive resonance. Pigment molecules need to be very close to one another for energy transfer by inductive resonance. "The local concentration of ... chlorophyll *a* probably is of the order of 0.1 M. The theory of inductive resonance further indicates that appreciably transfer from chlorophyll *a* ... to a pigment in very small concentration is possible." Thus, a consideration of the mechanism brought an estimate of the maximum distance apart for molecules that transfer energy so efficiently among themselves. These ideas were immediately translated into numerous models for the arrangement of chlorophyll molecules in photosynthetic membranes. But, several crucial elements were still unrecognized and models based only on these data had little chance of being correct.

Electron transport was a dominant theme of photosynthesis research in the 1950s. The work of van Niel was taken seriously enough by this time for people to look for electron transport components in photosynthetic materials. Among these investigators, Hill and Scarisbrick (14) in 1951 described cytochrome *f*. This new *c*-type cytochrome was purified first from elder leaves and shown to be associated with their chloroplasts. In 1955, Allen et al. (15) reported that isolated chloroplasts can fix carbon dioxide without the cooperation of any components of the cytoplasm. Whatley et al. (16) went on to show that the enzymes of CO_2 fixation were in the stroma while the thylakoids carried out photophosphorylation. Jagendorf (17) showed in 1956 that isolated illuminated thylakoids reduced $NADP^+$, and in 1958, San Pietro and Lang (18) discovered an NADP-reducing factor that was later identified as the iron-protein ferredoxin by Whatley et al. (19). Another important event of the decade was Kok's discovery (20) in 1957 of light-induced bleaching at 703 nm in the green alga *Scenedesmus*. He suggested that this pigment bleached when it absorbed energy either directly or via light-harvesting chlorophyll molecules. P-700 appeared to be at a reaction center. Some other photosynthetic electron transport components identified included plastoquinones, the copper protein plastocyanin, and $NADP^+$-ferredoxin reductase. Cytochrome b_6 was also found but its precise role remains unclear to this day.

In 1960 Hill and Bendall (21) reviewed the functions of cytochromes *f* and b_6 in photosynthesis by putting them on an electrical potential scale in relation to the positions of the oxygen electrode at +0.8 eV and of the NAD^+/NADH midpoint potential at about −0.4 eV. In photosynthesis, electrons from water, i.e., at the oxygen electrode potential, are raised to the NAD^+/NADH potential. Hill and Bendall considered the placement between these points of cytochromes b_6 and *f*, whose potentials are 0.0 and +0.37, respectively, as well as the potential of P-700. They concluded that "if cytochrome in chloroplasts is directly concerned in hydrogen or electron transfer, the system would require more than one light-driven reaction ..." This revolutionary position altered the view of photosynthesis as radically as van Niel's interpretation of the overall process had done almost 30 years earlier. The formulation by Hill and Bendall came to be known as the Z scheme. It found surprisingly quick acceptance because some older "red drop" data of Emerson and Lewis (22) and "chromatic transient" experiments of Blinks (23) that had seemed important but puzzling could now be fitted into place.

The overall scheme of electron transport (the continuous solid line running from H_2O to NADPH in Fig. 3 *top*) starts with the extraction of an electron from water at a reaction center called photosystem II (PS II) close to the oxygen potential at 0.8 eV as the result of the photo oxidation of PS II with the concomitant reduction of a carrier called Q at about +0.03 V. Q yields an electron which is then moved down the electrical potential scale through the carriers plastoquinone (PQ), cytochrome *f* (F), and plastocyanin (PC) to reduce the oxidized form of P-700 (at PS I) which, in an independent but related step, is oxidized after absorbing a quantum of light energy and transferring an electron to ferredoxin (Fd). Reduced ferrodoxin is at a potential high enough to reduce $NADP^+$. Thus, the energy of two photons—one absorbed by accessory pigments or directly by pigments of the PS II reaction center and one whose energy was converted at the PS I reaction center—would be added together. The puzzle of the potential drop from Q to P-700 without any apparent use remained; various pieces of

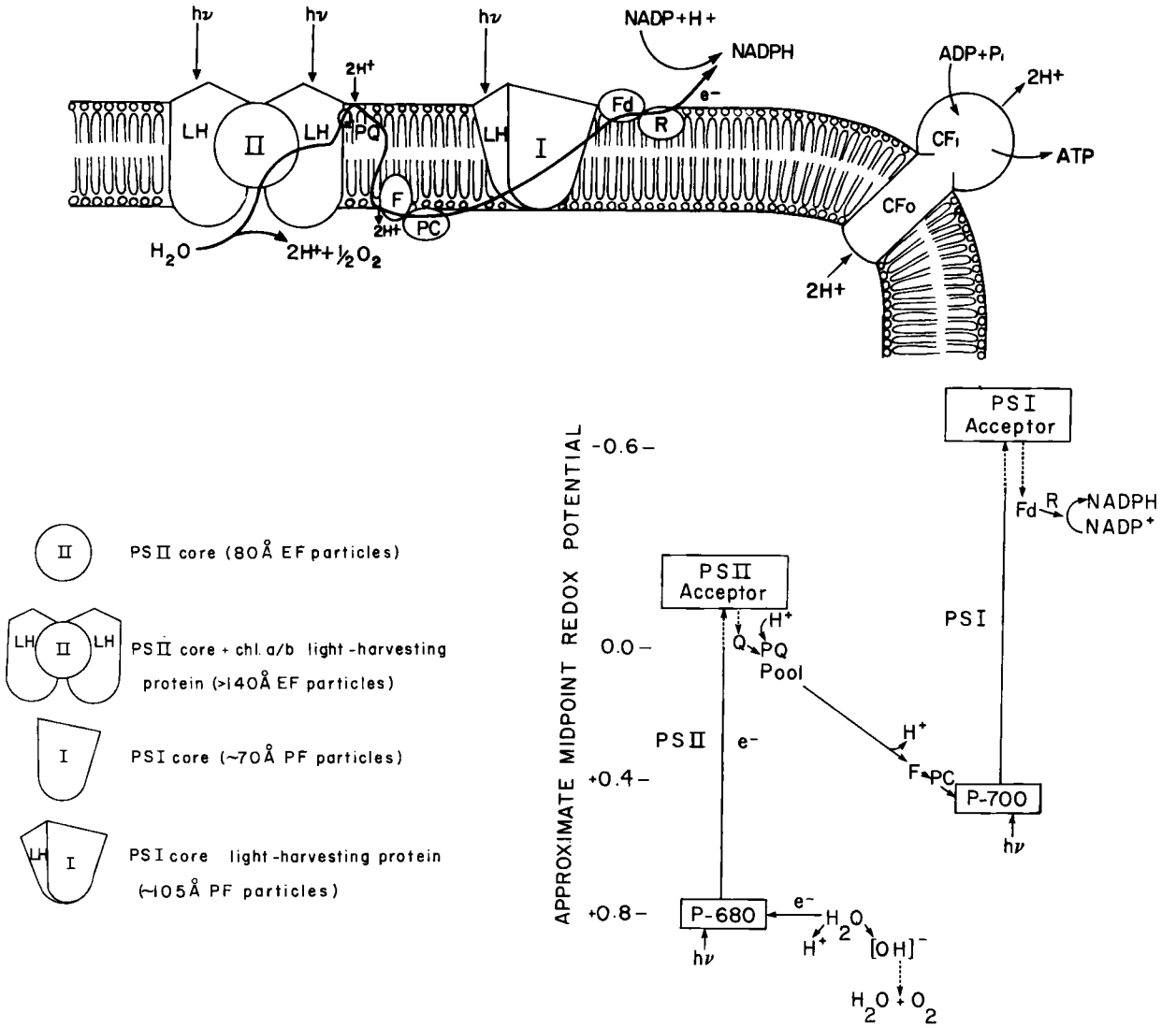


FIGURE 3 (*Top*) A diagrammatic representation of a chloroplast thylakoid membrane. The inside of the vesicle is in the lower part of the diagram. The flow of electrons is shown as a continuous solid line starting from H_2O, at the left side of the diagram, passing (to the right) through II (the 80-Å core of the photosystem II reaction center), to a group of quinones (PQ) which pick up protons from the stroma. The electron is passed to cytochrome *f* (F) and the two protons are discharged into the lumen of the thylakoid (together with protons released during photolysis of water.) The electrons are then passed to a plastocyanin (PC) and to the 70-Å core of photosystem I that can absorb light primarily through its associated light-harvesting (LH) proteins. The electron is passed then to ferredoxin (Fd) and, via the ferredoxin-$NADP^+$ reductase (R), is used to reduce $NADP^+$. The buildup of protons within the thylakoid lumen by the extraction of electrons from water and delivery by PQ molecules result in a gradient, relative to the stroma, which is discharged through the CF (right hand side of figure) with the production of ATP. The photosystem II core is seen as an EF particle on splitting of the thylakoid membrane; together with its associated chlorophyll *a/b* light-harvesting protein complexes it is more than 140 Å. The photosystem I core is a 70-Å particle which protrudes from the PF face in frozen-fractured thylakoids. Together with its associated light-harvesting protein, the particle is about 105 Å. (*Bottom*) A diagrammatic representation of electron flow in a chloroplast thylakoid membrane displayed along the scale of midpoint oxidation-reduction potentials of the electron carriers. The carriers are designated here in the same way as in the display of the electron flow in relation to the EF and PF surfaces of the thylakoid.

data suggested that this might have something to do with ATP formation.

The preface to a 1966 symposium on photosynthesis held at the Brookhaven National Laboratory (24) states that "the program was built around the currently popular "Z-scheme" in which light is fed in, and NADPH, ATP, and O_2 come out." At the symposium, one session was entitled "Phosphorylation, Ion Flows, and Conformational Changes." Peter Mitchell (25, 26) had previously suggested the chemosmotic hypothesis, in which it was proposed that a proton gradient could be built up by pumping protons to one side of a membrane, e.g., into a vesicle, and that the discharge of the gradient could be coupled to the phosphorylation of ADP. Jagendorf and Uribe (27) reported at the symposium results of an ingenious acid-base test of the chemosmotic hypothesis. Thylakoids of spinach were placed in a solution of succinic acid (pH 4), and an aliquot was transferred and diluted in a more basic solution containing ADP and inorganic phosphate. Even in darkness and the presence of poisons which blocked the electron transport chain of photosynthesis, ATP was formed. The un-ionized succinic acid molecules in the pH 4 solution move across the thylakoid membranes, and in this "acid phase," an equilibrium is estab-

lished between the inside and outside of the thylakoid. On transfer to a more alkaline solution in a comparatively large volume, the acid is ionized and the succinate molecules and protons outside of the thylakoids, but not those inside, are much diluted. This proton gradient energy is used somehow to produce ATP from ADP and inorganic phosphate. This process works only if the vesicles are intact. The chemosmotic hypothesis and the acid-base phosphorylation experiments show why chloroplasts contain thylakoids and not simply open membrane sheets, although both would be equally effective for capturing photons and converting molecular excitation energy into electrical potential energy.

The first structural feature of chloroplast thylakoids to be clearly associated with a specific photosynthetic function was the coupling factor (CF_1). Starting with the report by Vambutas and Racker in 1965 (28), Racker and his colleagues (e.g., 29, 30) showed that (*a*) calcium-activated ATPase activity is associated with chloroplast membranes, (*b*) this activity can be removed from the membranes by dilute solutions of EDTA, (*c*) thylakoid membranes that could carry on photosynthetic phosphorylation are incapable of doing so after extraction with EDTA, and (*d*) the capacity for photophosphorylation can be restored by adding, under proper conditions, the EDTA extract to preparations of the EDTA-extracted membranes. It was demonstrated by other workers (31–34) that the particles with a diameter of 90 Å visualized on the surface of thylakoids by negative staining are removed by dilute solutions of EDTA and can be seen in the extracts, and the particles can reassociate with thylakoids, which are then again capable of photophosphorylation and acid-base phosphorylation.

The Composition and Structure of Photosynthetic Membranes

Before thin-sectioning techniques were developed, the surfaces of chloroplast lamellae were examined after shadowing with heavy metals. In 1953, Frey-Wyssling and Steinmann (35) found that the surfaces are bumpy and argued that lamellae are made of globular units strung together into closed disks.

During the ensuing seven or eight years, thin-sectioning technology was developed for electron microscopy. In cross section, plastid lamellae in tissue stained with heavy metals appear as alternating dark and light, or i.e., electron-opaque and translucent, layers. Steinmann and Sjöstrand (36) in 1955, for example, estimated the thylakoid membrane to be approximately 140 Å in thickness and comprised of a 65-Å-thick electron translucent region bordered by electron-opaque portions, each about 30–40 Å thick. The strong influence of the unit membrane ideas of Robertson and others, as well as certain notions about the affinities of stains being used at the time, led to the strongly and widely held view that the lamellae are composed of alternating continuous layers of lipid and of protein.

In the early 1960s, there was a return to the idea that photosynthetic lamellae might be made up, at least in part, of groups of discrete particles rather than continuous smooth protein and lipid elements. In 1961, Park and Pon (37) rediscovered the rough nature of the surface of chloroplast lamellae and of some ordered structures (which later proved to be within the membrane). They suggested that the membranes might be comprised of lipoprotein building blocks. The entire lamellar structure was judged to be about 160 Å in thickness. Although the interpretations were not wholly correct, these data and those of Menke (38), based on x-ray diffraction data, raised serious doubts about the idea that thylakoid membranes are smooth, continuous, uninterrupted layers of lipid and protein. These doubts were reinforced by studies of replicas of frozen-fractured photosynthetic membranes.

The first electron microscope images of replicas of frozen-fractured and etched chloroplast membranes were published by two research groups in 1965 and 1966 (39–41). The images obtained by the two groups were virtually identical, but their interpretations were in sharp conflict. Mühlethaler et al. (39) considered that the fracture occurs between thylakoids—i.e., in the aqueous phase—and that the surfaces of the thylakoids are more or less smooth with globular elements embedded halfway into the membrane and half-protruding. At its thickest point, where two globular units are embedded opposite one another, the membrane will be expected to be about 80 Å in thickness. In this now-classical dispute, Branton (40) and Park and Branton (41) argued, correctly, that fracturing occurs in the hydrophobic phase within the membrane although the fracture face sometimes passes between thylakoids. They measured particles of 175 × 90 Å and 100 × 90 Å which, they argued, are exposed upon splitting within the membrane. The face toward the lumen of the vesicle (the EF face) and the face away from the lumen (later designated the "PF" or protoplasmic face) each have characteristic, partially embedded particles protruding. Electron micrographs of frozen-fractured and etched chloroplast thylakoid membranes that were taken in 1979–1980 (Fig. 4) are technically superior to those made in the mid- and late 1960s, but the same features can be discerned in micrographs of both periods.

A schematic illustration of the supramolecular organization of thylakoid membranes of a higher plant, modified from a 1980 paper by Staehelin and Arntzen (42), is shown in Fig. 3. The richness of interpretive detail in this drawing comes from (*a*) technical improvements that permitted more precise measurements of particles, (*b*) knowledge gained about photosynthesis in the intervening years, and (*c*) the kinds of experiments described below that were designed to determine the function of particles seen in replicas of frozen-etched thylakoids.

As noted above, the biochemistry of photosynthesis by the early 1960s had been sufficiently dissected to reveal a number of components of the photosynthetic electron transport system including, for example, the large pool of plastoquinones, cytochrome *f*, the copper protein plastocyanin, the P-700 at the reaction center of PS I, the nonheme iron protein ferredoxin, and ferredoxin-$NADP^+$ reductase. In addition, a number of partial reactions of photosynthesis had been identified and studied in vitro. Among these was the reduction of the dye 2,6-dichlorophenolindophenol using electrons removed from water at or near the reaction center of PS II—this reduction could go on even in the presence of 3-(3,4-dichlorophenyl)-1, 1-dimethylurea) (DCMU), a compound that blocks photosynthetic electron transport at a point before the reaction center of PS I (i.e., P-700), but somewhere after the oxidation of the reaction center of PS II. The dye serves as a substitute for the natural electron acceptor at some point prior to the site of action of DCMU and its reduction is a measure of PS II activity. Another partial reaction permits measurement of PS I. This is the reduction of $NADP^+$ by electrons from ascorbate introduced into the photosynthetic electron transport chain via the dye dichlorophenolindophenol and raised to the potential of ferredoxin by light energy absorbed by PS I.

It had also been learned that the action spectrum for PS II activity in green algae and higher plants includes a big contri-

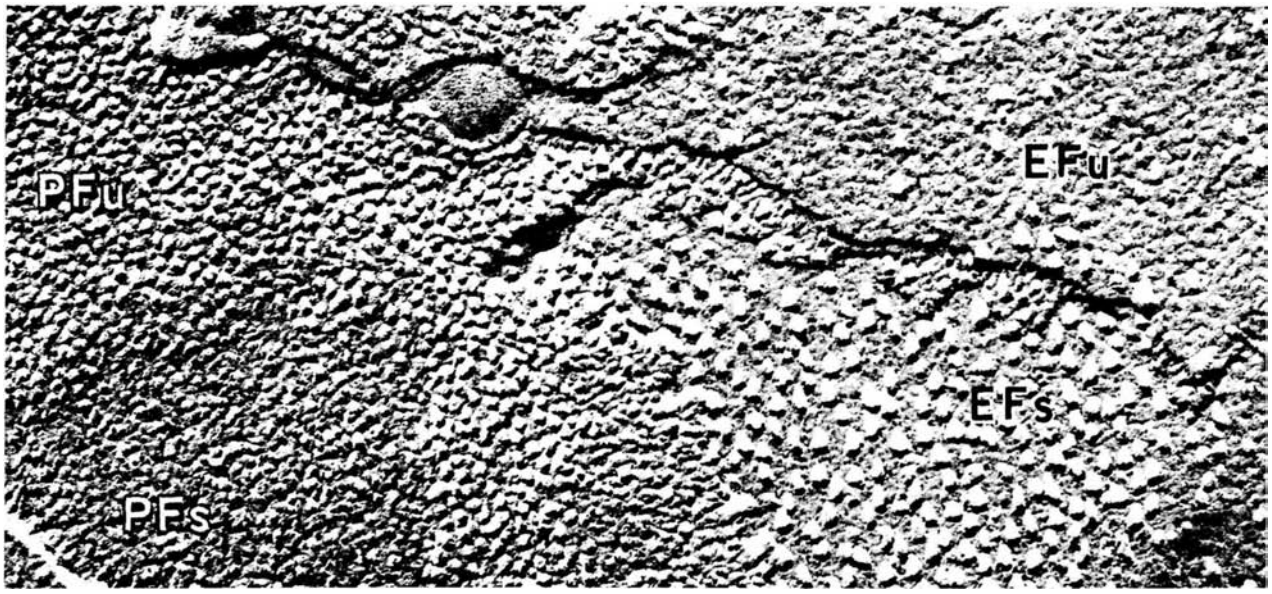


FIGURE 4 A freeze replica of fractured isolated thylakoids of barley. Besides showing the EF and PF particles, this micrograph also shows the effects of stacking of thylakoids on particle distribution. EFu shows a EF face in an unstacked region of the membrane. The comparable stacked region is designated EFs. The PFu face is shown adjacent to a PFs (i.e., stacked) face. This electron micrograph was kindly provided by K. E. Miller (43).

bution from chlorophyll *b*, i.e., chlorophyll *b* was shown to be the major light-harvesting pigment for PS II in these plants.

These various bits of information were exploited in an interesting series of experiments by Boardman and Anderson (44, 45) and Boardman (46). In 1964, they undertook to separate photosystems I and II physically and to determine which electron transport components are physically most closely associated with each photosystem. The detergent digitonin partially dissociates thylakoid membranes. After spinach thylakoids had been incubated with 0.5% digitonin for 30 min, differential centrifugation was used to separate solubilized chlorophyll-containing fractions into sedimentation classes. Two fractions were studied in most detail: one that sedimented at 10,000 *g* for 30 min (the 10-K fraction) and another that was pelleted at 144,000 *g* for 60 min (the 114-K fraction). The 10-K fraction had a low ratio of chlorophyll *a* to *b*, i.e., it was enriched for chlorophyll *b* compared to chloroplast thylakoids, but the 144-K fraction had relatively little chlorophyll *b*. This indicated that the 10-K fraction was enriched for PS II with its light-harvesting system whereas the 144-K fraction was depleted of this photosystem. The general picture that emerged from several assays, including the two partial reactions described above, was that the heavier, relatively easier-to-sediment 10-K fraction, was enriched in PS II whereas the 144-K fraction was enriched in PS I. Briantais (47–50) and Vernon et al. (51) used the non-ionic detergent Triton X-100 in similar experiments to disrupt chloroplasts into fragments with different ratios of chlorophyll *a* to *b*. An important aspect of this type of work for subsequent correlation of structure and function was the physical separation of the two photosystems from one another. Each enriched or purified photosystem preparation included its own reaction center, light-harvesting systems, etc. Boardman (46) has reviewed the large number of studies of this sort carried out between 1964 and 1969. But could any correlation be made between these separated fractions and the particles seen in fractured thylakoid membranes?

Arntzen et al. (52) examined digitonin-derived purified PS I (the 144-K fraction) preparations and PS II-enriched fragments (the 10-K preparation) by freeze-fracture and electron microscopy. Particles of 100 Å were found in the fragments enriched in PS I (144-K) whereas the PS II-enriched membrane fragments in the 10-K preparations were found to have 175-Å particles on most of their exposed faces. Now, what is the molecular composition of each photosystem—or at least of each enriched preparation?

Remy (53) compared the proteins associated with PS I and II preparations. He distinguished a total of 8–10 protein-containing bands after gel electrophoresis of wheat thylakoid proteins dissolved in a 0.3 M solution of the strong anionic detergent sodium dodecyl sulfate (SDS). Another sample was treated with the neutral detergent Triton X-100; it separated into two green zones by centrifugation in a 20–50% sucrose gradient. The material in each zone was dissolved with SDS for analysis of polypeptides according to size by polyacrylamide gel electrophoresis. Some differences in the number of bands and their relative intensities showed that each of the two green fractions had some unique polypeptides, but other polypeptides were common—perhaps from cross contamination. Levine et al. (54) obtained comparable results with fractions of spinach thylakoids produced by the methods of Boardman and Anderson. (In later experiments, [e.g., 55–58] as many as 50 or so polypeptides ranging in molecular weight from about 11 to 120 kilodaltons could be distinguished on polyacrylamide gradient gels of total thylakoid membranes completely dissociated with SDS.) Thus, each isolated photosystem is comprised of a complement of proteins, chlorophylls, lipids, etc.

The search for chlorophyll-protein complexes, another kind of attempt to identify specific components of the photosynthetic apparatus, goes back to the 1940s, but the new wave of this line of research began 25 years later. In 1966 Ogawa et al. (59) and Thornber et al. (60, 61) presented good evidence for the presence of two major chlorophyll-protein complexes in preparations of photosynthetic membranes solubilized with relatively low concentrations of SDS or SDBS (sodium dodecyl benzene sulfonate). On electrophoresis in detergent-containing polyacrylamide gels, three pigmented zones could be discerned.

The most rapidly moving zone, designated III, contained free chlorophyll. The complex of lowest electrophoretic mobility, designated I, had a chlorophyll *a/b* ratio greater than 7—resembling the photosystem I-enriched fractions of Boardman and Anderson. Complex II, on the other hand, had a chlorophyll *a/b* ratio of approximately 1.8—recalling the chlorophyll *a/b* ratio of the 10-K photosystem II-enriched fraction of Boardman and Anderson (44). The chlorophyll-protein complexes obtained by low SDS treatment could thus be related to the functionally characterized preparations of Boardman and Anderson. In a converse approach, Thornber et al. (60) showed that PS I- and PS II-enriched preparations of thylakoid membranes (produced by digitonin treatment according to the method of Boardman and Anderson) were enriched in chlorophyll-protein complexes I and II, respectively. This was determined by treating digitonin-generated chloroplast fractions with SDS and analyzing the completely solubilized proteins electrophoretically. Complexes I and II were judged to be small portions of photochemical systems I and II, respectively. Complex II was subsequently redesignated the "light-harvesting chlorophyll *a/b* protein" (LHCP). In the green alga *Acetabularia mediterranea*, the LHCP complex has an apparent molecular weight of 67,000 daltons and is composed of 23,000 and 21,500 dalton subunits in a molar ratio of 2:1 (55). In maize the light-harvesting chlorophyll *a/b* protein is composed of 27,500, 27,000, and 26,000 dalton polypeptides in a ratio of 8:4:1 (62). Multiple polypeptides have been shown to be associated with this complex in other species also.

Complex I generally migrates as a band of about 125,000 daltons, but on more thorough disassociation with detergent, a single 65,000–70,000 dalton polypeptide was observed in the earliest experiments. In more recent work both 66,000 and 68,000 dalton polypeptides have been seen (e.g., 55, 56, 63–65).

The concomitant enrichment of PS II activity, the larger EF particles, and the LHCP in the 10-K digitonin fraction implied that these features are related to one another functionally. This correlation was strengthened and amplified by studies of changes in the sizes of EF particles during chloroplast development carried out by Armond et al. (66).

Prolamellar Bodies and Primary Thylakoids

Plastids in leaves of dark-grown seedlings lack chloroplasts but develop to a characteristic etioplast stage (Fig. 5), at which they are arrested until leaves are illuminated (68). Each etioplast contains one or more paracrystalline prolamellar bodies. This structure, first discovered in 1954 by Heitz and by Leyon,

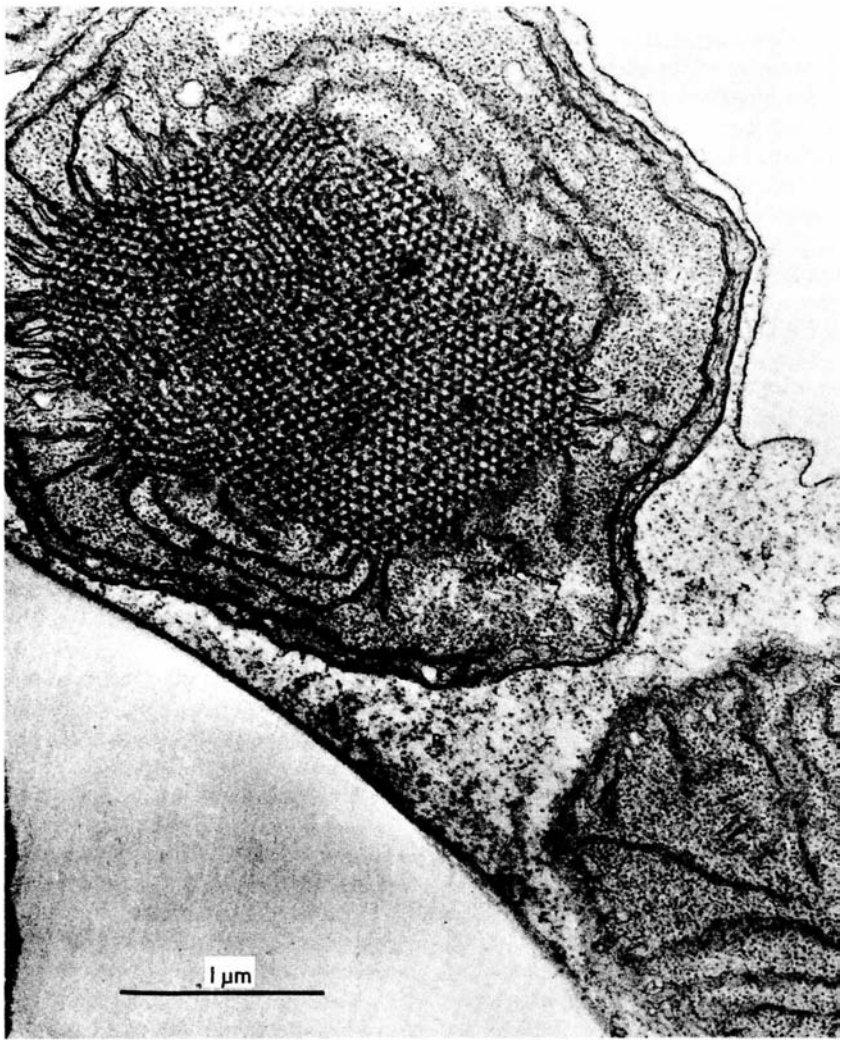


FIGURE 5 A cross section of an etioplast from a leaf of dark-grown maize. The most conspicuous feature is the paracrystalline prolamellar body (PB). Strands of DNA are visible in cleared areas within the plastid and ribosomes (R) are visible as small dots. (Ribosomes are also seen in the surrounding cytoplasm.) (67).

is the most conspicuous and characteristic feature of an etioplast. Etioplasts also contain at least a few perforated flattened vesicles that frequently appear to emerge from a prolamellar body. Ribosomes and DNA are also visible when sections of etioplasts are stained appropriately.

Upon brief illumination, some chlorophyll *a* forms from accumulated protochlorophyllide *a*, and prolamellar body elements are converted from the paracrystalline form to a less ordered state. For example, a total of 281 erg/mm^2 of 655-nm light given over a 10-min period (69) is sufficient for almost complete conversion of bean etioplast prolamellar bodies from paracrystalline to a disarrayed state. Under continuing illumination, many more flattened vesicles (primary thylakoids), now not perforated, appear. Some of them are stacked in the form of primary grana. In the electron microscope, the additional flattened vesicles seem to arise by dispersal and growth of vesicles derived from the gradually disappearing prolamellar body. In a day (or considerably less time depending on the species, light intensity, etc.) the photosynthetic membrane system devleops fully. New species of polypeptides and lipids are introduced during thylakoid membrane maturation.

Lütz and co-workers, (70, 71) have recently presented evidence that prolamellar bodies are comprised largely of saponins but do contain a few sizes of small polypeptides. Primary thylakoids appear to have a different set of polypeptides. The relationships among components of prolamellar bodies, primary thylakoids, and mature membranes are very uncertain at present. It is clear that etioplast membranes (prolamellar bodies plus primary thylakoids) contain many of the proteins present in photosynthetically functional thylakoids. Upon continuous illumination, numerous polypeptides are added, among them are those of the LHCP complex (e.g., 72).

Armond et al. (66) noted that all the EF particles in plastids of dark-grown, i.e., etiolated, leaves of pea were of a single size, 80 Å in diameter. At various times during greening under continuous illumination, EF particle size distribution became multimodal; in addition to the 80-Å core, particles of 105, 132, and 164-Å diameter were seen. Because the LHCP as well as chlorophyll appear during this time, it was judged that one, two, and four units of LHCP became associated with the 80-Å core in a series of steps during greening. The general idea of an EF particle core to which light-harvesting pigment protein is added came from the study of Miller et al. (43) of a barley variety lacking chlorophyll *b* and at least one polypeptide of the LHCP. The EF particles in the mutant were about 120 Å in diameter whereas those of the wild type were about 160 Å in diameter. The drawing in Fig. 3 shows PS II 80-Å EF particles associated with LHCP aggregates to bring their maximum sizes to more than 140 Å (or 164 Å from Armond et al. [66]).

During light-induced development, the PF particles prominent in PS I-enriched fractions, also increase in size: from 70 to 105 Å (50). This increase coincides with the production of a group of polypeptides of 21,500–24,500 daltons during continuous light-induced development of cucumber chloroplasts (73). The barley mutant lacking chlorophyll *b* shows another abnormality; it is also deficient in PS I polypeptides of 22,500, 23,000, and 24,500 daltons. The scheme in Fig. 3 shows a PS I 70-Å PF core particle together with light harvesting chlorophyll protein. Mullet et al. (65) estimate that their isolated 106-Å PS I particles contain 110 ± 10 chlorophylls/P-700 with a chlorophyll *a*/*b* ratio greater than 18. The 70-Å core complex is thought to contain 40 chlorophylls/P-700, and Mullet et al. suggest that the 22,500–24,500 dalton polypeptides of the mature PS I particle bind additional chlorophyll molecules to the structural complex. These would be light-harvesting units for PS I. A chlorophyll "special pair," perhaps associated with pheophytin (chlorophyll minus magesium), is believed to be at the heart of the energy conversion system of P-700 (e.g., ref. 74), presumably in the PS I core.

A number of uncertainties remain but the identification of particular partial reactions of photosynthesis with structural elements, e.g., the large EF particle as the core of PS II plus LHCP and the PF particle as a core plus probably light-harvesting elements of PS I, together with data on the vectorial arrangement of photosynthetic partial reactions outlined below yields an image of functional and structural arrangements in the thylakoid membrane.

In a series of very interesting experiments, Berzborn (75) showed that ferredoxin-NADP$^+$ reductase (R in Fig. 3), the enzyme that channels electrons between ferredoxin and NADP$^+$, is located on the same face of the thylakoid membrane as CF_1 particles, i.e., on the outside of the PF face. Rabbit antibodies against ferredoxin-NADP$^+$ reductase failed to precipitate thylakoid membranes unless antirabbit gamma-globulin was added as a second antibody. On the other hand, when lamellae prepared from *Anthirrinum majus* or broken chloroplasts of spinach were first washed with a concentration of EDTA which removes CF_1 particles, the membranes were agglutinated directly with the antiserum against the reductase alone.

Junge and Auslander (76) obtained good evidence that plastoquinone is reduced on the outer face of the thylakoid vesicle. They also were able to show by the use of dyes that there are two proton release sites in the thylakoid vesicle: one at the site of oxidation of water—and thus presumably associated with the PS II particle—and another at the plastoquinone oxidation site. The plastoquinone pool is known to be quite large and the transport of protons from the stroma into the thylakoid, i.e., across the membrane, is thought to occur through this population (Fig. 3). The presence of two proton uptake sites on the outer side of the membrane were shown by Schliephake et al. (77) and confirmed by Junge et al. (76, 78).

Thus, some of the particles within and on the photosynthetic membrane that had first been identified physically now have been shown to be associated with particular steps in photosynthesis: PF particles with PS I; EF particles with PS II; CF_1 with photophosphorylation. This information, plus other data (Fig. 3), assigns oxygen production to the inside of the thylakoid at PS II and the transport of electrons from PS II into the large plastoquinone pool that extends to the outside of the thylakoid membrane, where protons are accepted for transport to the plastoquinone oxidation site on the lumen face of the thylakoid membrane, close enough to the PS I particles for connection by the electron carriers (cytochrome, *f*, plastocyanin) that are present at much lower concentrations than are plastoquinones. Oxidation of PS I is coupled to the reduction of ferredoxin and NADP$^+$; the reductase is on the outer face of the thylakoid. The proton gradient built up inside the thylakoid by the water-splitting step at PS II and by plastoquinone oxidation is somehow discharged through (?) the coupling factor system and coupled to ATP synthesis.

Space limitations in this paper have prevented giving attention to some additional interesting and important problems. For example (Fig. 4), the individual scattered EF particles in unstacked membranes, such as the stroma lamellae or in grana

that have been disassociated into individual thylakoids under appropriate ionic conditions, appear as densely packed tetramers in stacked regions of thylakoids (79, 80). An intriguing feature of photosynthetic membranes is "spillover." In vitro the transfer of energy from light absorbed by the light-harvesting chlorophyll molecules of PS II to PS I is affected by the concentration of magnesium ions in the medium. Transfer is measured by following the fluorescence of light-harvesting chlorophyll pools associated with each of these two photosystems. The membrane stacking (and concomitant particle ordering) is closely correlated with spillover.

The limited amount of space together with the emphasis on structural-functional relationships has made it impossible to enumerate important contributions to the understanding of photosynthesis made over the past 25 years in the laboratories, for example, of Achim Trebst, H. W. Witt, Bessel Kok, Jack Myers, David Arnon, and Warren Butler who have studied, primarily, photosynthesis in higher plants and of Roderick Clayton, Martin Kamen, Britton Chance, William Parsons, and John M. Olson who have directed their studies at the stripped-down, but still complex, photosynthetic process in bacteria. For more of past and current problems, the reader is directed to more detailed reviews in advanced textbooks, symposium volumes, and annual reviews of progress in plant physiology and biochemistry. The sketchy view of the process of photosynthesis and of the organization of its biological machinery has skipped over many problems that are yet unresolved. This discussion has also omitted explicit references to the contributions that studies of photosynthetic bacteria and blue-green algae have made to our understanding of both structural and functional aspects of this subject.

The Molecular Biology of Plastids

Starting in the early 1960s, the older, generally equivocal, evidence for the presence of DNA and RNA in plastids was succeeded by firm knowledge that these nucleic acids are present and have unique properties. Early work, to about 1970, has been reviewed extensively (81–85).

Chloroplast Ribosomal RNAs (rRNAs) and Proteins

It seems especially fitting to note in this volume that it was in a report at the first meeting of the American Society of Cell Biologists in Chicago in 1961 that A. B. Jacobson described, in plastids of *Zea mays*, RNA-containing particles that were a little smaller in size than their counterparts—cytoplasmic ribosomes (86). At about the same time Lyttleton (87) showed that spinach chloroplasts contained 66S ribosomes in contrast to cytoplasmic ones estimated to be 83S. Plastids from a number of plants were subsequently shown to have ribosomes of about 70S (e.g., 88). The principal rRNAs of plastids are about 23S and 16S in contrast to 25S and 18S rRNAs of the 80S cytoplasmic ribosomes (e.g., 89, 90). Ribosomal RNAs of 5S (91) and 4.5S (92, 93) have been found in large subunits of ribosomes from chloroplasts of spinach and some other plants. Genes for 7S and 3S RNAs of undetermined function have been found between 16 and 23S rDNAs in *Chlamydomonas* (94).

22 different proteins have been separated by two-dimensional polyacrylamide gel electrophoresis of components of the *Chlamydomonas reinhardii* chloroplast ribosome small subunit; 26 from the small subunit of the cytoplasmic ribosome; 26 from the large subunit of the chloroplast ribosome; and 39 from the large subunit of the cytoplasmic ribosome (95). These numbers are minima, not necessarily final. They are likely to be altered by future refinements in techniques for ribosome isolation and methods of detection of proteins on gels. The molecular weights of the *Chlamydomonas* plastid ribosomal peptides range from about 12,500 to 54,000 daltons. Two pairs of proteins in the small chloroplast and cytoplasmic subunits and four pairs of proteins in the large subunits were found to have similar electrophoretic mobilities in the two dimensions. This establishes the upper limit to the number of proteins which may be common to each pair of ribosomal subunits but does not prove that any of these are pairs of identical polypeptides.

In the green alga *C. reinhardii*, some characters are transmitted biparentally and behave according to the classic rules of Mendel (96). These traits are considered to be carried in the nuclear genome. Other characters are transmitted uniparentally from only the plus mating-type parent. All of the uniparentally transmitted characters identified to date appear to be part of a single linkage group, i.e., the chloroplast genome. The difference in transmission makes it possible to determine rapidly whether a character is coded by a nuclear or chloroplast gene. This genetic approach has been used to trace genes for resistance to the protein synthesis inhibitor erythromycin, an antibiotic that binds to the large subunit of *Chlamydomonas* chloroplast ribosomes and blocks the activity of these ribosomes in vivo, but that neither binds to nor inhibits protein synthesis by the 80S cytoplasmic ribosomes (97, 98).

Some mutations to erythromycin resistance in *Chlamydomonas* are transmitted biparentally, indicating that they are coded by nuclear genes (97–101) whereas another mutation studied by Mets and Bogorad (97, 99) is transmitted uniparentally, indicating that it is coded for by a chloroplast gene. A chloroplast erythromycin resistance locus has been identified with an alteration in protein LC 4 of the chloroplast ribosome large subunit (99). Two of the biparentally transmitted resistance mutations have been identified with two different chloroplast ribosomal proteins (99, 100). The structural gene for chloroplast ribosomal protein LC 6 has been mapped to linkage group XI in the *Chlamydomonas* nuclear genome (100). These data, together with the fact that all chloroplast rRNAs tested, including those of *C. reinhardii*, hybridize to chloroplast but not nuclear DNA (with one or two exceptions that may be technical), provided clear evidence for what appears to be a principle of organelle biology: genes for components of multimeric organelle elements are dispersed between the organelle and the nuclear genomes (102). Two other good examples of this principle will be discussed below. The implications of gene dispersal for the evolution of eukaryotic genomes has been discussed elsewhere (102–104). Alterations in some chloroplast ribosomal proteins associated with mutation to resistance to streptomycin and some other antibiotics have also been reported (105–107).

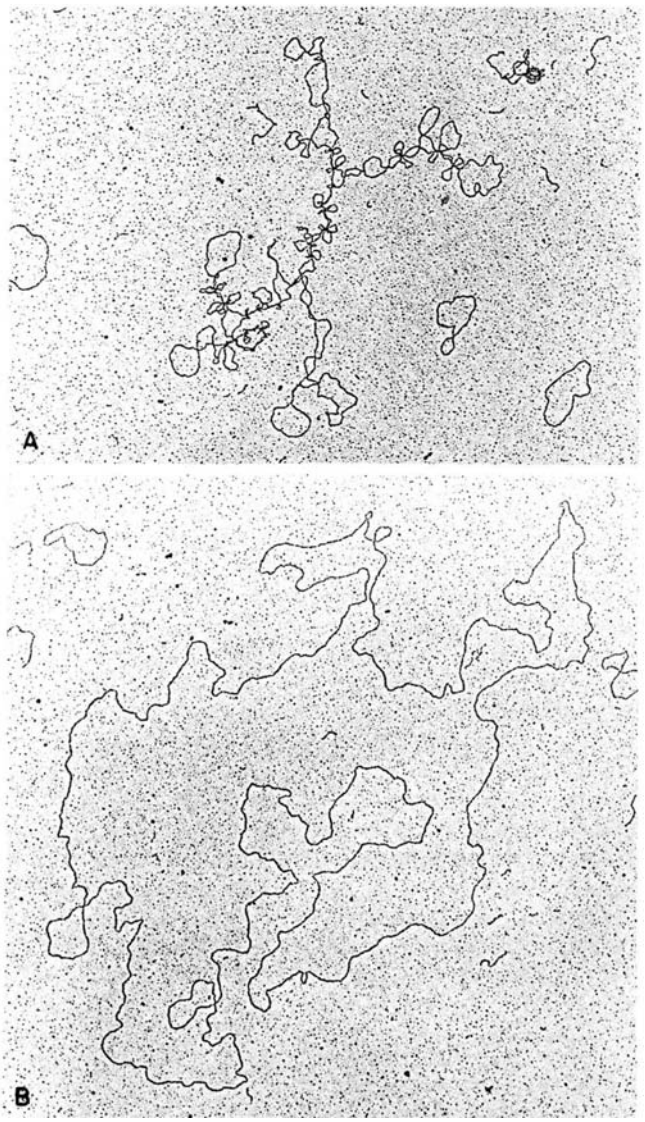
Plastid DNA

The modern era of study of chloroplast DNA began with the first definitive demonstration that it existed. In 1962 Ris and Plaut (108) showed that the single chloroplast in *Chlamydomonas moewusii* contained acridine orange-staining material which was removable by DNAse. In the electron microscope, 25-Å-thick strands could be seen in electron-transparent, i.e., ribosome-free, areas but were absent from DNAse-treated sections. Similar observations on chloroplasts of *Beta vulgaris*

were reported (109) in 1965, and since that time such DNA strands have been seen in plastids of many species.

In 1971 Manning and Richards (110) detected 40-μm-long circular chloroplast DNA molecules in lysates of *Euglena gracilis* chloroplasts. Circular chloroplast DNA molecules in this size range, including supercoiled molecules as shown in Fig. 6 (110–113), have been detected in preparations from a number of species of higher plants and from *C. reinhardii* (reviewed in ref. 114). Molecular weights calculated from measurements of electron micrographs of relaxed circles range from 91.1 × 10^6 daltons for *Zea mays* to 103.2 × 10^6 daltons for *Lactuca sativa* and 143 × 10^6 daltons for *C. reinhardii* (114). The significance of the size differences is not known. Each chloroplast contains enough DNA for about 10–60 circles; how many different kinds of circles are present in each plastid? The kinetic complexity of chloroplast DNA from most species is about 1 × 10^8 daltons, arguing that there is one type of circle present. (An exception is the case of chloroplast DNA from *Acetabularia* that shows a kinetic complexity of 1–1.5 × 10^9 daltons but the length of the molecule is not known [114].) Evidence that there is essentially a single type of circular chloroplast chromosome in a species comes from restriction mapping data as well (e.g., 115). In the species for which chloroplast DNA restriction maps have been reported, there is little if any evidence of heterogeniety.

Among special features of chloroplast DNA are (*a*) the absence of 5-methyl cytosine, a base which is present in plant nuclear DNA and (*b*) the presence of 12–18 ribonucleotides per chloroplast chromosome in at least three species of higher plants (116). Another feature, discovered by restriction mapping (115) and by electron microscopy (116), and subsequently observed in chloroplast DNA from many species examined but with some variations, is the presence of two or more large-to-huge repeated sequences. The 91.1 × 10^6 dalton *Zea mays*



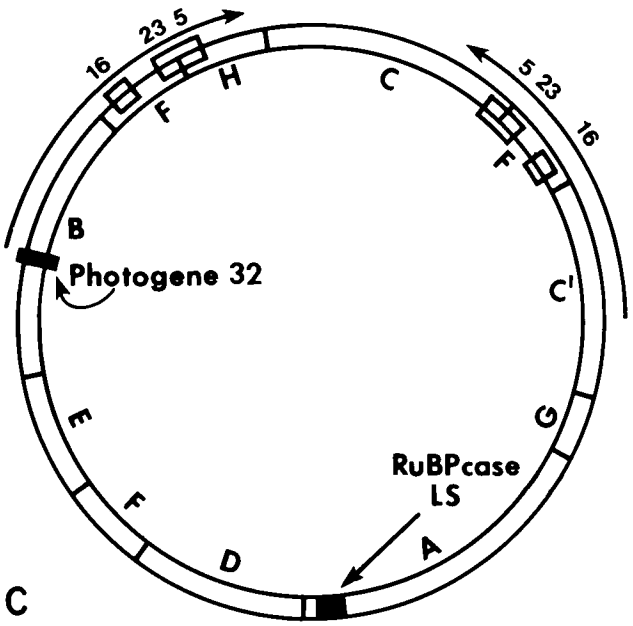


FIGURE 6 Electron micrographs of (A) a supercoiled oat chloroplast DNA molecule of molecular weight 92.2 × 10^6 and (B) of a relaxed circular maize chloroplast DNA molecule of 91.1 × 10^6 (111, 114). Electron micrographs were kindly provided by Richard Kolodner. (C) Diagram of a maize chloroplast DNA molecule. Recognition sites for the restriction endonuclease Sal I are indicated by lines connecting the concentric circles representing the two strands of DNA (115). The locations of the two large, inverted repeated sequences (115) are shown by the opposite facing arrows. The locations of genes for 16S, 23S, and 5S rRNAs are shown as open boxes (123). The positions of genes for LS RuBPcase (135–138) and of Photogene 32 (155) are shown by filled boxes. The letters inside of the circle are the names of the restriction fragments produced by Sal I (115).

chloroplast DNA contains two inverted repeats, each of 22.5 kilobase pairs, separated by a spacer region of 12.6 kilobase pairs (115, 117). Both spinach and lettuce contain a pair of inverted 24-kilobase pair repeats separated by unique sequences of 18.5 and 19.5 kilobase pairs, respectively (117, 118). *C. reinhardii* contains two inverted repeats of 19 kilobase pairs each (119). On the other hand, *Euglena gracilis* DNA contains three smaller, tandemly repeated sequences of 5.6 kilobase pairs each (120–122). In every case the repeats have been found to contain genes for chloroplast rRNAs (115, 118, 121–125). In *Euglena* an extra 16S rDNA is outside of the three complete repeats (126). In *Chlamydomonas*, the gene for 23S rRNA contains an intron of 0.94 kilobase pairs (124). Unlike the 23S rRNA gene in *C. reinhardii*, there are no introns in the rRNA gene in maize (123). The significance of these repeats is not known—some plants lack them. *Vicia faba* chloroplast DNA has a single set of rDNA genes (122a) and *P. sativum* chloroplast DNA, which had been reported to carry two tandemly repeated segments (117), has been shown to lack such repeats and to carry a single set of chloroplast rRNA genes (122b).

The 16S rDNA of a cloned maize fragment (123) has been sequenced in its entirety (127). Of the 1,154 residues of *E. coli* 16S rDNA, 1,104 positions (74%) are identical in maize 16S rDNA, although the maximum number of consecutive identical nucleotides is only 53. Also, in spite of the high degree of homology, fewer than a third of the cleavage sites for restriction enzymes are at identical positions.

Some tRNAs have been identified at various places on the inverted repeated sequence including within the spacer region between the genes for the large and small rRNAs (128–130a). The genes for 4.5 and 5S rRNAs are located at the 3′ end of the DNA sequence coding for 23S rRNA. One striking recent finding (130a) is the presence of a 949 long intron in almost the center of the gene for $tRNA^{Ile}$ found in the spacer region near the 16S rDNA in maize. Between the gene for $tRNA^{Ile}$ and the 23S rRNA gene is a gene for $tRNA^{Ala}$, which has an intervening sequence of approximately 806 bp. A gene for $tRNA^{leu}$ that contains a 406-nucleotide-long intron has been found on the maize chloroplast chromosone outside of the two large inverted repeats, but several other tRNA genes do not contain introns.[1]

Hybridization of tRNAs to total chloroplast DNA has been used to estimate the number of genes for these small RNAs on the chloroplast genome. Maize chloroplast DNA contains sequences complementary to about 20–26 tRNA cistrons changing at least 16 different amino acids (131) and 30–40 tRNA genes can be accommodated in pea chloroplast DNA (132). The most detailed mapping of tRNAs to positions on a chloroplast genome has been carried out in spinach (128). Genes for tRNAs are distributed on many parts of the genome although there are a few regions in which clusters are found. Genes for $tRNA^{His}$ and $tRNA^{Leu}$ have been located and sequenced on the inverted repeat of maize chloroplast DNA. Another gene for $tRNA^{Leu}$ and genes for serine and phenylalanine tRNAs have been sequenced as well. These two genes appear to be transcribed monocistronically and have mixtures of prokaryotic and eukaryotic features.[1]

The Gene for the Large Subunit of Ribulose Bisphosphate Carboxylase

Besides the gene for the 16S rRNA and some tRNAs in maize, the most thoroughly studied chloroplast gene is that for the large subunit (LS) of the carbon dioxide-fixing enzyme RuBPcase. This enzyme is comprised of eight identical small subunits of 12,000–14,000 daltons (depending upon the species) together with eight identical large subunits (LS) of 50,000–55,000 daltons (133). The gene for the small subunit of RuBPcase was shown by transmission genetics to be located in the nuclear genome of tobacco (134), but the large subunit of this enzyme is transmitted maternally—suggesting that the gene for the latter is located in the chloroplast genome. The gene for the large subunit has now been located on the maize chloroplast chromosome by physical means (135–137) and has been sequenced in its entirety (138). It has also been located on the *C. reinhardii* chloroplast chromosome (139).

Five nucleotides upstream from the initiation codon for methionine is the nucleotide sequence GGAGG (139), which is complementary to a sequence at the 3′ terminus of maize chloroplast 16S RNA (127). *E. coli* mRNAs similarly contain a sequence complementary to a sequence at the 3′ terminus of 16S rRNA that is believed to play a role in ribosome building (140, 141).

The structural DNA sequence for maize LS contains almost all the possible codons for amino acids (138), and the relatively small number of tRNA genes so far identified on maize chloroplast DNA raises some questions about the recognition systems that may be used in the chloroplast although the problem may be resolved by finding more chloroplast tRNA genes. The translation systems of yeast, *Neurospora crassa* and human mitochondria, recognize UGA as the codon for tryptophan rather than as the "universal code" signal for termination of translation. Yeast mitochondria translate the usual CUA leucine codon as threonine. Human and yeast mitochondria translate AUA as methionine rather than isoleucine (142–144). Furthermore, thus far only 23–24 tRNAs have been located in mitochondria, and it has been suggested that a simplified codon recognition system may be employed. As noted above, at least the pea chloroplast chromosome contains 30–40 tRNA genes (132)—enough to use the usual triplet code. Judging from codon usage for RuBPcase LS of maize, chloroplasts use the universal code.

Cyanogen bromide fragments representing approximately 50% of the RuBPcase LS gene of barley (145) and some fragments of the spinach polypeptide (146) have been sequenced. The gene for the maize LS contains codons for 475 amino acids (to give a molecular weight of 52,682). Only seven positions in the maize sequence have amino acids different from those in the known barley sequences, and in all cases the change could be due to an alteration in a single nucleotide. Five of the alterations could be considered neutral replacements. The peptide fragments from spinach LS that have been sequenced are thought to be near the catalytic sites (146) and these too, when aligned with the deduced amino acid sequence for the maize gene, show remarkable homology. The LS gene is highly conserved, at least in this limited group of angiosperms; this may be a property of genes for multimeric components generally and especially for those of organelles where the genes for the components are dispersed in two genomes.

A polypeptide 4,000–5,000 dalton larger than the mature form of the small subunit is produced in the cytoplasm by free cytoplasmic ribosomes and is then taken up by plastids and processed (147–150).

The gene for the LS RuBPcase is located about 300 base pairs away from another gene and transcription of the two genes is divergent (138). Maize is a C-4 plant. CO_2 is fixed into a four-carbon acid in mesophyll cells, and a derivative is

[1] Steinmetz, A. A., and L. Bogorad. Unpublished observation.

transported into bundle sheath cells where it is decarboxylated. The carbon dioxide that is liberated is refixed by RuBPcase. Mesophyll chloroplasts lack RuBPcase but bundle sheath plastids contain this enzyme. Mesophyll cells have very little if any LS mRNA, but it is abundant in bundle sheath cells (151). mRNA for the unidentified gene adjacent to that for LS is present in both bundle sheath and mesophyll plastids.[2] The nature of any DNA signal sequences and other aspects of the mechanisms for the control of transcription of these two adjacent genes is a matter of great interest.

Photogene 32 and Light-Harvesting Apoproteins

Angiosperm seedlings grown in darkness do not form chlorophyll; their plastids develop only to the characteristic etioplast stage, as mentioned in the discussion on PS I and II development. Etioplasts contain DNA and ribosomes but lack thylakoids. They have instead a characteristic paracrystalline prolamellar body. Upon illumination, the prolamellar body is rapidly altered in appearance, and in a few hours, the thylakoid structure begins to emerge as new proteins and lipids are added (e.g., 72, 152–154).

About 35% of the double-stranded weight of the chloroplast chromosome is transcribed in etioplasts and about 45% in green plastids.[3] A mixture of RNAs from the two plastid types hybridizes to about 50%. These data indicate that more of the chloroplast chromosome—more genes—is transcribed in chloroplasts than in etioplasts but that some genes transcribed in etioplasts are not read out in chloroplasts.

One maize plastid photogene (i.e., a gene expressed in dark-grown plants only upon illumination) has been identified by comparing RNA prepared from etioplasts and chloroplasts both in an in vitro translation system and by hybridization against fragments of maize chloroplast DNA (155) and by analysis of a chloroplast DNA fragment cloned in *E. coli*. The gene product is a 34,500 dalton polypeptide that is inserted into the thylakoid membrane and then processed down to a 32,000 dalton form (156).

The light-harvesting chlorophyll *a/b* apoprotein appears to be a product of a nuclear gene in barley and, like the RuBPcase small subunit, it is produced as a larger precursor which is processed after passage into the chloroplast (157, 158). The appearance of this mRNA is under the control of phytochrome (157).

Other Chloroplast Gene Products

Genes on only a very small fraction of any plastid chromosome have been mapped by transmission genetics plus biochemistry or by physical means: rDNAs, tDNAs, the LS RuBPcase gene, and photogene 32, the gene for ribosomal protein LC 4 of *C. reinhardii*. Of chloroplast constituents for which there is evidence for coding by a nuclear gene—small subunit of RuBPcase, apo-LHCP, genes for several ribosomal proteins—only the gene for ribosomal protein LC 6 has been mapped even by transmission genetics, but availability of cloned cDNA and nuclear genes should permit physical mapping.

Ellis (159) is largely responsible for the development and popularization of another approach to identifying plastid gene products. The assumption of the method is that transcription and translation occur in the same cellular compartment; i.e., messages translated in the plastid are transcripts of chloroplast DNA. Chloroplasts are isolated, incubated with radioactive amino acids either under illumination (as a source of ATP) or in darkness with ATP. The proteins that are made or completed are then analyzed. Among chloroplast components identified as products of plastid genes by this approach have been LS RuBPcase (159); CF_1 subunits γ, β, and ϵ (156, 160); cytochrome b_{559} (161), a protein of the photosystem I complex (161), translation elongation factors G ($EF\text{-}G_{chl}$) and Tu ($EF\text{-}Tu_{chl}$), (162), and cytochrome f (163). Most of these remain to be mapped physically on a chloroplast chromosome.

Transcription of the Plastid Genome

Two examples of regulation of gene transcription in plastid differentiation have been given: the regulation of photogene 32 (155) and differences in transcription in mesophyll vs. bundle sheath cells of the LS RuBPcase gene and the gene adjacent to it. The rate of chloroplast rRNA synthesis is accelerated upon illumination of etiolated leaves (152).

The maize chloroplast DNA-dependent RNA polymerase has been solubilized and shown to be distinct from nuclear polymerase II (154–156). The S factor, a 27.5 kilodalton polypeptide isolated from maize plastids, promotes the preferential transcription in vitro of some cloned maize chloroplast genes in supercoiled DNA (167). This in vitro system, using identified cloned chloroplast genes of different expression classes (e.g., photogenes vs. the LS RuBPcase gene of maize, etc.) as templates, seems a promising approach to the study of the regulation of gene expression. Perhaps other specificity factors will be found.

Complexes of DNA and protein capable of transcription prepared from *Euglena* chloroplasts (158) preferentially synthesize RNA complementary to restriction fragments of chloroplast DNA bearing rDNA. Hybridization with total chloroplast DNA by products of similar preparations from spinach chloroplasts (169) is 40% competed out by rRNA.

Summary and Prospectus

Since the early to mid-1960s, plastid molecular biology has progessed from the discovery of DNA and ribosomes to their detailed analyses.

The maize chloroplast gene for 16S rRNA is strongly homologous to that of *E. coli*, although identical stretches are short. The sequences of tRNAs and of genes for tRNAs now available show them to have a unique set of properties—each feature has been seen in some tRNA, but not in combination in other tRNAs.

The two mRNAs studied thus far are colinear with their genes, but introns have been observed in 23S rDNA of *C. reinhardii* and in tRNAs for isoleucine, leucine, and alanine in maize. The mRNA for maize LS RuPcase and what appears to be another maize mRNA both contain parts of the same short sequence complementary to the 3′ end of 16S rRNA in analogy to the bacterial situation.

In two regions of maize chloroplast DNA studied in detail, the only regions in any species except within and around the rDNA genes in *C. reinhardii*, transcribed regions are packed tightly. These and other features discussed above may be general characteristics of chloroplast chromosomes, but the number of species studied is still very small as is the fraction of any plastid chromosome charted to date.

Functional aspects such as the regulation of transcription in relation to development and differentiation are only now be-

[2] Link, G., and L. Bogorad. Unpublished observation.

[3] Haff, L. A., and L. Bogorad. Unpublished observation.

ginning to become amenable to experimentation. However, virtually nothing is known about several problems such as DNA replication and recombination.

A little is known about transport of proteins into plastids—two that are known, the small subunit of RuBPcase and LHCP, are synthesized in larger precursor forms, but so is the maize chloroplast photogene 32 product made within the plastid as a 34.5-kilodalton precursor. Yet to be identified are possible receptors and a transport apparatus in or on plastid outer membranes, for recognizing and importing proteins made in the cytoplasm, as well as processing enzymes for these and plastid synthesized components. The relative roles of nuclear and plastid genomes in the production of importing and processing systems must also be delineated.

Plastids divide, mature, differentiate for the production and storage of starch, oils, or carotenoids, and have genomes small enough to handle in studying the control of expression of identified genes. Plastids offer the opportunity to study mechanisms for the regulation of gene expression in a convenient form. However, students of this subject look forward to discovering the rules for gene dispersal and the mechanisms for intergenomic integration—the functional requirement imposed by the dispersal of genes for multimeric components in plastid and nuclear genomes. Still further ahead is the prospect of experiments to test theories of the origin and evolution of eukaryotic organelles and genomes.

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Microbodies: Peroxisomes and Glyoxysomes

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Microbodies were first reported at the ultrastructural level in the proximal convoluted tubule of mouse kidney by Rhodin in 1954 (1) and in hepatic parenchymal cells by Rouiller and Bernhard in 1956 (2) at about the time *The Journal of Cell Biology* was established. They were reported in plants by Porter and Caulfield in 1958 (3) and by Mollenhauer et al. in 1966 (4). Microbodies are now recognized as ubiquitous subcellular respiratory organelles in eukaryotic cells. Microbodies from all tissues appear morphologically similar and have similar enzymatic properties, but the metabolic pathways within this subcellular compartment vary, depending upon the tissue. Microbodies (peroxisomes and glyoxysomes) were one of the last major subcellular compartments to be recognized, and it was not until the end of the 1960s that their significance was established by several reviews. Most important were the following two summaries: "Peroxisomes (Microbodies and Related Particles)" by de Duve and Baudhuin in 1966 (5), and "The Peroxisome: a New Cytoplasmic Organelle" by de Duve in 1969 (6). The Nobel Prize that de Duve received was based on his pioneering work in the discovery and isolation of subcellular organelles, such as microbodies. Material in these two papers is essential reading for new students in the field. Also in 1969, the morphological literature was assembled into a book, *Microbodies and Related Particles* by Hruban and Rechcigl (7), which summarized the evidence for the widespread distribution of the particle. Another landmark in 1966, also from de Duve's group (8), was the development of procedures for isolating microbodies. The first research symposium, "The Nature and Function of Peroxisomes (Microbodies, Glyoxysomes)," was held in 1969 (9).

Recently there has been such a proliferation of papers about the many aspects of microbodies that in this article we cite only reviews or use only an initial reference to a specific subject. Some of the general reviews are on development and enzymatic content (10), microbodies in leaves (11–13), germinating seeds (14, 15), algae (16), fungi (17), and protozoa (18); other reviews will be cited with specific topics. Nevertheless, we have little knowledge today of the physiological role of microbodies in cellular metabolism. Properties and characteristics of microbodies are still incompletely described, and much of the recent literature has not been confirmed or well established by the few biologists working in this field.

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Nomenclature

From the titles cited above, it is apparent that there has long been uncertainty about naming this organelle. Morphologists sometimes used the nonspecific term microbody until a more specific name came to be established through functional and biochemical studies. At present, microscopists are continuing to use the term microbody for the organelle that is variously called, by others studying its biochemical properties, mammalian peroxisomes (5), leaf peroxisomes (12), glyoxysomes (12, 15), or glycerophosphate oxidase bodies (18). The term peroxisomes was proposed by de Duve for the organelle because it produced and consumed hydrogen peroxide. The in vivo potential for its catalase to metabolize peroxidatively other organic substrates with H_2O_2 that is generated internally by flavin oxidases, was used as an assay, based on the conversion of [^{14}C]HCOOH to [^{14}C]CO_2. Novikoff and Novikoff (19) have used the term microperoxisome to describe similar smaller particles. The use of the term leaf peroxisome was adopted by Tolbert (12) because the morphological and enzymatic properties of microbodies from leaves were consistent with de Duve's description of the organelle. In addition to meeting de Duve's criteria for peroxisomes, microbodies in germinating fatty seeds have as one of their metabolic pathways the glyoxylate cycle, and were termed glyoxysomes by Breidenbach and Beevers (20). Initially, Müller et al. (21) referred to those in *Tetrahymena* as peroxisomes (9), but the term glyoxysome is now generally used for microbodies containing at least malate synthetase or isocitrate lyase, the two unique enzymes of the glyoxylate cycle.

Microbodies from all sources have a somewhat similar appearance, but they will have different metabolic pathways, depending on the tissue and its function. In current usage, the term microbody is assigned to the particle that has not been biochemically characterized from a given tissue, or is used as a general term to include both peroxisomes and glyoxysomes. Peroxisomes are microbodies that are known to contain catalase and at least one flavin oxidase; glyoxysomes are microbodies that contain, in addition, isocitrate lyase and/or malate synthetase, two enzymes of the glyoxylate cycle. Abandoned terms for microbodies are phragmosome, cytosome, peroxidosome, and crystal-containing body.

Whereas microbody respiration must be significant and different from mitochondrial respiration or other O_2 uptake processes in the cell, there is no convenient method for measuring it specifically, nor is there a general physiological nomenclature for it. Because of big changes in the total number of microbod-

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ies and amounts of their substrates, this respiration must vary in different tissues, at different stages of development, and in plants at different periods of the day. Through spectrophotometric measurements of catalase turnover in perfused liver, a member of Chance's group (22) has estimated that hepatic peroxisomal respiration may account for up to 10% of the total O_2 uptake. In leaves, the term photorespiration, referring to glycolate biosynthesis in the chloroplasts and its oxidation in the peroxisomes and mitochondria, may be fivefold greater than dark mitochondrial respiration, but all of photorespiration cannot be designated as peroxisomal respiration because of the participation of other organelles in this process. Although precise figures are not available, glyoxysomal respiration in germinating seeds, which would be the O_2 uptake associated with the conversion of long-chain fatty acids to C_4 acids, probably is also much greater than mitochondrial respiration during this period.

Morphology

Microbodies are morphologically characterized as particles ranging in diameter from 0.1 to 1.5 μm (average about 0.5 μm), which are delimited by a single tripartite membrane and contain a finely granular matrix. In liver, an estimation of 1,000 microbodies per hepatocyte has been made (23). They show a close spatial relationship to the endoplasmic reticulum. Their catalase can be demonstrated cytochemically. Because microbodies without inner membranes must be pliable, they usually appear spherical or ovoid, but in the cell they may also be irregular in shape or show unusual projections. Hepatic (Fig. 1) and plant (Fig. 2) microbodies contain, in addition to the granular matrix, an electron-dense core (nucleoid) in which a series of parallel membranes or lattice structures are sometimes observed. De Duve and Baudhuin (5, 24) considered that urate oxidase in rat-liver peroxisomes is associated with the nucleoid. Newcomb's group (25) showed cytochemically that the core in plant microbodies is rich in catalase. Although usually structureless, fine branching filaments (4–5 nm) or short fibrils have also been described in the microbody matrix (7).

The hepatic microbody is delimited by a tripartite membrane approximately 6–8 nm in thickness. In contrast to lysosomes, the delimiting membrane appears to be thinner, and no electron-lucid zone is found between the membrane and the matrix of the microbodies (Fig. 1). Freeze-fracture replicas of hepatic microbodies revealed numerous particles (7–8 nm), often in clusters, on the protoplasmic face of the delimiting membrane, whereas the extracellular face is almost devoid of such particles (26). These features, which resemble those seen in other types of membranes, are also found on the corresponding fracture faces of these portions of endoplasmic reticulum which are located adjacent to the microbody membrane.

The microbodies of rat kidney frequently show tubular protrusions and circular profiles at the margin of the matrix (7). Barrett and Heidger (27) showed that the tubular protrusion rods were absent from rat renal microbodies when fixed by perfusion, but the circular and tubular profiles were consistently demonstrable. According to Tisher et al. (28), the morphology of the renal microbody can vary markedly, depending on the method of fixation. Recent freeze-fracture studies, which provide a three-dimensional view of these inclusions (100–125 nm), appear to confirm the impression that the tubular and

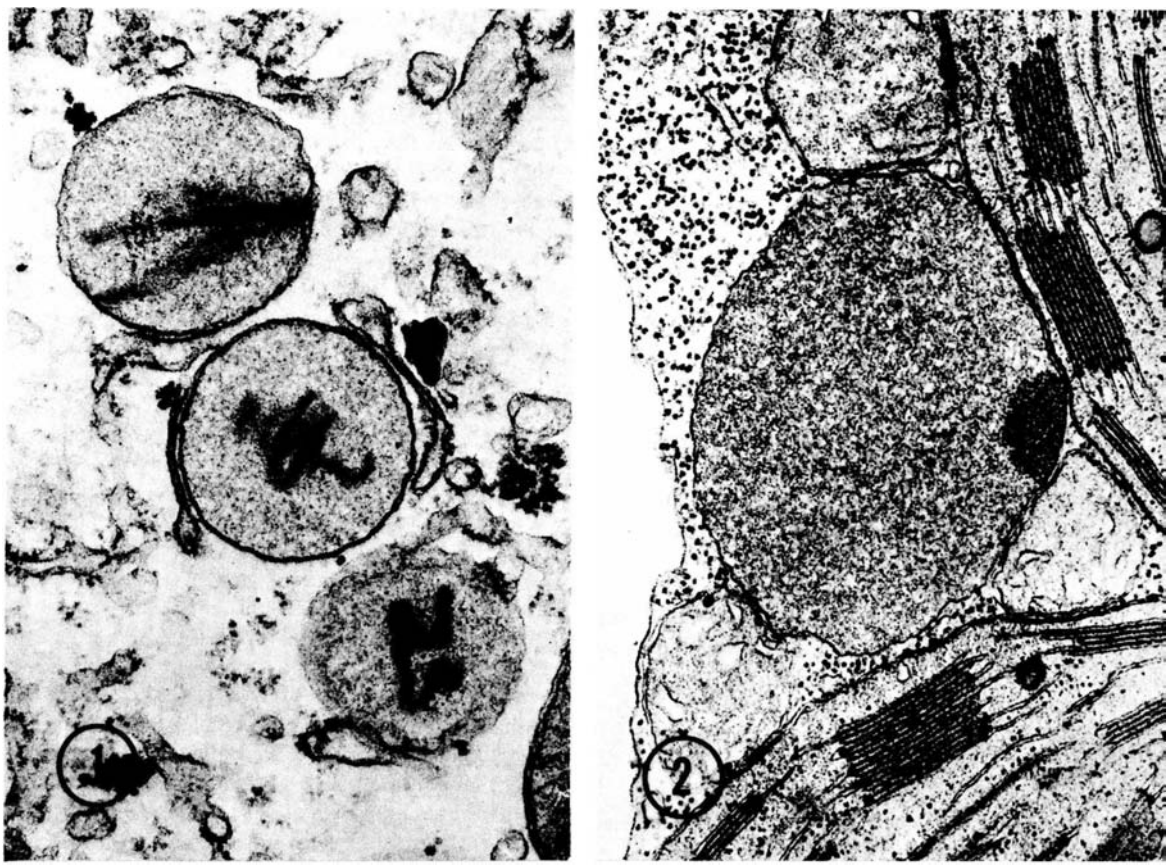


FIGURE 1 Peroxisomes in mouse liver. Note characteristic configuration of nucleoids. X 43,000.

FIGURE 2 Peroxisome in parenchyma cell of a tobacco leaf. Electron micrograph courtesy of Dr. S.E. Frederick. X 40,000.

circular profiles represent sections through different planes of the same elements (29). As in the case of hepatic microbodies, freeze-fracture replicas of renal proximal tubule cells reveal more particles on the protoplasmic face than on the extracellular face of the delimiting membrane.

Much interest has been focused on the central core, or nucleoid, of plant and hepatic microbodies that consists of homogeneous, membranous, or lamellar forms. In hepatic microbodies, the lamellar type may appear as a crystalloid consisting of a polytubular substructure. Hruban and Rechcigl (7) examined the form and complexity of crystalloids in microbodies in various species, and attempted to classify microbodies according to the presence or absence of a nucleoid and to determine the pattern formed by the elements of the crystalloid. In many species these can be grouped into the following two categories "coarsely polytubular" or "finely polytubular." The nucleoids of hepatic microbodies were isolated by Tsukada et al. (30) and were shown to consist of parallel bundles of highly dense, hollow tubules, which, in cross section, have a honeycomb appearance. In the microbodies of guinea-pig liver, which correspond to the second type, the nucleoid consists of microtubules, approximately 4.5 nm in diameter, arranged in a regular hexagonal lattice with spacings of about 11 nm between the axes of contiguous tubules (5, 24).

In 1964, Hruban and Swift (31) reported that the microbody nucleoids of rat hepatocytes and of certain transplantable rat hepatomas were similar in structural organization and dimensions to commercial preparations of urate oxidase from hog liver, and suggested that the core was composed of the crystalline enzyme. However, Baudhuin et al. (24) felt that structures seen in the urate oxidase preparations probably represented preformed microbody nucleoids that had been concentrated by the purification procedure, rather than crystals of the enzyme. The question was reexamined by Lata et al. (32) with sections through highly purified preparations of rat-liver urate oxidase that revealed a polytubular structure similar to that present in the microbody nucleoids of the same species and to that described by Hruban and Swift (31). Comparative ultrastructural studies also suggest a correlation between the presence or absence of a nucleoid and the level of urate oxidase activity. The occurrence and substructure of nucleoids and the presence or absence of urate oxidase activity were correlated by Afzelius (33) and Shnitka (34) in hepatic microbodies of various species. Among rodents, carnivores, and ungulates, there was a positive correlation between the presence of a nucleoid and the presence of urate oxidase activity. Humans and birds, whose hepatocytes contain anucleoid microbodies, are without hepatic urate oxidase activity. Although urate oxidase is now considered a reasonably constant component of the nucleoid, the functional significance of the structural variations encountered among different species remains unclear. Shnitka (34) suggested that such differences may be related to conformational differences in the folding of the peptide chains of the enzyme or to differences in the amino acid composition.

Another characteristic structure sometimes observed in microbodies is the "marginal plate." It is usually located at the microbody periphery and consists of a relatively straight, thickened region that sometimes shows periodic substructure. Marginal plates have been described in microbodies of liver and kidney from several species (7). The functional significance of the marginal plate remains obscure.

In 1958, Porter and Caulfield (3) described membrane-bound bodies ("phragmosomes") in dividing onion root cells that measured 0.25–0.5 μm in diameter and contained a granular matrix. Organelles of similar appearance were subsequently identified in other higher plants, algae, and fungi by Mollenhauer et al. (4), who also drew attention to the similarity of these bodies to the microbodies of animal cells. A delimiting tripartite lipid bilayer, 6–7 nm thick, and a granular matrix are virtually constant features of microbodies in plant cells (for reviews see references 25 and 35). As in animal cells, plant microbodies show a close spatial relationship to the endoplasmic reticulum. The presence of a marginal plate has also been described in fungal microbodies. As in liver and kidney, plant microbodies may be grouped into three categories: anucleoid microbodies, those containing noncrystalline cores, and those with crystalloids characterized by an organized substructure. Examples of anucleoid microbodies have been found especially in meristematic and differentiating plant cells, such as root cells (4, 35), and those with crystalloid cores were first reported in oat coleoptiles (36) and, subsequently, in various other plant cells (25). The crystalloid core of plant microbodies gives a strong cytochemical reaction for catalase and has been variously interpreted as layers of parallel sheets, or as tubules which are organized into hexagonal, tetragonal, or rectangular patterns (7, 25, 35).

Cytochemistry

A number of attempts have been made, with varying degrees of success, to localize enzyme activities within microbodies by cytochemical means. In 1965, Graham and Karnovsky (37) localized uricase (urate oxidase) activity in microbodies by light microscopy. Horseradish peroxidase was used to catalyze the oxidation of 3-amino-9-ethylcarbazole by H_2O_2 generated at the sites of urate oxidase activity. Allen and Beard (38) reported on the light-microscope localization of α-hydroxyacid oxidase in renal peroxisomes by use of a method based on reduction of nitro blue tetrazolium. Although subsequently modified, this method was not useful for electron-microscope localization (39). Shnitka and Talibi (40) introduced a method for the light- and electron-microscope localization of α-hydroxybutyrate oxidase. In this reaction, ferrocyanide, produced by enzymatic reduction of ferricyanide, is captured by copper to yield insoluble, electron-dense, cupric ferrocyanide. Although subsequently modified to yield more reproducible results (41), the method was unsatisfactory, in part because of the sensitivity of the enzyme to glutaraldehyde and the prevalence of nonspecific precipitate. An electron-microscope method for demonstrating NADH oxidase, based on the use of cerium ions, has now been modified for the localization of D-amino acid oxidase (42). The cytochemical localization of peroxisomal oxidases has been reviewed recently by Hand (43).

Although catalase had been demonstrated in hepatocytes by direct assay and immunocytochemical techniques, the limitations of the methods provided only hints of possible localization in microbodies. In 1968 Novikoff and Goldfischer (44, 45) described a procedure for the cytochemical visualization of microbodies at the light and electron microscope levels using a modification of the diaminobenzidine (DAB) procedure originally described by Graham and Karnovsky (46) for peroxidase (Figs. 3 and 4). This modification made it possible for Novikoff and Goldfischer (44, 45) to confirm the identity of catalase in particles considered to correspond with microbodies in various animal cells, and has facilitated the analysis of their number, distribution, and relationship to other structures, particularly the endoplasmic reticulum. The cytochemical localization of microbody catalase was also reported independently by Hirai (47) and, subsequently, by Fahimi (48). In tissues such as liver,

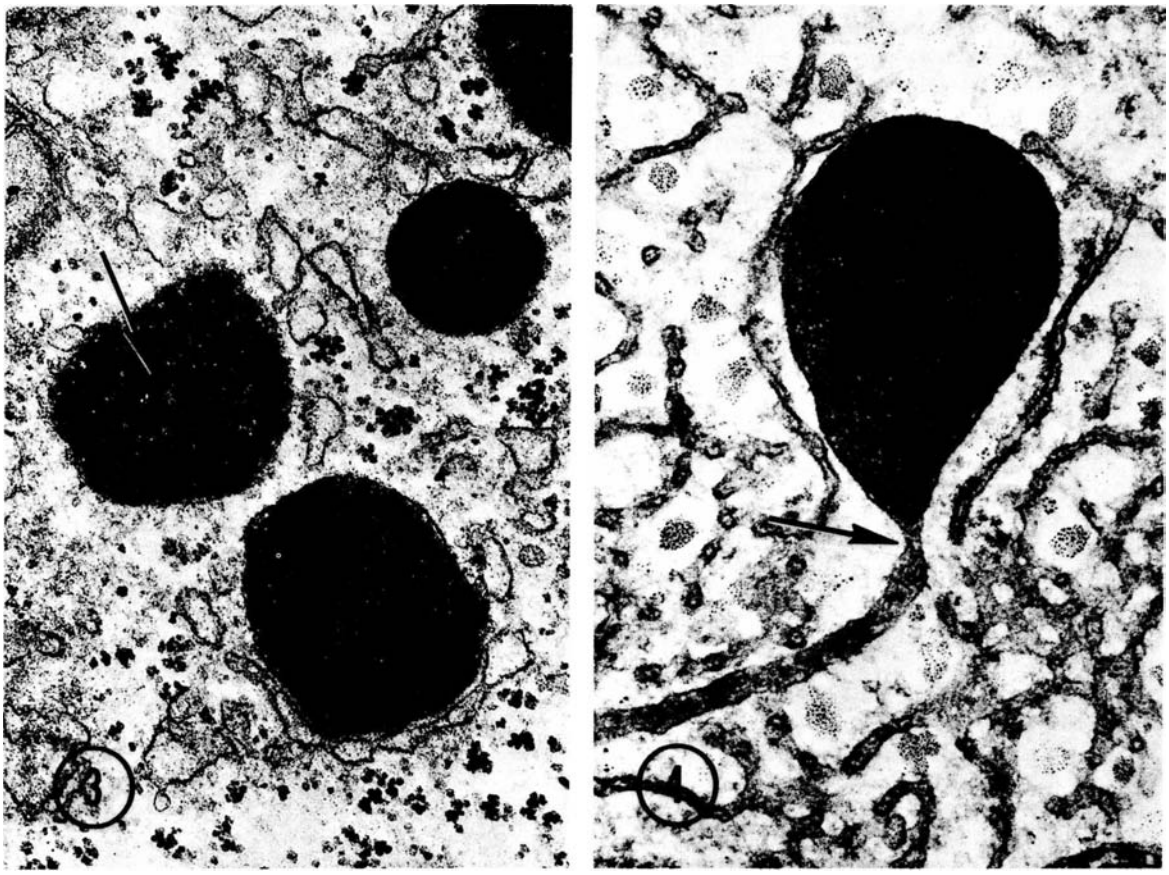


FIGURE 3 Peroxisomes in rat liver after incubation in diaminobenzidine medium. Catalase reaction product is localized in peroxisomes, partially obscuring the nucleoids (arrow). X 40,000.

FIGURE 4 Peroxisome in hepatocyte from rat treated with hypolipidemic drug, nafenopin. Tissue was incubated in diaminobenzidine medium. The delimiting membrane of a catalase-positive peroxisome is continuous (arrow) with that of smooth endoplasmic reticulum. Electron micrograph courtesy of Dr. P. M. Novikoff. X 51,000.

the identification of microbodies after incubation for catalase activity was unequivocal, because the tubular substructure of the nucleoid was still recognizable. Microbody staining is almost completely inhibited by aminotriazole, a potent and relatively specific inhibitor of catalase. Distinguishing the peroxidatic activity of catalase from that of peroxidase by cytochemical means has been investigated by a number of workers (for a review see Fahimi [49]). Frederick et al. (25) and Vigil (35) used the alkaline DAB medium and also demonstrated an aminotriazole-sensitive staining of the granular matrix of peroxisomes in leaf cells, as well as a localization of catalase in the crystalloid of microbodies in oat coleoptile cells and in endosperm cells of germinating castor beans.

Incubation of tissue in the alkaline DAB medium results in deposition of reaction product in the matrix of the microbody and over the central nucleoid, if present. Although the procedure is now widely used, the mechanism of the reaction remains unclear. It is assumed that catalase, acting peroxidatively, oxidizes the DAB to a conjugated double-bond structure that binds large amounts of OsO_4 and appears opaque by electron microscopy. Fahimi (48) has suggested that the alkalinity of the medium serves to enhance the oxidation of certain substrates. It has been suggested by Goldfischer and Essner (50) that the alkaline medium causes dissociation of microbody catalase into subunits, which, despite loss of catalatic activity, have enhanced peroxidase activity, as occurs with preparations of hepatic catalase. Additional evidence that microbody catalase is dissociated into peroxidatic subunits came from studies of mutant acatalasemic mice. The catalase in these mutants is temperature-sensitive, and its catalatic activity is rapidly destroyed at 37°C. Goldfischer and Essner (51) found that hepatic and renal microbodies of these mice exhibited stronger staining in DAB medium, even at neutral pH, than did those of the wild-type tissues, and suggested that the microbody catalase in the mutant existed in vivo in a partially degraded form that showed enhanced peroxidase activity.

Microperoxisomes

Small microbody-like organelles, usually lacking a nucleoid, had been observed in various tissues by a number of earlier electron microscopists. In 1968, Kuhn (52) described small particles in the dog perianal gland, which were similar to microbodies and which sometimes showed a marginal plate or a dense nucleoid and had continuities with the endoplasmic reticulum. Hruban et al. (53) used the alkaline DAB method to demonstrate small, anucleoid microbodies in a variety of vertebrate cell types and noted their prominence in cells that were engaged in the metabolism of cholesterol, steroids, and lipids. By using a modification of the DAB procedure for the demonstration of catalase activity, Novikoff et al. (54) were able to identify similar particles in a variety of cell types (55) (Fig. 5). They drew attention to the fact that these particles were generally smaller than the nucleoid-containing peroxisomes, and showed frequent continuities of their delimiting membrane

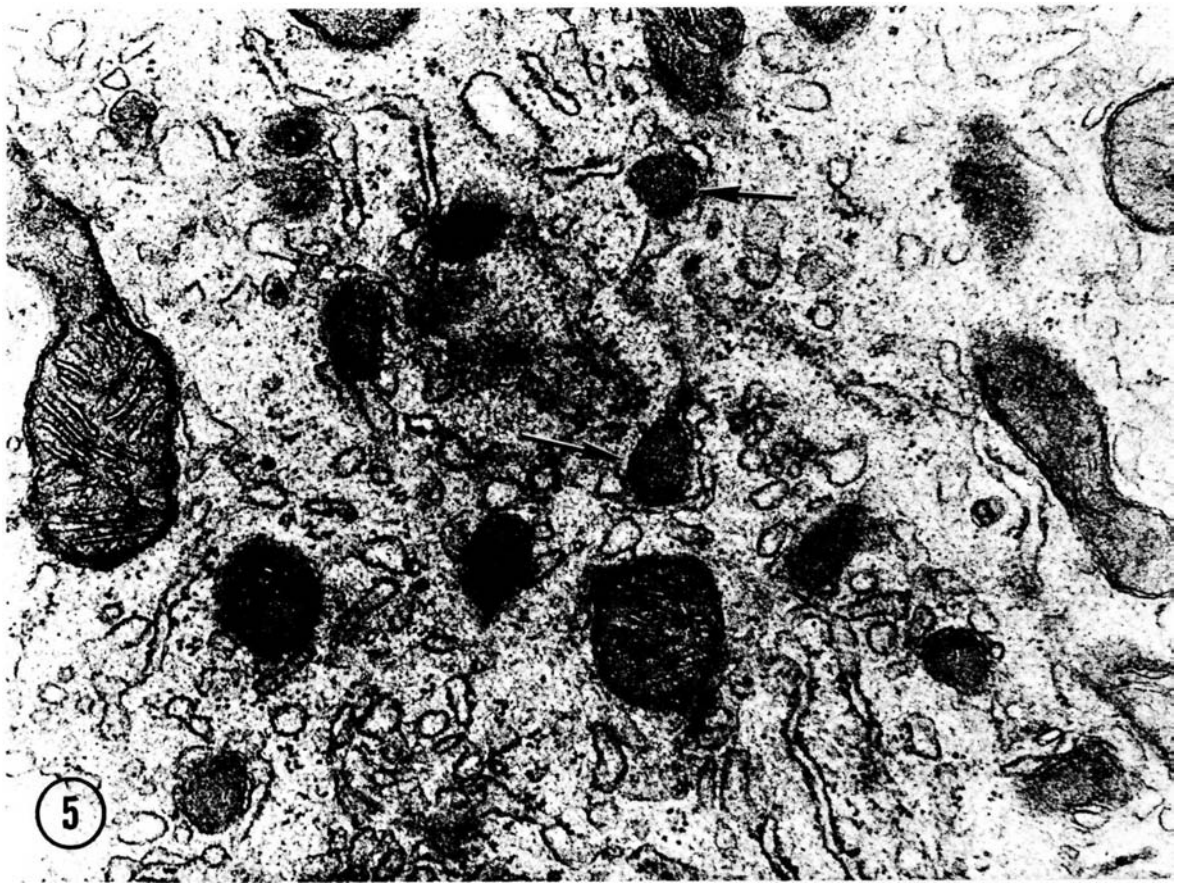


FIGURE 5 Absorptive cell from guinea pig duodenum showing cluster of microperoxisomes (arrows) in proximity to smooth endoplasmic reticulum. Electron micrograph courtesy of Dr. P. M. Novikoff. X 43,000.

with that of the endoplasmic reticulum. Based on these hallmarks, they suggested the term "microperoxisome" (19). Microperoxisomes are found in virtually all cells with the exception of erythrocytes and, possibly, other specialized end-stage cells (55). Although their functions are not known, microperoxisomes appear to be involved in the metabolism, transport, and storage of lipid. Relatively large numbers of these particles are found in cells engaged in lipid metabolism and, in some instances, may show a close spatial relationship to lipid droplets (56) or to lipofuscin granules (57). Organelles with the appearance of microperoxisomes also have been described in root meristem cells and in differentiated plant parenchyma cells, where they are found together with nucleoid-containing microbodies (25). For a brief review of microperoxisomes see Novikoff and Novikoff (58).

One of the most significant and characteristic features of microperoxisomes is their frequent, often multiple, continuities with the endoplasmic reticulum (ER). These connections are wider and therefore easier to demonstrate than are the slender connections seen between microbodies and ER in liver and kidney. As is the case for microbodies, the continuities are regarded as local dilatations of the smooth ER that lack ribosomes and in which peroxisomal constituents accumulate. Whether microperoxisomes exist separately from the ER, are always in continuity with it, or are in a state of change between the two phases is not known. According to Novikoff et al. (57), microperoxisomes are also found in liver and kidney, where they may represent the progenitors of the larger microbodies. The process would involve enlargement of the microperoxisome together with formation of an electron-dense area (nucleoid) in which a tubular substructure later develops. The sequence may be similar to that described for the transformation of anucleoid to nucleoid microbodies in the liver during late fetal development (59, 60) and to the process described by Frederick et al. (25) in root parenchyma cells. Further possible evidence for the role of microperoxisomes as progenitors of microbodies comes from studies of liver from rats treated with clofibrate (see Development section). Many of the large numbers of microbodies formed in response to administration of this agent have the morphological characteristics of microperoxisomes, such as multiple continuities to and occurrence in clusters.

Isolation and Assay

The development of buoyant density or isopycnic centrifugation was essential for the isolation of microbodies sufficiently separated from other particles for examination of enzymatic composition. Because the subject is reviewed in this volume by de Duve, only some salient points relative to microbody isolation are mentioned here. Between 1964 and 1968, de Duve's group (8), using a zonal rotor designed by Beaufay, developed a complex procedure for partial purification of microbodies that has been the basis of all subsequent procedures. The development of commercial zonal rotors (B-29 and B-30) and centrifuges during the same period was based on Anderson's (61) adaptations of buoyant density centrifugation for the isolation of biological particles. Swinging-bucket rotors, such as Beckman's SW 25.2, for 10- or 55-ml tubes have been in use for small-scale preparations of microbodies. Several advantages of the zonal rotor were discussed by Anderson, such as a larger

volume and size of preparation, and good separation of the bands of organelles. The earlier work by de Duve's group showed that the "light mitochondrial" particles obtained by rate sedimentation were a mixture of mitochondria, lysosomes, and microbodies, as well as a good deal of contaminating ER; however, the term light mitochondrial fraction is no longer used extensively. Preparations of the light mitochondrial fraction from differential centrifugation may be used for partial separation of microbodies before isopycnic centrifugation, but such manipulations decrease the yield of the fragile microbodies. More recently, *Methods in Enzymology* (particularly volume 31) have devoted many chapters to isolation of subcellular particles, and several discussions of the isolation of microbodies are available (62–66).

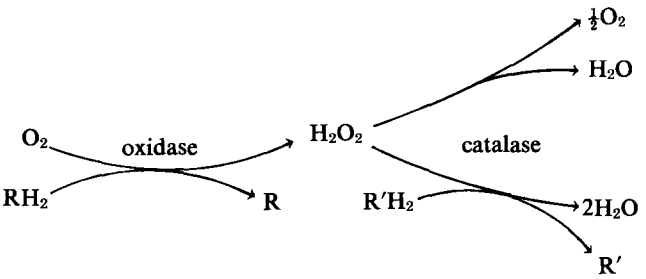
The final equilibrium density of a particle is dependent on its composition (protein, lipid, and bound water). In addition, microbodies and lysosomes, which have only a single bounding membrane, may more rapidly lose bound water to the sucrose solution of the gradient than do the mitochondria. Thus microbodies sediment to the highest density in the gradient because of their low lipid composition and dehydration. Sucrose gradients have generally been used, because in gradients of large molecular weight-compounds, microbody isolation is based more on rate sedimentation. The equilibrium density of microbodies from all sources is generally about 1.24 g-cm^{-3} (about 51% sucrose) but it must be remembered that these isolated particles have lost bound water and probably many soluble components.

The separation of the hepatic microbodies and lysosomes of nearly similar equilibrium density was accomplished by de Duve's group by injecting a detergent, Triton WR-1339, into the rat two days before sacrifice. Lysosomes containing the engulfed detergent then had a lower equilibrium density than the microbodies. However, for other biological tissues, and now for most mammalian peroxisomal preparations, investigators use no detergents and rely only on marker enzyme profiles to evaluate their microbody purity. Plant lysosomes (vacuoles) do not generally survive grinding. Liver lysosomes distribute over two major areas: a small (10%) part of the lysosomes bands at the edge of the peroxisome peak and the rest among the ER. Although the specific activity of peroxisomal enzymes is increased 10- to 50-fold by isopycnic centrifugation, the reported specific activity values would be still higher if all the contaminating organelles could be removed. Besides lysosomes, the other major contaminant in the microbody peak is a small part of the total ER. This ER almost seems to be attached to and dragged down into the dense sucrose by the microbodies, and it represents a more serious contamination than the few mitochondria tailing into the microbody fraction. For these reasons, unbiased electron-micrograph observations of isolated microbodies for purity ought to be, but rarely are, done for each gradient.

The location of the microbodies in the gradient is established by a profile for the catalase or urate oxidase activity. Marker enzymes for the other particles must also be assayed in order to calculate their contamination in the microbody fraction. Any other enzyme activity in the gradient that exactly coincides with the profile of peroxisomal catalase distribution is judged to be in the microbody. Because broken microbodies may lose their various enzymes at different rates, a ratio of the total activity in the peroxisomal peak to the activity in the soluble fraction cannot be used. Investigators have reported their gradient profiles on the basis of relative enzyme activity, such as the fraction of the total activity on the gradient or a ratio to that in the microbody peak, whereas others have used units of enzyme activity per fraction volume or as specific activity on a protein basis. The total amount of activity per gradient never represents the total activity in the tissues because of losses from incomplete grinding and previous differential centrifugation. For total activity, a separate analysis should be run on a totally homogenated fraction.

Enzymatic Composition and Metabolic Pathways

CATALASE AND FLAVIN OXIDASES: The terminal oxidase for O_2 uptake by microbodies from all tissues examined has been a H_2O_2-producing flavin oxidase associated with catalase. In addition, some microbodies have copper-containing urate oxidase. The flavin oxidase varies with the tissue and with the available substrates. An exception is that glyoxysomes from some fungi and molds may contain only part of the glyoxylate cycle and no terminal oxidase system. In 1966, de Duve (5) illustrated this concept by the following scheme (slightly modified), which indicates one oxidase reaction and two possible catalase reactions with the H_2O_2:



The identity of the various substrates, RH_2, for the oxidase will be cited later for each metabolic cycle. Whether a second substrate, $R'H_2$, is peroxidatively oxidized in vivo is not established. However, the original in vitro assay (8) was the quantitative peroxidative oxidation of [^{14}C]HCOOH to [^{14}C]CO_2. More recently, isolated peroxisomal fractions have been quantitated by peroxidation of volatile [^{14}C]CH_3OH to a nonvolatile product (67). Catalase has been estimated to be about 33% of the hepatic peroxisomal protein (5), so it is present in the particle in great excess. This important enzyme for peroxisomal respiration was exhaustively characterized and studied by Chance (68), Theorell (69), and others before the realization that it resided in a subcellular organelle. In the reaction mechanism for catalase, a stable complex, compound 11, of catalase H_2O_2 is first formed; it then reacts peroxidatively with either another H_2O_2 or $R'H_2$. The amount of the catalase H_2O_2 complex formed is increased by high catalase concentration, as in the peroxisomes. The complex has a characteristic absorption spectrum (22), which has been monitored in vivo by Chance's group, who used perfused liver provided with substrates (RH_2) for H_2O_2 generation by peroxisomal oxidases (glycolate or urate). These measurements indicated that hepatic peroxisomal respiration could be as much as 10% of the total respiration.

The extent of peroxidative metabolism of a second substrate, $R'H_2$, remains to be established by physiological experiments. In support of a peroxidative mechanism, methanol detoxification in rats (but not in monkeys) was reduced when catalase was poisoned by aminotriazole (70). However, ethanol detoxification was not inhibited, probably because of the alternative alcohol dehydrogenase reaction. During photorespiration in leaf peroxisomes or during the glyoxylate cycle in seed glyox-

ysomes, a very large flow of carbon to glyoxylate occurs, and the glyoxylate is converted to glycine or malate. In solution, glyoxylate is oxidized extremely rapidly by H_2O_2 to CO_2 and HCOOH, but this does not occur in plant microbodies in vivo, as if the peroxidase mechanism was actually inhibited in these microbodies.

De Duve (6) envisaged subsequently that the flavin oxidase of microbodies might be coupled to reduction of the oxidized substrate R back to RH_2 by dehydrogenases so that the peroxisomal system could serve as a terminal oxidase linked to reduced pyridine nucleotides in the cell. No proof for this has been forthcoming, and in fact the example he suggested, glycolate oxidase coupled to glyoxylate reductase, has been shown to be invalid because the latter enzyme does not exist in hepatic peroxisomes (64), and in leaf peroxisomes it functions instead as a hydroxypyruvate reductase (71). A detailed evaluation of plant photorespiration suggests that the total cycle is indeed a terminal oxidase system, but so complex (involving three organelles) and controlled that it can not be simplified as a single peroxisomal terminal oxidase system (12).

One generality has been that the presence of catalase in all eukaryotes is indicative of microbodies. Two exceptions to this are the immense amount of catalase in erythrocytes and the large portion of catalase found in any soluble cytosolic fraction after cell breakage. It is not yet established which portion of this "soluble" catalase fraction is the result of broken or leaky microbodies or of a real cytoplasmic pool of catalase. It is of interest that "extraperoxisomal" catalase has been demonstrated cytochemically in the liver of several species (72).

α-HYDROXYACID OXIDASE AND D-AMINO ACID OXIDASE: These flavin-containing oxidases are present to some extent in most microbodies and represent the characteristic peroxisomal flavoproteins that form H_2O_2. The α-hydroxyacid oxidase catalyzes the oxidation of glycolate to glyoxylate, L-lactate to pyruvate, and L-α-hydroxycaproate to the corresponding α-ketoacid. Characterization (73) of this enzyme started before its localization in the microbody. Like other microbody enzymes, it was thought to be in the cytoplasm, as harsh grinding procedures then in use broke the microbodies. Studies with isolated microbodies and cytochemical tests (74) indicate that the enzyme is always present in the organelle. The peroxisomal glycolate pathway of metabolism to glycine, part of leaf photorespiration, may exceed the rate of mitochondrial respiration fivefold, but it does not occur in the dark when there is no photosynthetic formation of glycolate (12). The role of hepatic and renal peroxisomal α-hydroxyacid oxidase is unknown. The enzyme is present in relatively low activity (1–10 nmoles/min/mg protein in the homogenate), but its substrates, glycolate and lactate, are present in substantial amounts. The renal peroxisomal α-hydroxy acid oxidase does not oxidize the short-chain acids, but it does oxidize C_8 acids, although the function of this activity is unknown.

GLYOXYLATE CYCLE: This modification of the citric acid cycle was elucidated by Kornberg and Krebs (75). The glyoxylate cycle was later localized, not in the mitochondria, but in microbodies of *Tetrahymena* by Müller et al. (21, 76) of germinating castor bean endosperm by Breidenbach and Beevers (20), and of yeast by Szabo and Avers (77). Today these microbodies may be called glyoxysomes including those in *Tetrahymena* (which were called peroxisomes by their discoverers) if they contain the two unique enzymes, isocitrate lyase and malate synthetase, of the glyoxylate cycle. Both enzymes have been highly purified and characterized. Whereas the citric acid cycle oxidizes acetyl CoA to 2-CO_2, the glyoxylate cycle condenses 2-acetyl CoA to a C_4 dicarboxylic acid (succinate). In the glyoxysomes of germinating castor beans, all the enzymes of the glyoxylate cycle are present. Glyoxysomes of *Tetrahymena* contain isocitrate lyase and malate synthetase, but the other enzymes of the glyoxylate cycle that are common with the citric acid cycle are only in the mitochondria. Certainly a partial spatial separation of the citric acid and glyoxylate cycles has been achieved by the two organelles.

The glyoxylate cycle is substrate-inducible and so are the glyoxysomes in eukaryotes. Glyoxysomes are greatly repressed by growth on sugars or substrates not metabolized by them. Growth on acetate, malate, glycolate, or larger molecules that are cleaved to these C_2 and C_4 compounds induces the glyoxylate cycle and glyoxysomes. Glyoxysomal development and metabolism in fungi (13), which obtain such compounds from the host, has also become an important glyoxysomal research area.

So far there are two exceptions to the above generalities about glyoxysomes. They may not contain catalase, as there is no flavin oxidase in the glyoxylate cycle (17). Second, Trelease's group (78) have found that isocitrate lyase and malate synthetase are in the mitochondria of the roundworms *Turbatrix aceti* and *Ascaris suum,* and catalase is either soluble or associated with mitochondria, with no distinct microbody particle for these enzymes. Besides posing questions about the requirements for regulating two competing pathways (citric acid and glyoxylate cycles) within the same organelle, the presence of this unique form of compartmentation in organisms of the phylogenetically highest animal group in which the glyoxylate cycle has been observed is something of an enigma, especially when one considers that other nematodes contain glyoxylate cycle enzymes in particles that do separate from mitochondria on sucrose gradients.

FATTY ACID β-OXIDATION: Glyoxysomes were found among the lipid bodies of a germinating seed, and glyoxysomal enzymes reach maximum activities after four to five days of germination, at the time of rapid conversion of stored lipids into sucrose for seedling growth with 90% retention of the carbon (14). These glyoxysomes contain a complete β-oxidation system for converting fatty acids to acetyl CoA (79). In this case, the flavin-linked oxidase is a fatty acyl CoA oxidase that forms H_2O_2 and is linked to catalase. Likewise, microbodies are abundant among the lipid bodies during fungal spore germination (17). Castor bean glyoxysomes also contain a lipase and an ATP-requiring fatty acyl CoA synthetase. More recently, Lazarow and de Duve (80), Lazarow (81), and Osumi and Hashimoto (82) have reported that hepatic peroxisomes also have a fatty acid β-oxidation system that is different from the mitochondrial β-oxidation and that preferentially oxidizes long-chain fatty acyl CoA's (81, 84), whereas the mitochondrial system oxidizes short-chain as well as long-chain substrates. The hepatic peroxisomal enzymes for β-oxidation are being investigated currently. The fatty acyl CoA oxidase, as predicted by comparative biochemistry, is a flavin-linked H_2O_2-producing system (67, 79, 81, 82). The other steps of β-oxidation in the hepatic peroxisomes are apparently catalyzed by slightly different enzymes from those in the mitochondria. The distribution of fatty acid β-oxidation between peroxisomes and mitochondria has yet to be worked out and can certainly be expected to be a complex process based on many considerations, such as transport and mechanisms of enzyme regulation. According to maximum enzyme capacity,

the hepatic peroxisomal system is about one-fourth to one-third as active as the mitochondrial system in the rat (85), and the two pathways are about equal in activity in the mouse (10, 86). Speculatively, the mitochondrial β-oxidation may proceed via acetyl CoA to CO_2 and ATP, whereas the peroxisomal system, which only degrades the fatty acids to C_8 or C_6 acids, may provide acetyl CoA or acetylcarnitine for other synthetic processes in the cell. Such exciting developments certainly indicate that microbodies are essential organelles.

ALCOHOL AND ALKANE OXIDASES: When yeast is grown on methanol, the cells contain mainly gigantic microbodies containing a flavoprotein, methanol oxidase, and catalase (87, 88). As expected, growth on a sugar represses these microbodies. Growth on long-chain alkanes (oils) also induces the development of microbodies containing an oxidase to initiate alkane oxidase (89).

UREIDE METABOLISM: Enzymes for ureide metabolism, including xanthine oxidase, urate oxidase, allantoinase, and allantoicase have been found in microbodies of some tissues. The H_2O_2-producing oxidase, indicative of microbodies, is urate oxidase. Indeed urate oxidase (see Morphology) is a major peroxisomal enzyme from liver of rat and presumably all ureotelic animals. Scott et al. (90) originally reported that the whole ureide pathway was in avian hepatic peroxisomes, but the results have not been confirmed, There is now one report of the ureide pathway of metabolism in peroxisomes from fish liver (91). The end products of this pathway are CO_2, NH_3, and glyoxylate, and the formation of glyoxylate is also indicative of a microbody system (see next section on Glyoxylate). Xanthine oxidase in liver generally appears to be in the cytoplasm, and there are no publications, except for the one on avian hepatic peroxisomes, that locate it in the microbodies.

GLYOXYLATE METABOLISM AND AMINOTRANSFERASES: Many, if not all, reactions involving glyoxylate biosynthesis and metabolism are compartmentalized in microbodies. This is true for the glycolate pathway of photorespiration in plants, for the glyoxylate cycle, for glycolate oxidase in the liver, and for ureide metabolism. Likewise, the aminotransferases of microbodies are relatively specific for glyoxylate as the amino acceptor resulting in the formation of glycine. The equilibrium of the aminotransferases lies almost totally in the direction of glycine formation. In leaf peroxisomes, there are two different, active aminotransferases: a glutamate:glyoxylate, and a serine:glyoxylate enzyme (92). In hepatic peroxisomes, a leucine:glyoxylate aminotransferase was reported from rats (93), and an alanine:glyoxylate aminotransferase from humans (94). All aminotransferases in microbodies seem to be able to use alanine to some extent as an amino donor for glyoxylate.

Glyoxylate oxidation by glycolate oxidase in the peroxisomes to oxalate is considered a side reaction of C_2 metabolism. Possibly the glyoxylate may be peroxidatively oxidized to CO_2 and formate. Much earlier work in the metabolism of added glyoxylate by animals ought to be reevaluated with the knowledge that it is an excellent substrate for the abundant lactate dehydrogenase and for other aminotransferases of the cytoplasm, but it is doubtful whether glyoxylate is ever formed in vivo outside of the metabolic pathways of the microbodies.

METABOLIC GENERALIZATIONS: From the current biochemical knowledge about microbodies, a few tentative generalizations can be made. (*a*) The metabolic pathways within the microbody are catabolic; however, the end products of microbody pathways, e.g., acetylcarnitine or a C_4 acid, may be used for synthetic processes elsewhere in the cell. (*b*) Some of the enzymes of microbodies from different tissues vary greatly, depending on physiological parameters and tissue function. That is, there is no constancy for the same metabolic pathway in microbodies from different tissues as there is in mitochondrial composition. (*c*) The metabolic pathways so far described in microbodies represent one of dual or alternative pathways for metabolism of a substrate. Examples are the two β-oxidation systems, the citric acid cycle versus the glyoxylate cycle, and postulated peroxidation of alcohol versus alcohol dehydrogenase. Some of these differences may be essential because of different use of the end product of the metabolic pathway in the microbody. (*d*) The unique microbody enzymes are those associated with O_2 uptake, namely the flavin oxidases and catalase. Other enzymes are either slightly different or isoenzymic with their counterparts elsewhere in the cell; however, they are never identical. Examples are isoenzymes of NAD:malate dehydrogenase in leaf peroxisomes versus leaf mitochondria and different fatty acyl CoA enoylhydratases in hepatic peroxisomes and mitochondria.

Transport, Shuttles, Latency, and Outer Membrane

Active membrane transport by translocases, as in the mitochondria and chloroplasts, has not been discovered so far in microbodies. One concept is that microbody substrates and products may diffuse passively across the single bounding membrane. Microbodies may be simply compartments for clustering enzymes for specialized metabolic pathways associated only with catabolism. De Duve (5, 6) was of the opinion that most enzymatic assays showed no latency or initial lag during assay with isolated microbodies, as if the rates were not limited by membrane diffusion. This is a difficult problem to assess because fully intact particles may never be isolated or they may be broken at the beginning of the subsequent enzyme assays. Assays in dense sucrose are diffusion-limited, whereas dilution and handling before assay damages the organelle. In the author's (N. E. Tolbert) laboratory, assays are performed after dilution into a buffered detergent (Triton X-100) to dissolve the particle for maximum enzyme activity.

It seems likely that shuttles of organic and amino acids may exist between the inside of the microbody and the cytoplasm, but still the actual membrane transport could be by passive diffusion. Several such microbody shuttles have been investigated. A malate, oxaloacetate, aspartate shuttle has been proposed for plant microbodies similar to the one for mitochondria (12). Both leaf peroxisomes and seed glyoxysomes contain large amounts of an isoenzyme of NAD:malate dehydrogenase that is unique to microbodies. Except for catalase, this is the most active enzyme yet measured in leaf peroxisomes (about 50 μmol $\cdot$ $min^{-1} \cdot mg^{-1}$ peroxisomal protein). Such a shuttle seems to be the only way to oxidize NADH produced in the microbody, because no NADH oxidase has been detected in them. Hepatic peroxisomes do not contain malate dehydrogenase, but they do contain a small part of the total NAD:glycerol-P dehydrogenase (95). The existence of a hepatic peroxisomal glycerol-P shuttle has not yet been fully elaborated.

Fatty acids are transported across the mitochondrial membranes as acylcarnitine derivatives, for which there are three enzymes — a carnitine acetyltransferase, a carnitine octanoyltransferase, and a carnitine palmitoyltransferase. Rat liver peroxisomes, but not renal peroxisomes, contain considerable amounts of the short-chain and medium-chain transferases (96, 97). The function of these two peroxisomal transferases is not

clear, particularly since they are located in the peroxisomal matrix and not in the membrane. Palmitoyl CoA oxidation by isolated hepatic peroxisomes is not stimulated by carnitine. A working hypothesis at the present is that during hepatic peroxisomal β-oxidation, acetyl CoA and octanoyl CoA are formed and converted to the carnitine derivatives to conserve intraorganellar CoA, and that the carnitine derivatives then diffuse out of the peroxisomes to the cytoplasm and the mitochondria.

The composition of the single tripartite peroxisomal membrane is similar to that of the ER, from which it is presumed to arise by budding. The peroxisomal membrane contains phosphatidylcholine, phosphatidylethanolamine, plus phosphatidylinositol (98, 99) and some antimycin A-insensitive cytochrome b_5 reductase. At first, it appeared that an isolated peroxisomal fraction would be an easy way to obtain a pure membrane fraction. This has proven not to be the case, because even the best peroxisomal preparations when examined by electron microscopy, contain a significant amount of ER relative to the small amount of peroxisomal outer membrane. Several groups of investigators (15, 100) have broken microbodies gently by osmotic shock and observed occluded matrix protein in the ghosts, which may be related to preferential retention of certain enzymatic activities with the ruptured particles. Such enzymes are readily solubilized from this "membrane fraction" by $MgCl_2$ solutions. Perhaps differential rates of loss of matrix enzymes from microbodies may account for some puzzling results, including the very rapid loss of catalase. Muto and Beevers (101) have clearly shown, however, that a monoglyceride lipase remains with isolated glyoxysomal membrane from germinating castor bean seeds.

Biogenesis and Development

ASSOCIATION WITH ER: Almost from the time of the initial descriptions of the microbody, the question of their mode of origin has occupied the attention of numerous investigators. In earlier studies, microbodies were thought to originate from the Golgi apparatus, multivesicular bodies, mitochondria, or dense bodies (lysosomes) (see reference 7), but none of these modes of origin has been substantiated. Evidence for nucleic acid in microbodies has so far been negative. For over a decade, however, evidence for the origin of animal and plant microbodies from the ER has been accumulating (Fig. 4). Early electron microscopists noted "projections" of smooth ER associated with the microbody membrane (reviewed by Hruban and Rechcigl in reference 7). In 1964, Novikoff and Shin (102) studied rat hepatocytes after partial hepatectomy and demonstrated numerous continuities, which often appeared in ringlike or hooklike configurations, between the delimiting membranes of microbodies and those of the ER. They suggested that the moderately opaque material characteristic of microbodies is deposited within dilated portions of smooth ER that further enlarge to form microbodies. These then separate from the ER or remain attached via narrow, tortuous connections. So frequently were these continuities observed that the authors raised the possibility that microbodies are always attached to smooth ER in vivo. Such connections might, however, be broken during homogenization. In fetal mouse liver, Essner (59) described continuities between the ER and anucleoid microbodies, which form late in gestation, and suggested that the microbody constituents accumulated in regions of the rough ER. After dilating and losing ribosomes, a nucleoid formed within the bulge. A similar sequence was described by Tsukada et al. (60) in fetal rat liver. These authors also found that the specific activity of urate oxidase in isolated nucleoid fractions from liver was significantly lower at earlier stages of postnatal growth (when anucleoid forms are found) than at later stages. Following a report by Hess et al. (103) that the number of microbodies increased after administration of the hypolipidemic drug clofibrate, Svoboda and Azarnoff (104) described irregular dilatations of the ER that contained material similar in appearance to the microbody matrix. The relationship between microbodies and ER was documented in a series of papers by Svoboda, Reddy, and co-workers (see Reddy [105] for a brief review).

In 1970, Rigatuso et al. (106), who studied hepatocytes of clofibrate-fed male rats, described small, catalase-positive, smooth-walled "vesicles" that contained a microbody-like matrix and were adjacent to or in continuity with microbodies. They suggested that microbodies proliferated by a process of fragmentation or budding from preexisting microbodies. In addition, cytochemically demonstrable catalase activity was detected in association with both the membrane surface and portions of attached ribosomes of the ER that were adjacent to the microbody matrix. In subsequent studies (107) these observations were expanded, and the findings were interpreted to indicate that catalase was synthesized on regions of the rough ER adjacent to the microbody membrane and, after accumulating in the surrounding cytosol, was transferred directly into the microbody without having entered the cisternae of the ER. In 1972, Novikoff et al. (108) demonstrated that DAB reaction product (oxidized DAB) could diffuse from sites where it had been deposited originally, especially if such sites contained heavy accumulations, and that DAB was adsorbed to other sites, such as ribosomes, which normally lack oxidative activity. They considered it likely that staining of ribosomes was caused by diffusion and subsequent adsorption of oxidized DAB, rather than of the enzyme itself. Ribosomal staining after intravenous injection of horseradish peroxidase was also described by Bock (109). He and also Seligman et al. (110) argued in favor of diffusion of the hemoprotein, rather than of oxidized DAB. In 1974, Fahimi (111) demonstrated that when glutaraldehyde-fixed tissue was stored in buffer for prolonged periods of time, catalase diffused from microbodies and adsorbed to adjacent ribosomes, as well as to mitochondria and ER. It is now evident that ribosomal staining, whether caused by diffusion of oxidized DAB or of catalase, represents an artifact and cannot be offered as evidence for the synthesis of the enzyme on ribosomes.

A general concept for microbody biogenesis is that the enzymes, after synthesis on the ribosomes, move to the budding or developing microbody. Whether protein synthesis is by bound or free ribosomes and whether the transport is through the ER channel or through the cytoplasm to the microbodies by some selective mechanism, continue to be investigated. Recent data from Goldman and Blobel (112) indicated that catalase and uricase were immunoprecipitated from translation products directed by the free polysomes, but not from products of membrane-bound polysomes. Their data are taken to mean that those two peroxisomal enzymes could neither be synthesized by ribosomes bound to the ER nor selected during cotranslational segregation by the microsomal membranes. Rather, a mechanism of "posttranslational" transfer from the cytoplasm during passage through the peroxisomal membrane would have to be involved. Further insight into this process

will greatly contribute to the understanding of the physiological phenomena described in the next section.

DEVELOPMENT: A large body of physiological literature during the past decade has focused on the development of microbodies. One generality is that microbodies form during tissue development and differentiation. The near absence of microbody enzyme activities in young tissue or in poorly differentiated hepatomas, such as the Morris 3683, needs to be further explored. Another generality has already been discussed in the section on Metabolism, namely that microbody development and enzyme content may be substrate-dependent or induced.

During seed germination, after RNA and ER proliferation, development of glyoxysomes begins on day 2, and glyoxysomal activity reaches maximum at days 4 and 5 during lipid degradation. When the seedling continues to develop in the dark, the glyoxysomal enzymes and particles disappear on days 6 to 8. During glyoxysomal development there is *de novo* synthesis of its proteins. The most recent review concerning the rise and decline of the glyoxysomal population is by Beevers (15). Another example from plants is leaf peroxisomes, which greatly increase in activity in the light during greening of a new etiolated leaf. This development is largely independent of chloroplast development, but both processes seem to be controlled in part by phytochrome (15). Fatty seeds have cotyledons that develop in the light into cotyledonary leaves. Consequently, in the light, there will be an increase in leaf peroxisomal activity whereas glyoxysomal enzymes decrease. These two biochemical classes of microbodies are morphologically similar and cannot be separated by centrifugation. Thus, these changes during development can only be observed by enzyme assays of the microbody fraction. There has been much speculation as to whether the existence of two different biochemical populations of microbodies were a result of *de novo* formation of leaf peroxisomes or whether the glyoxysomes were being changed into peroxisomes by an alteration of their enzymatic composition. Because no evidence could be found for two populations of microbodies during *de novo* labeling of the newly formed enzymes, the possibility had to be considered that the microbody enzymes all change from glyoxysomal to peroxisomal types in the whole population of microbodies.

Postnatal development of hepatic peroxisomes has been described in terms of enzymatic composition (60, 113), but not in a molecular or physiological context. Peroxisomes and all peroxisomal enzymes in the rat liver are very low or not detectable at birth. The peroxisomal enzymes for β-oxidation and catalase increase rapidly during the first two postnatal weeks, whereas urate oxidase increases more slowly over a four-week period.

The development and turnover of hepatic peroxisomal catalase has been extensively investigated by several laboratories. De Duve's group (114, 115) has observed that, during catalase biogenesis, an apomonomer is formed in the extraperoxisomal pool with a half-life of about 14 minutes. The addition of heme and tetramerization of catalase takes place in the peroxisomes. The intracellular site for the apomonomer pool is unknown, but it is in the soluble fraction after cell breakage. It has been proposed that liver peroxisomes are all interconnected through the ER channels so that any alteration of the enzymes would be distributed to all the peroxisomes. Thus one would not detect differences between young or old, and large or small, hepatic peroxisomes. Such a scheme would be comparable also to the conversion of glyoxysomes into peroxisomes in greening cotyledons. Masters and Holmes (10) and Rechcigl and Heston (116) have examined the isoenzymic forms of catalase and phenotypic changes induced by structural gene mutations. Masters and Holmes (10) in genetic studies have also utilized polymorphisms of α-hydroxyacid oxidase in different strains of mice. They conclude that the peroxisomal enzyme loci in mice are not closely localized on a linkage group and are not associated in the form of one operon regulating peroxisomal enzyme synthesis. This conclusion may be consistent with the multiple metabolic pathways and rates of development of microbodies in different tissues.

Because metabolic activity of microbodies seems to be very readily modified, like some ER oxidase systems, investigators of microbodies have utilized chemical treatments as a way to elucidate microbody function. In the earlier work of Reddy, Svoboda, and Azarnoff (104, 105), it was discovered that feeding certain hypolipidemic agents, particularly clofibrate (CPIB or ethyl-*p*-chlorophenoxyisobutyrate), increased the number of peroxisomes or microperoxisomes in liver of male (but not female) rats by two- or threefold within two weeks. Upon withdrawal of clofibrate, the number of peroxisomes returns to normal. The mechanism of action is totally unknown, but this compound has been repeatedly used when measuring peroxisomal activity. More recently, several other analogues of clofibrate have been reported to be equal or more potent stimulators of peroxisomal number, although they may be toxic to man (Fig. 6). In general, the effect of all hypolipidemic agents on peroxisomes ought to be examined. Hashimoto's group (82) has used the plasticizer di(2-ethylhexyl)phthalate, which is mildly hypolipidemic, to induce hepatic peroxisomes in their studies of peroxisomal β-oxidation. Clofibrate, or the plasticizer in relatively large dosage, increases the total activity of the enzymes associated with β-oxidation in the hepatic peroxisomes of both male and female rats about tenfold. This includes the enzymes for the β-oxidation reactions, the two carnitine acyltransferases, and glycerol-P dehydrogenase. It also increases the mitochondrial β-oxidation activity two- or threefold. Clofibrate does not greatly alter the total catalase and urate oxidase activities. Thus, clofibrate may be increasing hepatic peroxisomal fatty acid oxidation capability without affecting other peroxisomal activities and, in the case of the female rat, without causing peroxisomal proliferation in number. The effects of other drugs and hormones on peroxisomal activity have not yet been reported.

Function and Metabolic Diseases

It is presumed that microbodies must have important functions because of their ubiquitous distribution in eukaryotic cells. The exact relationship of microbody respiration to the rest of the cell is beginning to appear to be a very complex interrelationship with the whole cell. Although several metabolic diseases could be cited as related to peroxisomal metabolism, no significant disease-oriented research has yet developed based on peroxisomes. Studies by Goldfischer's group (86, 117) reported the absence of peroxisomes in hepatocytes and renal proximal tubule cells associated with the fatal cerebrohepatorenal syndrome of infants.

In his first reviews, de Duve (5, 6) speculated that microbodies might be a primitive respiratory organelle. We now know they are present in most aerobic eukaryotic cells and are absent in prokaryotes. Leaf peroxisomes are in all photosynthetic cells of higher plants, whereas most unicellular algae do not have

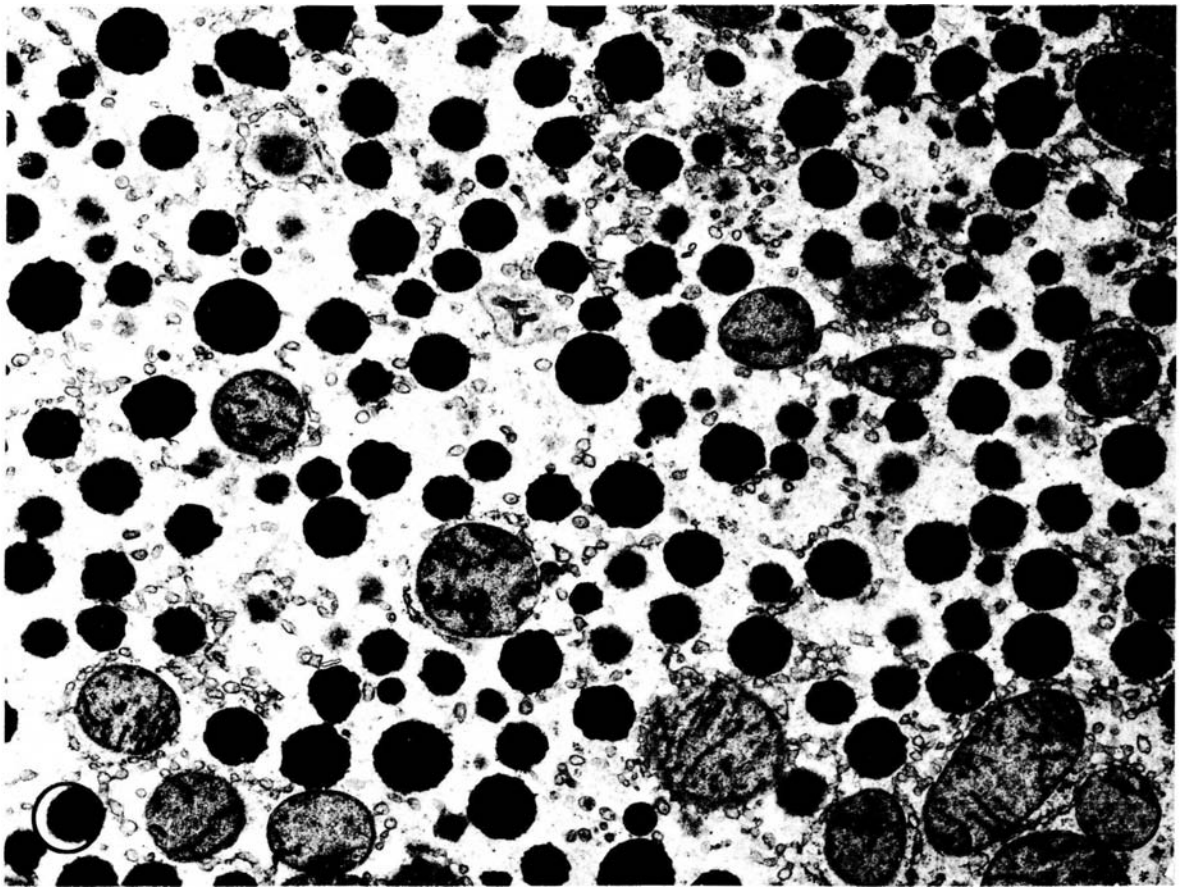


FIGURE 6 Increased numbers of peroxisomes are shown in hepatocyte from male rat fed the hypolipidemic drug, gemfibrozil. Electron micrograph courtesy of Dr. J. K. Reddy. × 21,000.

peroxisomes or nearly as much catalase, but oxidize glycolate by a dehydrogenase not linked to O_2 uptake and H_2O_2 production (16). Such data do not support de Duve's original hypothesis. Based on the fact that peroxisomes seemed to be present in gluconeogenic tissue (liver, kidney, and leaves), de Duve also called for consideration of the role of microbodies in gluconeogenesis. This hypothesis has been supported in plants, where seed glyoxysomes are active in the conversion of the stored lipid reserves into sucrose. During photorespiration in leaves, the carbon flow in the peroxisomes eventually leads to resynthesis of the sugars. There has been little support for this hypothesis in animals.

In leaf peroxisomes, photorespiration accompanying photosynthesis in high O_2 has suggested that microbodies are part of the protective processes against excess oxygen (11, 12). In this protection they may also participate with superoxide dismutase, which converts O_2^- to H_2O_2 that must, in turn, be removed in the microbody. In photorespiration, the primary benefit seems to be for the chloroplast electron transport system, which is kept from becoming overoxidized by continuous photorespiration involving carbon metabolism in the peroxisomes. None of these data indicate that microbody respiration removes a significant amount of the large excess of O_2, but rather that it functions in some manner to balance the cellular redox potential by respiration.

All microbody metabolic pathways to date are degradative and include one irreversible, flavin-oxidase, H_2O_2-producing step. These pathways often duplicate or complement another reversible metabolic sequence linked by pyridine nucleotide dehydrogenases for energy transfer. In the microbody pathway, the metabolic function seems to direct or to push carbon flow into a given sequence at the expense of the energy lost at the initial flavin oxidase step. Other reactions of a given metabolic pathway in the microbody are generally not energy-wasting. Thus, it can be calculated that the oxidation of a fatty acid initiated by the β-oxidation system in hepatic peroxisomes will cost only about 6% to 7% of the energy that would have been conserved as ATP if all the fatty acid β-oxidation had occurred in the mitochondria. Certainly the microbody flavin oxidase/catalase system has no energy-conserving mechanisms comparable to the coupling in the mitochondria to electron flow and ATP generation. But the initial concept that microbody respiration was simply wasteful or a way to lose energy is too simplistic to be substantiated by recent data. It is true that if leaf peroxisomal photorespiration is blocked, the microbodies photosynthesize and grow twice as fast. The increase in hepatic peroxisomal β-oxidation by clofibrate is also consistent with this hypothesis, but treated animals seldom lose much weight (97). In a series of studies of genetically obese mice (118), the total hepatic peroxisomal β-oxidation activity was actually twice that in the liver of the lean litter mates. In this case, the obese syndrome could not be blamed on lowered peroxisomal activity, but is probably a result of excess fatty acid synthesis.

Because the microbody enzymes vary immensely, depending on the tissue, it is not yet possible to discuss microbody function in tissues of the body other than liver. Renal peroxisomes do not contain a β-oxidation system, and the α-hydroxyacid oxidase is different from that in the liver. Microbodies (microperoxisomes) from the intestinal mucosa, myocardium, skeletal muscle, retinal pigment epithelium, and other tissues where

they abound will all have to be isolated and each group characterized enzymatically.

Little is known about metabolic imbalance and diseases that can be attributed to microbodies. Alterations in hepatic peroxisomal β-oxidation may be related to the many disease aspects of fat and lipid metabolism. But to date no direct peroxisomal alteration has been related to a metabolic disorder of this nature, except that the obese mouse is not deficient in hepatic peroxisomes (118). Some changes in hepatic peroxisomal activity have been recorded during starvation or diet change. Feeding long-chain fatty acids induces more long-chain substrate specificity for β-oxidation by the hepatic peroxisomes (84). From numerous reviews (119) on ethanol metabolism, there are at least two pathways: a cytosolic alcohol dehydrogenase with a low K_m and a peroxidative pathway with H_2O_2 and catalase; however, the latter pathway has not been investigated as a distinct peroxisomal system. Similarly, hepatic peroxisomal metabolism involves glycolate oxidation to oxalate or conversion to glycine, so that oxaluria and glycinuria might be examined as peroxisomal diseases.

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VI. Avenues to Information

Electron Microscopy and Ultramicrotomy

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> "The old adage 'to travel hopefully is better than to arrive' scarcely applies to microscopy, because in a sense science never arrives, the road going on and on from any temporary stopping place. Moreover, hope alone is not enough except sometimes to counteract despair. We need tenacity and the will to cling on against odds to reach something we believe to be important."
>
> Irene Manton, 1978 (1)

It is commonplace to recognize that the depth to which we explore ourselves and our environment is frequently determined by the development of new instruments and the creation of techniques for their use. Usually in such developments, one can recognize a time when fragments of information, acquired previously, are ready to be used to satisfy a concept or an urge to do or see what had not seemed possible before. So it was in the early 1930s that a group of physicists and engineers, mostly in Berlin, found conditions right to create an electron microscope. Max Knoll and his students, Ernst Ruska and Bodo von Borries, had available the knowledge that electrons would move through a vacuum and be deflected in their motion so as to be focused by solenoid lenses. It was mostly engineering skills that were needed to generate a microscope. Interest in the applications of the first microscopes naturally followed and, by the late 1930s, electron micrographs of recognizable value to biologists were being published.

Any consideration of the pace at which biological electron microscopy then developed must take into account worldwide events and constraints related to the outbreak and prosecution of World War II. Hitler invaded Poland on September 1, 1939. The very first Siemens & Halske AG electron microscope made for commercial sale was delivered in that year, only a few months before the War actually started (2). However, since the political alignments of Axis and Allied countries had been substantially established the year before, after the annexation of Czechoslovakia's Sudetenland, it is not suprising that not a single Siemens & Halske microscope was ever delivered to countries outside of Axis control. None was in Allied hands until one microscope was captured intact and brought to England after the 1944 Normandy invasion.

In the United States, the Radio Corporation of American was not ready to deliver its first commercially available electron microscopes, the RCA–EMB models, until 1941. The "Lend-Lease" program had started earlier in the year and developed quickly as a massive aid program to England. About one-third of all RCA-EMB instruments ever made were shipped to England.

About 40 Siemens & Halske microscopes seem to have been manufactured during the war years, and about 60 RCA/EMBs (2). On both warring sides, most of the applications of the new instruments were directed towards the research needs of the military. We are aware of only one RCA-EMB instrument in the United States that was available primarily for biological research: a microscope at the Massachusetts Institute of Technology in the laboratory of Cecil E. Hall, who was already recognized as an important pioneer in the original development of prototype instruments at the University of Toronto. A second EMB instrument, installed in the research laboratories of Interchemical Corp. in New York, was made available in 1943 to Albert Claude and Keith Porter at the Rockefeller Institute (3). Steward Mudd, a bacteriologist at the University of Pennsylvania, also had substantial access to RCA instruments at the RCA manufacturing plant in Camden, New Jersey, where Thomas F. Anderson worked on biological problems as an "RCA Fellow."

The situation in Germany for biologists during the early war years seems not to have been more advantageous. In April of 1940, Siemens & Halske AG sponsored an interdisciplinary meeting where the most prominent users of the new electron microscopes, as well as scientists who had had access to prototype instruments, reviewed the achievements of nonmilitary applications. R. Siebeck discussed medical applications in Germany (4), and there were other reviews of botanical and bacteriological applications. Biological work necessarily had been limited mainly to examining silhouettes of bacteria, viruses, fibrous proteins, and other organic objects that could be studied *in toto*. By this time, both bacterial flagellae and the repeating periodicity of collagen had been seen. Metallic mesh, suitable for grids, was available from photoengravers, and collodion support films had been introduced by Helmut Ruska in 1939 (5).

In the United States, Canada, and England, biological discoveries closely paralleled the German efforts at first. E. F. Burton and W. H. Kohl (6) reviewed the applications of electron microscopy that took place during the war years on this side of the Atlantic. Work with bacteria and viruses was soon underway, particularly at the RCA Laboratories, under the inspiration of L. Marton, Mudd, Anderson and W. M.

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Stanley. The M.I.T. Laboratory group, consisting at first of Hall, Francis O. Schmitt and Marie Jakus, particularly pursued studies of proteins that could be isolated by tissue fragmentation (collagen and muscle). But the war years also saw two important technical developments: first, in 1942, the replication of surface topography with Formvar films by Schaefer and Harker (7), and second, in 1944, shadowing by Williams and Wyckhoff (8). Then, in 1945, Porter et al. (9) demonstrated that whole-cultured tissue cells could be brought to the stage of the EM and examined profitably. While bathed in balanced salt solution (Tyrode's, pH 7.4), the cells in these early studies were fixed with vapors of OsO_4. The advantages of this reagent for the faithful preservation of cultured cells had been described in 1927 by Strangeways and Canti (10). For several years after 1945, and until thin-sectioning became a reality, these thinly spread cultured cells provided the only access to knowledge of cell fine structure, and contributed to observations on the endoplasmic reticulum and the intracellular presence of viruslike particles in cells from chicken tumors and mouse mammary tumors.

Quite apart from theoretical considerations, the limited availability in vitro of many kinds of tissue cells convinced biologically oriented electron microscopists of the need for ultrathin sectioning. In 1934, Marton (11) had examined osmium-fixed, 15-μm sections of plant material. Naturally he had little success, even at a magnification of only ×450. Later, von Ardenne (12) attempted to cut tapering wedges of tissue so that at least some parts of their areas would be adequately thin. Richards et al. (13) and Sjöstrand (14) continued this approach, but only the Richards' group had sufficient success to warrant publication of micrographs. A considerable historical hiatus in sectioning techniques followed, lasting until 1948, as the War and the recovery years took their toll. There was, however, one diversion during that interval into ultra-high-speed microtomy.

In 1943, O'Brien and McKinley (15) developed the hypothesis that, at high sectioning speeds, specimen inertia should restrict strain distribution so as to localize it very closely to the knife edge. They reasoned that there would be no time for plastic flow, and that thermal expansion would be negligible. They therefore designed a microtome with a steel knife supported just beyond the circumference of an 8-inch wheel. The wheel originally was driven at 12,500 rpm, and produced a cutting speed equivalent to 140 feet per second. The block was moved into this whirling blade at a rate calculated to deliver 0.1-μm sections. Their original article was not illustrated with micrographs of successful sections. However, according to a report by Gessler and Fullam (16), a year later, at the 1944 Annual Meeting of the Electron Microscopy Society of America, O'Brien and McKinley did show some micrographs of reasonably good sections, which inspired Fullam and Gessler to begin their own work with high-speed microtomy. (They also reported that by 1944, O'Brien and McKinley had almost doubled the original speed of their microtome to 22,500 rpm.) Subsequently, Fullam and Gessler (17) produced a microtome which operated at 57,000 rpm, delivering a cutting speed of 1100 feet per second. They used fragments of razor blades as knives, and sectioned a variety of plastics, as well as tissues. They demonstrated considerable success in cutting the plastics, and even some in sectioning tissues (3, 17). In view of this, it is curious that they did not explore the potential of embedding with plastics. Instead, they focused their attention on embedding media that would volatilize after sectioning was completed, and experimented with such substances as camphor, resorcinol, naphthalene, etc., and eutectic mixtures of these and related compounds, almost all with boiling points below 85°C, and some with boiling points as low as 32°C. They did try paraffin embedments, but recognized problems in its subsequent extraction from the sections. Their first article was illustrated with only one micrograph of a tissue section, that of liver fixed with osmium tetroxide. Fairly severe artifact was evident.

Ernest F. Fullam's venture into biological microscopy followed an earlier acquaintanceship with Claude and Porter, who then were developing an interest in the potential of electron microscopy for cytological research. Claude wanted especially to identify the cytoplasmic origin of microsomes. In 1945, Claude and Fullam published a joint paper (18), illustrating osmium-fixed liver that was sectioned at high speed (49,000 rpm). Their embedment was specifically characterized as a eutectic mixture of camphor and naphthalene with a melting point of 32.5°C. Dry sections, which literally flew from the knife, were collected on a strip of copper mesh that was coated with a Formvar film. Areas of promising sections were selected with a light microscope, and then suitably positioned grids were punched out of the mesh. They strove for sections 0.3 to 0.6 μm thick. The published micrographs showed substantial artifacts, i.e., many artificially-created holes. The authors at least partially recognized this, and took the position that the major problems limiting effective biological ultrathin microtomy related not so much to the sectioning itself as to a need for refined fixation and embedding.

Although the War ended in 1945, and RCA was ready to begin marketing their newly designed EMU series microscopes at the end of that year, few instruments were available to biologists until well into the 1950s. By today's standards, those machines were rudimentary: the first of the new RCA microscopes did not even have a biased gun. Objective apertures were not introduced until 1950. Although Hillier and Ramberg (19) had recognized the need and means for lens correction as early as 1947, stigmators were not added to production instruments until 1953; externally controllable compensation was not available for RCA microscopes until Canalco Co. of Bethesda, Maryland, marketed a kit in 1956; and Siemens did not introduce its well-equipped, postwar "Elmiskop" until 1954. Thus, it took about a decade after the end of the War for electron microscopes to evolve to include features we now regard as absolutely essential for biological work, such as stable performance, astigmatic lenses, and excellent contrast. Only at the midpoint of that decade did ultramicrotomy and its associated techniques also mature; images of sectioned material were produced that would still be regarded as acceptable. Earlier, there simply were too many disparate problems to permit rapid progress towards a total solution.

Dr. Claude's 1945 experience with Fullam convinced him that very high-speed microtomy was not going to be essential. He therefore started work with Joseph Blum (then Director of the instrument shop at The Rockefeller Institute for Medical Research) to develop a microtome that operated at a more modest speed. The prototype, described by Claude (20), incorporated some of the design features of the earlier Fullam-Gessler instrument, but was operated simply by hand-turning a flywheel. The arrangement of the pulley system undoubtedly produced speeds that now we would regard as excessive. However, in this instrument, the knife did not move past the fixed specimen, as in the earlier high-speed microtomes; instead, the specimen was mounted at the edge of a turning and advancing

disk, which advanced by small increments toward a fixed knife. This permitted the use of a trough in association with the knife so that sections could be collected as a ribbon on a fluid surface. Claude's published report of this microtome was not illustrated with micrographs and he re-emphasized that "the task ahead is to find better ways for the preparation and preservation of the specimen."

In January, 1948, in New York, Claude delivered a Harvey Lecture on "Studies on Cells," in which he summarized his ongoing efforts, including his work on thin-sectioning techniques to improve electron microscopy of cells. The lecture reached mainly an audience from that city and the manuscript, unfortunately, did not appear in print until 1950. Thus, Daniel Pease and Richard Baker (21), working at the University of Southern California, had no inkling of the work in progress at The Rockefeller Institute when they published their own account of some success with low-speed microtomy. L. H. Bretschneider (22) in Holland was also attempting to produce half-micron sections, apparently unaware of the developments at The Rockefeller.

Pease and Baker (21) were influenced in their efforts to obtain ultrathin sections for electron microscopy by a suggestion of Prof. F. Kiss from Hungary, who had been associated with Prof. St. Apathy. The latter, working at Cluj, Rumania, during the last years of the nineteenth century and the early years of the twentieth, contributed much to the development of conventional microscope techniques, including double embedments of paraffin and collodion. In a personal communication, Dr. Kiss indicated that it was almost commonplace for members of that school to section small, double-embedded blocks in the submicron range of thickness by using conventional microtomes at normal operating speeds. This encouraged Pease and Baker (21) to change rather simply the advance mechanism of a standard Spencer 820 microtome by a factor of ten so that the nominal increment of specimen advance was reduced to 0.1 μm. More important for success, however, were the realizations that an adequate embedment had to be much harder, and offer more support, than conventional paraffin, and that section size had to be reduced by at least an order of magnitude from that commonly employed for conventional sectioning. This led them, first, to infiltrate tiny tissue blocks with as much collodion as possible, and then, second, to add hard paraffin. Subsequently, Pease (23) hardened blocks still further by a triple-embedding procedure, which involved incorporating Damar resin between the nitrocellulose and wax infiltration steps. Also, the paraffin was hardened additionally with bayberry or carnauba wax.

At first, Pease and Baker (21) collected dry sections individually with a camel's hair brush, and so transferred them to grids. The sections then were flushed with xylol in order to remove only the paraffin component, thus leaving the nitrocellulose network in place to provide specimen support. (The partial extraction was deemed necessary for want of an effective stain to provide adequate contrast.) In retrospect, the residual collodion was inadequate to prevent fairly serious collapse of fine-structural detail. However, it seems fair to say that these results, when first published, finally demonstrated that adequately thin sectioning could be achieved with relatively simple instrumentation and with low cutting speeds. Thus, the work served as a stimulus for other laboratories, and within the next four years, a rash of modifications of old microtomes, as well as rather simply designed new microtomes, were announced. At the same time, Bretschneider (22) independently began efforts to achieve ultrathin sectioning without resorting to new instrumentation or high speeds. He realized that the unit of advance of the "Cambridge Rocking Microtome" might produce sections as thin as 0.6 μm or, with simple modifications, even thinner. The basic design of this fundamentally simple and mechanical instrument dates from 1885, and is attributed to H. Darwin (23). Later, the designers of the eminently successful Porter-Blum ultramicrotome unwittingly incorporated some of its design features in the mechanism whereby the specimen arm was suspended and advanced.

Bretschneider realized, as had Pease and Baker, that a principal problem with ultrathin sectioning lay in the softness of the conventional embedding media. He therefore used paraffin with a melting point of 65°C and operated his instrument at 10°C. The micrographs he published indicated successful sectioning in the submicron range. Unfortunately, he had not preserved his tissue with osmium tetroxide, but rather with the more conventional fixatives of the day, including Bouin, Champy, Carnoy, bichromate-formol, alcoholic sublimate, etc. Also, as a final step, he extracted the paraffin. Thus, although the specimens demonstrated some electron transparency, they were full of artifacts. At least two other European laboratories (Danon and Kellenberger [25] in Geneva, and Oberling, Gautier, and Bernhard [26] in Paris) also made serious efforts to use rocking microtomes for ultrathin sectioning, and had enough success to warrant publication. In 1952, Bretschneider (27) published a comprehensive review of ultramicrotomy, which included references in tabular form of what he thought to be the entire literature through 1951 on the results of ultrathin sectioning: the list included only 36 papers.

In the critical years immediately after 1948, other key developments permitted fairly rapid technological advances. The introduction in 1949 by Newman, Borysko, and Swerdlow (28, 29) of polybutylmethacrylate (and later, mixtures of butyl and methyl methacrylate) as an embedding medium served as a great stimulus, although the botanical material in their micrographs generally was not well preserved. Originally, these investigators advocated the extraction of the polymerized methacrylate by an organic solvent such as acetone, toluene, or amyl acetate. At the time, the latter step seemed necessary to provide adequate contrast in lieu of any effective "staining" procedure other than that provided by an initial fixation with osmium tetroxide.

Another important advance was the 1950 introduction of glass knives by Latta and Hartmann (30). These immediately replaced the use of steel knives, which had always posed serious and largely unresolved problems. Apparently, most investigators had been using disposable razor blades which were ground with such an acute angle as to be undesirably flexible. Heavy knives, made for conventional microtomy, had to be resharpened before every use, at least if exposed to a trough fluid that visibly discolored (oxidized) edges within a few minutes. Very little had been published about how heavy knives might be sharpened easily and reliably (but see Hillier [31] and Ekholm et al. [32]). Perhaps this was because few investigators believed they had achieved anything approaching perfection. At best, the inherent grain structure of steel presumably would always have limited true uniformity and standardization. Fernández-Morán's (33) introduction of diamond knives in 1952 ultimately became an interesting success story, but these have proved to be more of a convenience than a necessity.

In 1950, Gettner and Hillier (34) formally introduced the useful and important technique of spreading and collecting

sections on and from aqueous surfaces in troughs attached to knives, although Claude (20) had suggested this technique earlier. During this period also, various laboratories experimented with heavy metal stains but had only limited success. However, it became obvious that phosphotungstic acid was useful as a stain, especially after OsO_4 fixation, and without the necessity to extract methacrylate embedments. The acid gained widespread use in an alcoholic solution. The usefulness of phosphotungstic acid had been partially realized and exploited earlier, notably by the group at the Massachusetts Institute of Technology, in work with whole mounts of fibrous proteins, etc. (However, it was not until 1955 that Hall [35] recognized, and deliberately used, phosphotungstic acid as a negative stain.)

From these early attempts at microtomy it became apparent that "single-pass" microtomes were a necessity, in order to take advantage of methacrylate embedments, sectioning with glass knives, and the collection of sections on fluid surfaces. Otherwise, sections often were lost on the return stroke of the microtome, or the face of the block was damaged. This influenced all subsequent designs of instruments made specifically for ultramicrotomy.

Many individual efforts to develop microtomes specifically for ultramicrotomy were made in the early 1950s. These included modifications of conventional microtomes, and also some ingenious original designs to minimize or eliminate problems with the bearings and lubricating films of moving parts. Thus, flexible rods and leaf springs were sometimes incorporated into the design to permit movements without bearing surfaces. Substantial efforts were made to increase the mass and decrease the elasticity of the machines. Design features that were finally to appear in commercial microtomes included, in addition to mechanical advance mechanisms, thermal expansion systems that originally were introduced by Newman, Borysko, and Swerdlow (28, 29). The list of ultramicrotome designs that have been published, but never reached commercial development, is long. In his 1955 paper, Sitte (36) appended an extensive bibliography of the pertinent information available at that time, and in 1956 Gettner and Ornstein (37) wrote a splendid review. Porter (38), in 1964, and Sjöstrand (39), in 1967, published considerable detailed information on the design features of early microtomes, particularly of those that reached commercial production.

For the truly rapid expansion of the developing field of ultramicrotomy to occur, a suitable, *commercially available* microtome was an obvious necessity. This was realized in 1953 with the introduction of the Porter-Blum instrument with a mechanical advance, manufactured and eventually marketed by Ivan Sorvall, Inc. of Norwalk, Connecticut (40). This was followed, also in 1953, by the Sjöstrand (41) thermally advanced microtome, manufactured by L.K.B.-Producter AB, Stockholm. For a time the latter microtome dominated the European scene (but eventually was taken out of production), while the Porter-Blum instrument became widely used in the United States and elsewhere. The simplicity and the reliability of the MT-1 Porter-Blum microtome soon made this the instrument of choice, and it is still manufactured to this day, despite the competition of second- and third-generation microtomes that are fully automated. As might be expected, this microtome went through several model changes before the commercial design was established. The most interesting of these incorporated a horizontal steel bar, which was suspended in a gimbel at one end and held the specimen in a chuck at the other. It had no mechanical advance, but relied on thermal expansion with heat from a reading lamp to move the specimen toward the knife. In its simplicity, it is still a charming and reliable instrument.

During the winter of 1954, an extraordinary workshop on microtomy was held at the New York Academy of Sciences. Designers of microtomes from up and down the East Coast came to the meeting with their creations. Altogether, 10 or 12 different instruments were shown. Irene Manton, 25 years later, recalled the occasion as follows: "It was my privilege, soon after arrival in New York, to attend a meeting at the New York Academy of Sciences at which an array of devices for thin sectioning were displayed, some crude, others almost comically complex, but only the Porter-Blum behaved perfectly, cutting a clean ribbon of serial sections of the right thickness to order, from a methacrylate block (1)".

In 1955, H. Sitte designed a thermal-advance microtome, which then was manufactured and marketed by Reichert AG, Vienna; its derivative commercial models have enjoyed a continuing success. Four years later, A. F. Huxley (42) introduced a mechanical-advance microtome, which was first produced by the Cambridge Instrument Company, and a cosmetically improved and motor-driven version continues to be built and sold by L.K.B. In addition to these microtomes of early design that reached commercial production (most of which are still being manufactured), inevitably others were introduced, only to disappear without leaving an important heritage.[1]

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[1] We know that the following microtomes for ultrathin sectioning were advertised as being in commercial production. Historically first, in the late 1940s, was the Fullam and Gessler very-high-speed microtome, advertised with the suggestion that the investigator could protect his investment by an easy conversion to an ultracentrifuge. The American Optical Co. of Buffalo then marketed a version of the adaptor for their "Spenser 820" rotary microtome that had been developed by Pease and Baker. After L. H. Bretschneider's use of the "Cambridge Rocking Microtome," the device was advertised specifically as an instrument suitable for the electron microscopists. "Minot" microtomes, redesigned according to plans by B. B. Geren and D. McCulloch, were sold for some time by the International Equipment Co. of Boston.

Other microtomes of substantially new design then began to appear on the market. A. J. Hodge, H. E. Huxley, and D. Spiro produced prototype instruments that were intended for manufacture by the Scientific Equipment Corp., Waltham, Mass. For a number of years, Ernst Leitz, of Wetzlar, Germany, marketed a succession of models based upon a design of H. Fernández-Morán, and its subsequent improvements. Philips, Inc., Eindhoven, produced microtomes designed by H. B. Haanstra. J. L. Farrant and S. E. Powell developed a microtome sold through Schuco Scientific Co., New York. B. von Borries, J. Huppertz, and H. Gansler introduced a microtome manufactured by Sartorius-Werke of Göttingen. D. Damon, at the Weizman Institute of Science, Rehovoth, Israel, marketed a commercial microtome through the Y.E.D.A.-Research and Development Co. associated with the Institute. M. E. Gettner made an effort to sell a microtome of his design through the Process and Instruments Co., Brooklyn, N.Y. Georg Jacob KG, Leipzig, offered a microtome patterned after an instrument first built by W. Niklowitz.

In addition to these designs of investigative scientists, the engineering staff of LKB-Produckter, Stockholm, anonymously developed a succession of substantially different designs. The Sorvall Division of DuPont Instruments Co., Newtown, Conn., is now beginning to do likewise, and, at least twice, the Japan Electron Optics Laboratory Co. of Tokyo has marketed ultramicrotomes without design credits. In addition, Jose Delville, Saint Germain-en-Laye, France, has recently introduced a new instrument. It is possible that still other microtomes of which we are unaware may have appeared in the market-place. It is evident,

As one reviews the published micrographs of the early years of ultrathin sectioning, it is apparent, in retrospect, that poor fixation—often bordering on the utterly inadequate—was a major source of difficulty. There were almost no guidelines except perhaps for that of Heidenhain; in a well-known essay in 1911 on "Plasma und Zelle," he had emphasized that osmium tetroxide was the only known fixative that preserved delicate tissues such as nerve axons without "enormous shrinkage" (43). Also, the faithfulness of osmium tetroxide had been dramatically demonstrated in 1927 by Strangeways and Canti (10) in their studies of cultured cells by dark-field light microscopy. It was this display that led Porter to use OsO_4 in the fixation of cultured cells in 1945. Certainly these considerations also influenced Pease and Baker's (21) original choice of this fixative. Subsequent uses of osmium tetroxide were influenced by the quality of those early preparations. Nonetheless, how to use it to best advantage in the fixation of tissues was not immediately evident. Its poor penetration through tissue was already notorious and it did not perfuse well. At first overly large tissue samples were used, which then were immersed in unbuffered solutions. The difficulties with this fixative were recognized and, in 1952, when Palade (44) first reported and demonstrated the value of pH control, the work was heralded as a landmark by all investigators in the field. In retrospect, no doubt the "Palade Pickle" worked as well as it did partly because he refined tissue-mincing to produce truly small blocks while the tissue was immersed in the fixative. In dissolving osmium tetroxide in Veranol buffer solution, Palade made no attempt to employ a physiologically compatible vehicle, for he did not think it was important. Most subsequent investigators have also ignored physiological compatibility, even when selecting other buffers. By contrast, in the mid-1950s, Rhodin (45), Zetterqvist (46), and Sjöstrand (47) advocated the use of a balanced salt solution with only minor buffering properties as the fixative vehicle, and also succeeded in fixing tissue remarkably well for the time. Unfortunately, since then this approach has been used only sporadically.

In the years 1952–54, the various U. S. National Institutes of Health came to recognize that the essential tools finally were available to utilize ultrathin sectioning techniques effectively to explore cellular structure and function. The NIH became generous in establishing new laboratories and in supporting existing ones. Electron-microscope installations proliferated. Many talented young investigators changed their research direction. In January 1954, a new journal, the first designed specifically to accommodate the expanding information relating to cellular fine structure, was launched under the aegis of The Rockefeller Institute. This was the *Journal of Biophysical and Biochemical Cytology* (JBBC), later to become the *Journal of Cell Biology* (JCB). In January of 1956, Keith Porter organized a "Conference on Tissue Fine Structure," which had the financial support of the Morphology and Genetics Study Section of the National Institutes of Health. This meeting produced an extraordinary volume, published in 1956 as a supplement to Volume 2 of the JBBC. The conference presented a good overview of what had been accomplished in the short period of time since satisfactory microtomes had become commercially available, osmium-tetroxide fixation had become reasonably well understood, and methacrylate embedding had become routine. One-hundred and nine investigators participated, including many from abroad, and 75 papers were presented. Despite the high quality of many of the micrographs presented, two papers spoke of impending problems. Borysko (48) had come to recognize "polymerization damage" that somewhat capriciously, but seriously, could change cellular fine structure. In addition, Morgan, Moore, and Rose (49) showed convincing evidence that the sublimation of methacrylate in the electron beam (previously recognized), could result in severe cytological artifacts, including damage to both cytomembranes and protein particulates. However, the full extent of the limitations of methacrylate embedments was not—and could not have been—fully appreciated until comparative evaluation was possible, after the development of cross-linked plastics as embedding media. Before that development, various palliative measures were devised to minimize tissue damage in methacrylate. These included the partial polymerization of methacrylate mixtures before initating the embedment (Borysko and Sapranauskas [50]), the use of more exotic catalysts than the original benzoyl peroxide (azodiisobutyronitrile, Shipkey and Dalton [51]); and the inclusion of traces of substances that could possibly serve as nucleation centers (uranyl nitrate, Ward [52]). In addition, to prevent, or at least to minimize, sublimation artifacts, Watson (53) proposed sandwiching methacrylate sections between two supporting films.

It was, however, the work with epoxy resins, begun by Maaløe and Birch-Anderson (54), that finally disclosed the full limitations of methacrylate embedding. It became apparent that these cross-linking resins do not liquify or decompose in the electron beam as does polymethacrylate, and that potentially destructive surface-tension forces could be avoided entirely through their use. Originally, Maaløe and Birch-Anderson used an unspecified "highly viscous epoxy compound," with diethylene triamine as the "hardening" agent. Soon after, Glauert et al. (55) introduced Araldite M. At about the same time, Kellenberger et al. (56) started to explore cross-linking polyester resins as embedding media, which led Ryter and Kellenberger (57) to settle on Vestopal W as their final choice. All of this original work was concerned with improving the preservation of bacteria, and it might have had a more immediate impact if more complex cellular morphology had been presented. A particular difficulty arose with Araldite M, for the American-made product turned out to be different from the English one, and there were problems with its infiltration into tissue. This also delayed its general acceptance as an embedment, and it really was not until Luft (58) introduced Epon 812 in 1961 as the resin of choice that electron microscopists worldwide had an easily obtainable and reasonably reliable cross-linking embedment.

Investigators faced another problem when they started to use epoxy resins, that of inadequate specimen contrast. A principal difficulty was that cured epoxies are themselves very dense substances, and there is no sublimation of material during electron bombardment to enhance a contrast differential. Furthermore, cured epoxy resins are quite hydrophobic, so that the aqueous, heavy-metal stains that had been used successfully with polymethacrylate did not always penetrate well and did not produce adequate contrast. Fortunately, Watson (59, 60) introduced an alkaline lead stain in 1958 that proved to be highly effective with epoxies, and, with its variants, is still by far the most valuable general-purpose stain.

however, that very few ultramicrotomes have had long competitive existences, and even these, with time, have undergone extensive modifications. The "improvements" have added to automation and to costs, but not necessarily to the ultimate quality of the sections they have produced.

Actually, Watson did all of his original work with methacrylate embedments. It seems to have been simply fortuitious that the alkaline-lead stain worked so well with the epoxy embedments. These embedments and an effective staining technique were the next-to-last step toward reaching the goal that we now recognize as "standard operating procedure."

The final step was to be fixation. In spite of the cytological detail that obviously could be preserved with osmium tetroxide, it was suspect for a number of reasons. Its chemical reactivity, particularly in relation to proteins, was poorly understood, even though Porter and Kallman (61) and Bahr (62) had reported on numerous model experiments. These had made it clear, however, that by no means all cytoplasmic macromolecules were rendered sufficiently insoluble to withstand leaching in subsequent processing steps. Furthermore, it was generally recognized that OsO_4 destroyed essentially all enzymatic activity, so that cytochemical reactions could not be demonstrated after its use (Sabatini et al. [63]). Thus, protein configurations were recognized as being severely damaged. Also, and quite unfortunately, there was no other fixative known in the 1950s that could be used for comparison with OsO_4 to help evaluate the quality of its ultrastructural preservation. Thus, formaldehyde had proved to be completely inadequate in methacrylate embedments, and although acrolein, as introduced by Luft (64), was recognized as an improvement, its noxious toxic properties discouraged its widespread use and delayed experimentation.

The "discovery" of glutaraldehyde as the primary fixative of choice by Sabatini, and Bensch, and Barrnett (63) immediately demonstrated consistently good and uniform tissue preparation, particularly of proteins. Cytological structures not generally seen before, such as cytoplasmic microtubules, now were routinely observed. Many tissues could be readily perfused because glutaraldehyde does not contract vascular smooth muscle as did OsO_4. A first approximation of the protein chemistry involved in glutaraldehyde fixation appeared to be relatively simple and understandable. To a considerable extent, proteins and other macromolecules often were so gently denatured that histochemical and immunological specificities were preserved. Fortunately, glutaraldehyde could be used with osmium tetroxide, as well as with uranyl salts, so that double fixation with the addition of heavy metals proved to be possible, and demonstrated particularly well-preserved cytomembrane systems. With this somewhat belated recognition of the great value of glutaraldehyde, ultramicrotomy finally could be said to have completed at least the first phase of its historical development.

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A Short History of Tissue Fractionation

CHRISTIAN DE DUVE and HENRI BEAUFAY

The period immediately following the end of World War II will be remembered in the history of cell biology as that of the great breakthrough. As with many scientific advances, new tools, not new thoughts, rendered possible the massive invasion of the subcellular world that was launched at that time. The availability of the electron microscope, and the development of procedures allowing the examination of biological samples with this instrument, made the cell accessible to detailed morphological exploration. At the same time, the introduction of chromatography, of radioisotopes, and of spectrophotometers and other refined physical instruments, enhanced enormously the power and incisiveness of biochemical analysis.

Revolutionary as these developments were, they would, nevertheless, not have sufficed in themselves for the construction of a true cell biology. What was needed, in addition, was a bridge between morphology and biochemistry, a junction between the essentially parallel avenues opened by these two disciplines, a hybrid methodology whereby the visible and the measurable could be correlated into a unified picture of the living cell. Tissue fractionation provided this indispensable link.

Like many important scientific advances, tissue fractionation owes its development to the vision and effort of a few innovators. It is, however, very far from being a monolithic construction, built according to carefully conceived plans. Rather it has arisen in a somewhat haphazard and untidy fashion from the meshing together, sometimes intended, sometimes accidental, of a remarkable diversity of interests. Physical chemists, engineers, biochemists, virologists, molecular biologists, have all had their input, in addition to the cytologists themselves. Their combined contributions have produced a vigorous and highly successful hybrid, which nevertheless still betrays traces of its mixed parentage in the uncertainties that surround some of its concepts and applications. Part of this haziness is due also to the nature of the object of tissue fractionation. Any reductionist approach to the complexity of the living cell must perforce proceed by successive approximation. This kind of progress is very apparent from the crude fractionations of yesteryear to the sophisticated dissections that are being carried out in many laboratories today. Clearly, the process of growth is still continuing, and we must be prepared to revise our rules and to refine our methods as our understanding of cellular organization becomes deeper and more detailed.

In writing this chapter, we have made no attempt to provide a comprehensive review of the whole field. This would have been quite impossible, in any case, within the space made available to us. On the assumption that our readers will be more familiar with the present than with the past, we have chosen to dwell mostly on the early history of tissue fractionation. It is our hope that we may in this way give the young generation of cell biologists a certain feel for the manner in which what is now textbook knowledge was actually uncovered, reminding them at the same time of certain fundamental principles which still remain true today, even though they were laid out some 30 years ago.

Broadly speaking, the history of tissue fractionation can be divided into three parts. First, there is the long period of germination, much of it subterranean and not recognized as such until later, ending with the publication of the two historical papers by Albert Claude in 1946 (1, 2). Then comes a period of explosive growth and luxurious blossoming, which more or less terminates with the untimely death of George Hogeboom in 1956. After that date, the pace quietens again, and we enter into an era of slow, progressive maturation, which is still continuing.

Early Preparative Attempts

Friedrich Miescher is generally given the credit for having first used a centrifuge to isolate a cell organelle. In 1869, he separated nuclei from human pus cells stripped of cytoplasm by peptic digestion (3). His techniques were crude, and his preparations would hardly pass muster today. But the outcome turned out to be a major one because it resulted in the discovery of a new class of biological constituents, which Miescher called "nucleins" as a reminder of their nuclear origin, and Altmann renamed "nucleic acids" when their acidic character was recognized.

Since these historical experiments, the isolation of pure nuclei has been pursued by numerous investigators using a variety of techniques. Unfortunately, such attempts were long hampered by the occurrence of an unknown degree of cytoplasmic contamination, which could be neither avoided nor properly assessed. Consequently, most of the analytical work performed on isolated nuclei until the early 1950s, especially that relating to enzyme activities, is practically valueless today. As a matter of fact, much remains to be known concerning the

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enzymic equipment of nuclei, and opinions are still divided on the extent to which soluble proteins may be lost by diffusion through the nuclear envelope in the course of the isolation of these organelles (4, 5).

This difficulty was recognized at an early stage by Martin Behrens, who devoted a long series of painstaking investigations to the isolation of nuclei from powdered lyophilized quick-frozen tissues fractionated in nonaqueous media (6). Although his procedure was later adopted in the laboratory of Alfred Mirsky (7), it never gained wide acceptance. The damage inflicted on cell constituents by freezing and by exposure to organic solvents is generally considered prohibitive. As a means of keeping ions and small molecules trapped inside cell organelles during isolation, quick-freezing and fractionation in nonaqueous media will, however, continue to serve a limited, but indispensable use (4, 5). The work of Behrens also deserves to be recalled for another reason. He was the first to separate cell components by equilibration in a density gradient, a technique which, transposed to aqueous media, became a major fractionation tool 20 years later.

Other landmarks in the history of tissue fractionation are the attempts made in the laboratory of R. R. Bensley to separate and to analyze pure mitochondria (8), and the isolation of microsomes by Claude (9). It is interesting that the latter discovery concerned a cell component that could not be seen as such in the light microscope and was, therefore, not known to exist (except in those cells where it forms large basophilic masses, or ergastoplasm). Claude's aim was actually to purify the agent of the Rous sarcoma, and he discovered the microsomes in the course of a control run carried out on uninfected chick embryos.

An important prewar development, without which tissue fractionation would perforce have remained very crude and incomplete, was the construction of more powerful centrifuges. The great pioneer in this domain was the Swedish scientist, The Svedberg, who not only built the first high-speed centrifuges, but also worked out a detailed theory of the behavior of macromolecules subjected to a centrifugal field and applied it to the measurement of molecular weights. Reviewed in the masterful treatise by Svedberg and Pedersen (10), this work became an inspiring source of information to those who later attempted to move centrifugal fractionation from the empirical to a more scientific basis.

Svedberg's ultracentrifuge, however, was essentially an analytical instrument, specifically designed for the accurate recording of sedimentation boundaries. Its conversion for preparative use would have been impossible, for the simple reason that its rotor axis was horizontal. It rested on bearings that were lubricated and cooled by circulating cold oil and was driven by two oil turbines situated at each end of the shaft. Heat exchange from the rotor was facilitated by a stream of hydrogen. The sample cell had transparent windows, which allowed the continuous observation of the sedimentation boundary while the centrifuge was running.

The transition from this analytical instrument to modern preparative ultracentrifuges came in very indirect fashion through the efforts of Emile Henriot, a French physicist established in Belgium, who, independently of Svedberg, had been able to achieve very high rotational speeds by means of a bearingless top, driven and supported at the same time by compressed air (11). The uses of this machine remained, however, limited, until Jesse Beams and Edward Pickels, in the United States, conceived the idea of adapting it to drive a larger rotor suspended by a steel wire (12). This device had the additional advantage that it permitted the rotor to be maintained in a vacuum chamber, thereby reducing considerably the braking and heating effects of frictional forces.

This new centrifuge still served only an analytical purpose. What prompted its further transformation into a preparative instrument was a demand from investigators interested in the isolation of viruses. This demand brought Pickels from the University of Virginia to the laboratories of the International Health Division of The Rockefeller Foundation, which at that time was located at The Rockefeller Institute for Medical Research in New York (13). There, with Johannes Bauer, Pickels built the first "high speed vacuum centrifuge suitable for the study of filterable viruses" (14), essentially a modification of the air-driven analytical centrifuge of Beams and Pickels (12). Later, he went on to develop a much more convenient electrically driven ultracentrifuge (15).

It is of some historical interest that these important technical developments seem to have had little influence on the efforts that were made, at the same time and literally under the same roof, to adapt centrifugation to quantitative tissue fractionation. Apparently, the Pickels machines were not used by Claude (1, 2, 9), nor by his immediate pupils (16) at The Rockefeller Institute, who relied for all their high-speed centrifugations on the so-called multispeed attachment of the International centrifuge. This was a rather primitive device, which allowed a small angle-head to be driven at up to 18,000 rpm by a belt-and-pulley connection with the main centrifuge shaft. It developed a maximum field of about 18,000 *g* at the bottom of the tubes. it also developed a considerable amount of heat, its temperature rising to 15°C in an ordinary cold room (1), and to 10°C in a −5°C cold room (17), during the 90-minute runs needed for the isolation (incomplete!) of the microsomal fraction.

Eventually, however, the two lines of research converged, paradoxically when Pickels left The Rockefeller Institute to found Specialized Instruments, Inc., in Belmont, California, whence came the famous Spinco analytical Model E, and preparative Model L, ultracentrifuges. Those who were engaged in cell fractionation at that time will never forget their sense of wonder and delight, almost of reverence, when they first unpacked the slick new instrument which completely changed their lives.

One last landmark deserves to be recalled, seemingly humble, but of immense practical importance, namely the construction by Carl Ten Broeck (18), of a hand-operated coaxial tissue homogenizer, which served as model for the motor-driven device later developed by Potter and Elvehjem (19), now in universal use. In fact, the credit for this discovery, as was pointed out by Potter himself (20), should really go to W. A. Hagan, who designed a very similar instrument in 1922, at a time when its utility was not yet perceived (21). Be this as it may, there is no doubt that popularization of the Potter-Elvehjem device served for the first time to inculcate in biochemists some measure of respect for biological structures. The standard procedure in those days was to release every cell constituent that could be made soluble by the whirling blades of a Waring blender, and then to throw the insoluble "residue" down the drain.

Development of Quantitative Analytical Fractionation: The Turning Point

Such, approximately, was the state of the art in the early 1940s. The idea that cell organelles could be separated for

analysis by centrifugal methods was obviously shared by a number of leading cytologists, and several attempts in this direction had already been carried out. Furthermore, improved instruments that could be used successfully for this purpose were becoming available. But something was still missing, namely the *analytical approach.* As exposed elsewhere (22), the development of this approach represents Claude's most important contribution. The point is a subtle one and deserves to be reemphasized.

Until then, investigators had followed mostly a preparative approach, trying to answer the question: WHAT IS IN . . . this or that subcellular entity that can be seen in the microscope? The question is, of course, a valid one, and the logical way of trying to answer it is by purification of the object of interest, followed by analysis. Unfortunately, centrifugal separation methods, especially those that were available 40 years ago, are really very crude. On the other hand, most subcellular organelles are, for a variety of reasons that need not be gone into here (see, for instance, references 22–24), very difficult to isolate in good yield and in a satisfactory state of purity and integrity. Compounding these difficulties was the fact that there is no way of ascertaining the purity of a subcellular preparation without the help of an electron microscope and/or of appropriate biochemical markers, both of which were unavailable at that time. Therefore, the preparative approach was bound to give equivocal results, as it did, for instance, in the case of nuclei.

What Claude did, when he enlisted the collaboration of Rollin Hotchkiss and of George Hogeboom in the investigation of the enzyme content of his subcellular fractions, was to ask a new kind of question, namely: WHERE IS . . . this or that enzyme that can be measured in the test tube? To answer such a question, a new approach had to be developed, in which "special emphasis was attached to the quantitative aspects of the results and efforts were made, whenever possible, to express the enzymatic activity exhibited by each fraction in terms of the total activity possessed by the unfractionated liver extract" (25). These were the guiding principles that led Claude to develop the *quantitative* fractionation procedure that is described in detail in the two seminal papers he published in 1946 in *The Journal of Experimental Medicine* (1, 2). The analytical approach, much more than the technical details—these were, in fact, never adopted by anyone—represents the essential contribution of these papers.

Indeed, less than two years after it was first described, Claude's method was largely reshaped by his young collaborators, George Hogeboom, Walter Schneider, and George Palade (16). For incomplete grinding by gently "rubbing the cells against each other" with a mortar and pestle, they substituted quantitative cell breakage by means of a Potter-Elvehjem homogenizer. This allowed separation of a nuclear fraction—discarded with unbroken cells and gross debris in Claude's original procedure—and of a more representative cytoplasmic fraction. Instead of "physiological" saline, they used hypertonic (0.88 M) sucrose as medium, which minimized agglutination of particles and also preserved the elongated shape of mitochondria. Thanks to this property, and with the additional help of Janus green staining, the workers were also able to identify Claude's "large granules" decisively as consisting mostly of mitochondria. The high viscosity and density of hypertonic sucrose did, however, greatly complicate and prolong the manipulations, and Schneider (26) soon published an alternative procedure using 0.25 M sucrose, which became widely adopted.

The years that followed publication of these historic papers witnessed a remarkable flourishing of enzyme distribution studies. The four-fraction scheme was rapidly adopted by the biochemical community, and soon became part of the regular enzymological arsenal. The findings obtained generated tremendous excitement. Meeting upon meeting was organized around the topic. Chapters and reviews were written almost every year. Heated discussions opposed the tenets of different doctrines, each side accusing the other of "artifact." In short, the biological world was in ferment, having realized—quite rightly—that history was being made.

In this crucial period, Hogeboom emerged as a major leader. In collaboration with Schneider, he investigated the distribution of numerous enzymes, keeping track at the same time of the results of others, which he surveyed comprehensively and critically in a number of reviews. Later, with Edward Kuff, he pioneered the use of density gradient centrifugation, and showed that this technique could be used both for the determination of molecular weights in crude solutions and for the analytical fractionation of subcellular particles. Especially, he championed relentlessly the proper adherence to Claude's analytical approach, insisting, sometimes in the face of severe opposition, on the "need of establishing balance sheets in which the summation of the activities of the tissue fractions is compared with that of the whole tissue" (27), and drawing attention time and again to the erroneous interpretations that were introduced into the literature as a result of poor techniques and sloppy thinking. His death in 1956, at the early age of 43, was an irreparable loss to cell biology. The second volume of *The Journal of Biophysical and Biochemical Cytology* is dedicated to his memory. His biography and list of publications, which appeared in that issue, are recommended reading to anyone interested in tissue fractionation (28).

By the time of Hogeboom's death, tissue fractionation had definitely come of age. It was used in many laboratories, and a certain consensus had emerged on methodology and nomenclature. As witnessed by a number of contemporary reviews (27, 29–33), fractionation of homogenates into "nuclei," "mitochondria," "microsomes," and "supernatant" was almost universally adopted. Even when workers departed from this scheme, they tended to discuss their results in the framework of an essentially dual division of cytoplasmic particles (34, 35). The danger of doing so was recognized by some investigators, for instance by Van Potter, who wrote in 1951: "The misuse of the words nuclei, mitochondria and, microsomes is . . . regrettable, although all of us are guilty in varying degrees" (36).

How treacherous this trap actually could be was revealed to us at an early stage in some experiments that were undertaken in 1949 by our group at the University of Louvain in Belgium. Attracted to tissue fractionation by the apparent insolubility of the hepatic enzyme glucose-6-phosphatase, we made two sets of observations that turned a passing interest in the methodology into a lasting commitment. The first one was that glucose-6-phosphatase comes down largely with the microsomal fraction and most likely is associated exclusively with microsomal particles, to which it is firmly attached (17). This finding, together with the similar conclusion reached by Hogeboom et al. (37) with respect to the exclusively mitochondrial localization of cytochrome oxidase, opened the way to the use of these enzymes as *markers* of their host organelles. Furthermore, the heterogeneity of their distribution, unless stringent precautions were taken, emphasized for us the important distinction between a cell constituent and the subcellular fraction in which it is concentrated.

Our second observation concerned acid phosphatase. First, we found that in freshly prepared homogenates this enzyme occurs largely in a latent, sedimentable form, which biochemical experiments helped to characterize as a saclike particle containing the enzyme within the confines of a substrate-impermeable membrane. Further, we found the particle-bound acid phosphatase to be distributed between the sedimentable fractions in a manner that differentiated it clearly both from the mitochondrial cytochrome oxidase and from the microsomal glucose-6-phosphatase. These findings prompted the development of a new fractionation scheme (38), which, with the help of the marker-enzyme hypothesis, eventually led to the recognition and characterization of lysosomes, and later of peroxisomes, as components of the mitochondrial fraction (22, 23, 39, 40).

Density Gradient Centrifugation and Other Refinements

An important development of the early 1950s was the introduction of density gradient centrifugation into the tissue fractionation arsenal. It is of some interest that here again, the first impetus came from virus research. The pioneer of the technique was a plant virologist, Myron K. Brakke, working at the Brooklyn Botanic Garden (41, 42). He developed zonal sedimentation through a sucrose gradient for the separation of potato yellow-dwarf virus, using a rotor that we are told in a footnote was designed on special order by Josef Blum (the head of The Rockefeller Institute instrument shop), and built by Ivan Sorvall, Inc.

The first application of Brakke's method to tissue fractionation was made by Heinz Holter and his co-workers at the Carlsberg Institute in Copenhagen (43). Thanks to the availability of the newly developed Spinco SW-39 swinging-bucket rotor, capable of withstanding fields as high as 165,000 *g* at the bottom of the tube, they were able to bring both mitochondria and microsomes close to density equilibrium in a sucrose-"Diodon" gradient. Diodon, a densely iodinated solute, was incorporated in the sucrose gradient in order to allow the required densities to be reached in media of lower viscosity and osmotic pressure than can be done with sucrose alone. This example was not followed, and most workers subsequently went on with Kuff and Schneider (44) to use sucrose alone for the construction of gradients. The principle of the Diodon method has, however, been revived recently by the introduction of Metrizamide (an iodinated carbohydrate derivative made by Nyegaard and Co. A.S., Oslo, Norway).

A different application of Brakke's technique was developed at the Argonne National Laboratory by John Thomson and collaborators (45–47), based, as in Brakke's own virus work, on incomplete zonal sedimentation through a stabilizing density gradient. By introducing some simplifying assumptions into Svedberg's equation, these workers were able to derive the approximate size of the sedimenting particles from the position reached in the gradient by their associated enzymes. The same type of information was obtained by Kuff et al. (48) in yet another form of density gradient centrifugation, first employed by Kahler and Lloyd (49) to analyze the sedimentation of polystyrene latex particles, by using a swinging tube rotor of their own design. In this method, the sample subjected to analysis, instead of being layered above the gradient, is actually incorporated homogeneously in it, and the appropriate size distribution of the sedimenting particles is derived from the position and shape of their sedimentation boundary, as in conventional analytical centrifugation. An advantage of this technique is that it is not subjected to artifacts due to "drop sedimentation," a well-known phenomenon whose importance was pointed out by Anderson (31).

Biochemists were quick to appreciate the power of density gradient centrifugation in preparative rotors, which extended vastly the domain of applicability of analytical ultracentrifugation. After the first attempts by Hogeboom and Kuff (50), and the refinement of their technique by Martin and Ames (51), preparative centrifugation became widely used for the determination of the molecular weight of enzymes and of other materials that could be measured by specific methods. Zonal sedimentation is now part of the standard arsenal of molecular biology, especially in the analysis of nucleic acids and ribosomal preparations. A particularly elegant application of isopycnic centrifugation is that developed by Meselson, Stahl, and Vinograd (52), who used self-generating gradients of cesium chloride to separate DNA species of different density. The demonstration of semiconservative replication by Meselson and Stahl (53) was an early triumph of this technique.

In many such applications, the fact that sedimentation occurs through a density gradient, rather than in a homogeneous medium, introduces relatively minor complications. This is not so, however, in the case of tissue fractionation. When membrane-bounded subcellular particles move through a gradient of sucrose or of some other low molecular-weight solute, their density increases progressively, owing to permeation of solute, or to osmotic loss of water, or to both phenomena occurring simultaneously. Because sedimentation is a function of the difference between the density of the particle and that of the medium, the continuous increase in density suffered by the sedimenting particles causes them to move far beyond the level in the gradient corresponding to their normal density, and to become arrested only at a final equilibrium position, which is a complex function of their physical characteristics. In addition to the injuries dehydration may inflict on the particles, this phenomenon forces them to traverse layers of such high viscosity that the centrifugation time may become prohibitively long. Increasing the centrifugal field, as has become possible with modern titanium rotors, has not proved as helpful in resolving the latter problem as might have been hoped, because of another complication. As first shown by Wattiaux et al. (54), and directly confirmed by Bronfman and Beaufay (55) with a high-pressure chamber, particles may undergo extensive damage when subjected to the high hydrostatic pressures that are generated in high-speed rotors.

Searching for a solute that would avoid some of these difficulties, Holter and Max Møller (56) first tried a number of commercially available macromolecules, and finally had one made to order, according to the following description: "sufficiently high molecular weight to insure low osmotic activity even in highly concentrated solutions, sphaerical molecules to insure low viscosity, high solubility in water and weak salt solution, chemical inertness and stability to autoclaving, lack of toxicity, absence of nitrogen (so as not to prevent Kjeldahl analysis)—and finally, as high specific gravity as possible." A substance meeting most of these specifications was synthesized in the Swedish firm Pharmacia by B. Ingelman and P. Flodin, and marketed under the name "Ficoll." It has one additional advantageous property, which was not included in the order: with a molecular weight of only 50,000, it does not have the high sedimentation coefficient that complicates the use of many other macromolecules.

We encountered the latter problem in our own use of glycogen as a solute (57). Nevertheless, we were able to investigate experimentally the effect of sucrose concentration on particle density by means of isopycnic centrifugation in isosmotic gradients of glycogen made up with sucrose solutions of different concentrations (57). The results obtained in these experiments, which also included a number of trials in gradients made in D_2O, to assess particle hydration, were fitted to a theoretical model (58, 59). From this analysis, estimates could be obtained for a number of typical properties of rat-liver mitochondria, lysosomes, and peroxisomes, including: density and hydration of the particle matrix, size of the sucrose-accessible space, and content in osmotically active solutes (39, 57, 59). Once the dependence of particle density on sucrose concentration was known, the distribution of particle size could be derived accurately from the analysis of sedimentation boundaries (60) or of sedimenting zones (61). Results obtained in this manner were found to be in good agreement with those of other approaches, including quantitative morphometry (62, 63).

As has been pointed out repeatedly elsewhere (22, 23, 39), such experiments are nothing but an extension of the original Svedberg analytical approach from the macromolecular to the submicroscopic, and even microscopic, range. They do, however, depend on the availability of some method for the quantitative evaluation of the particles in the subfractions separated from the gradient at the end of the experiment. Unless the particles have been first purified, which is rarely the case, one requires for this purpose a characteristic biochemical constituent, usually an enzyme, uniquely located in the particles under consideration (postulate of unique location). In addition, the relation of this constituent to the total particle mass or protein content must be known, or some such relation must be assumed. The simplest assumption is that the content of a particle in a specific biochemical marker is proportional to particle mass or protein content, which is another way of saying that all the particles in a given population have the same biochemical composition, irrespective of size (postulate of biochemical homogeneity). Within the limits of present experimental techniques, both postulates have been verified reasonably well for several markers, thus serving their purpose (64, 65). But this does not mean that the marker-enzyme hypothesis should be in any way generalized, or extended beyond its purely operational limits. It never was, and exceptions to its underlying postulates were recognized from the very beginning of its application (38). The fact that it does apply to a number of membrane-bound enzymes is, however, remarkable, and raises interesting problems of subcellular organization. These will be alluded to at the end of this chapter.

The increasing interest in density gradient centrifugation has also spurred a great deal of instrumentation research. The pioneer in this domain is undoubtedly Norman Anderson, who, as early as 1955, started experimenting with a variety of devices designed to avoid or to minimize the artifacts that are caused by wall effects, drop sedimentation, mechanical convections during acceleration and deceleration of the centrifuge, and other disturbing phenomena inevitably associated with the operation of swinging-bucket rotors (66–68). Thanks to the availability of the unique engineering facilities of the Oak Ridge National Laboratory, his efforts have culminated in the development of a number of entirely automatic zonal centrifuges, of which several are now produced commercially (69).

In our laboratory, the main efforts have been devoted to the construction of automatic rotors of a different design (70) that combine a high efficiency with a low hydrostatic pressure, and are particularly well suited, therefore, for isopycnic centrifugation. Much ingenuity also has been expended in the development of various accessories, such as gradient makers, sampling devices, rotor stabilizers, graded-speed accelerators and decelerators, centrifugal field integrators, etc. (see, for instance, references 31, 58, 69). Commercial firms have joined in this effort, and workers now have available a rich choice of instruments that represent the technological returns of tissue fractionation.

Another key advance of the late 1950s was the development and generalization of techniques for the examination of subcellular fractions in the electron microscope (48, 71). These were eventually complemented by the introduction of quantitative morphometric procedures (62, 63). It thus became possible to confront directly the biochemical and the morphological properties of subcellular fractions.

Thanks to these various developments, the progressive dissection and biochemical characterization of the different parts of rodent-liver cells, first initiated by Albert Claude, has now been extended to most morphologically recognizable cell components, and a large number of other tissues and cell types have been similarly investigated. As already mentioned, Hogeboom et al. (16) found that Claude's "large granules" consist mostly of mitochondria. Later, the occurrence of nonmitochondrial particles as "contaminants" of this fraction was recognized in our laboratory. This work led to the biochemical characterization of lysosomes (38) and of peroxisomes (57, 72), and allowed the morphological identification of the former as the "pericanalicular dense bodies" (73, 74), of the latter as the "microbodies" (74), described by electron microscopists. The mitochondria themselves were successfully subfractionated by Parsons et al. (75) and by the late Jack Greenawalt and his coworkers (76), allowing the separate biochemical characterization of the outer membrane, the inner membrane, the intermembrane space, and the matrix of these particles.

Other important landmarks were the discovery by Palade and Siekevitz (71, 77) that the microsome fraction is made up largely of vesicles derived from the endoplasmic reticulum, and the subsequent subfractionation of these vesicles into luminal content, membrane constituents, and ribosomes (71, 78–80). Further knowledge about cytomembranes was provided by the development of methods for the isolation of plasma membranes (81, 82) and of Golgi components (83–85), which allowed the identification of characteristic biochemical markers for these cell components, for instance 5′-nucleotidase for plasma membranes (86) and galactosyltransferase for Golgi elements (83, 87, 88). These findings, in turn, have made possible a detailed analytical subfractionation of the microsomal fraction, which has revealed that vesicles derived from the smooth and rough endoplasmic reticulum account for only about 75% of the total microsomal proteins (89). The remainder belongs to plasma membrane fragments, Golgi elements, and a third component, probably originating from torn-off mitochondrial outer membranes, with a minor contribution from larger cytoplasmic particles.

How these and the numerous other discoveries that have arisen from tissue fractionation have opened the way to the elucidation of such fundamental cellular processes as oxidative phosphorylation, intracellular digestion, protein synthesis, bulk transport in and out of cells, secretion, organelle biogenesis, and the underlying metabolic processes and their control, is illustrated by many of the other chapters in this book.

Future Prospects

Today, cell biology has come to display so many exciting vistas, and to offer so many sophisticated tools to explore them with, that primary biochemical mapping of cells by quantitative tissue fractionation has lost some of the glamour and appeal it had 25 years ago. This is understandable, but nonetheless regrettable, because the job is still far from finished. There are many enzymes whose intracellular location is unknown, or is believed to be known, but actually would bear reinvestigation. Easily forgotten in this connection is that much of our knowledge of enzyme localization is really derived from fairly primitive experiments that lack many of the controls that are available today. Fatty acid β-oxidation is a good example. Classically ascribed to mitochondria, this process has now been found to take place in both peroxisomes and mitochondria (90, 91).

Even the best-established data may deserve closer scrutiny. Tissue fractionation remains a relatively gross tool, whatever the sophistication of the techniques used, and it can reveal only the major site or sites of localization of enzymes and other specific biochemical constituents, not minor ones. For these, special methods are needed, usually based on some sort of cytochemical or immunochemical form of visualization. Other difficulties, not to mention artifacts, may, however, complicate the interpretation of the results of such approaches. Lack of quantitation is one; accurate identification of the "decorated" structure is another.

Modern work on cytomembranes serves to highlight these uncertainties. It is becoming increasingly clear that extensive fusion takes place continuously in living cells between membranes belonging to different domains, for instance in the course of secretion, of endocytic uptake, and of other cellular processes requiring bulk transport. Such fusion events should be detectable morphologically by the coincidence of markers of the two domains concerned on the same continuous piece of membrane. Not only should these markers be seen side by side, but they might also be expected to intermingle by lateral diffusion in the plane of the membrane. Considering, in addition, the intensity of this kind of traffic as revealed, for instance, by estimates of membrane recycling, a considerable degree of randomization of markers would appear quite likely, except, of course, that they would not be identified as markers anymore.

In the light of these considerations, the manner in which certain enzymes remain confined to a specific membranous domain is quite remarkable. Presumably some fuzziness occurs at fusion boundaries, but it has not proved important enough to be detectable by fractionation techniques. On the contrary, any improvement in resolution has served only to sharpen the distinction between different domains. This point is illustrated by the results of Amar-Costesec et al. (92), and, even more dramatically, by some recent findings by Ito and Palade (93). By using a specific immunoadsorption method, these workers were able to remove selectively from a highly purified Golgi fraction vesicles containing the endoplasmic reticulum marker glucose-6-phosphatase, from vesicles bearing the typical Golgi marker galactosyltransferase. Interestingly, glucose-6-phosphatase was accompanied by two other enzymes with which it is associated in the endoplasmic reticulum, NADH cytochrome *c* reductase and NADPH cytochrome *c* reductase. As a matter of fact, the antibody used in the separation was directed against the NADPH-specific reductase.

This interesting experiment thus demonstrates that even in a purified preparation, where fuzziness might have a better chance of being detectable, it did in fact not show up. Some vesicles in the preparation had the composition of endoplasmic reticulum, as indicated by three enzyme activities, and others that of Golgi membranes, as revealed by at least one enzyme. The authors insist that both vesicles were "reliably identified" as Golgi elements, on the basis of morphological criteria. This, however, raises a semantic problem. What name should one give to a structure that looks like A, but has the chemical composition of B?

This is but one of the many problems that are left for future generations to solve. The role tissue fractionation will play in this continuing adventure may tend to be overshadowed by the numerous new cytochemical tools that have become available. But it would be a great mistake to abandon this valuable approach, which alone can provide the kind of documented and quantitative biochemical information needed both for our knowledge of cellular organization and for the development of many of our cytochemical techniques.

An important lesson of the past 35 years lies in the magnitude of the effort that has gone into the development of better instruments, new materials, and more refined approaches, based on a deeper understanding of the physical parameters involved. This effort should be pursued, for it is as true today as it was 35 years ago that "further advance has to await the accident of technical progress" (94).

It is doubtful, however, that centrifugation alone will provide the new tools that are necessary, for we have come close to the point where distinct components of the cell cannot be separated because they do not differ sufficiently from one another in size and in density. Then, one has to find means of modifying selectively the properties of one component, or one must take advantage of other properties by which the components differ sufficiently to achieve their separation.

Examples of the first approach are the selective changes in density that can be produced in lysosomes by injection of such substances as Triton WR-1339 (95, 96) or dextran (97), which accumulate in these particles; in mitochondria by the nutritional rationing of an essential phospholipid building block (98), or by active uptake of calcium (99); in cholesterol-rich membranes by digitonin binding (92, 100); and in rough endoplasmic reticulum vesicles by removal of the ribosomes (92). These procedures, however, inherently alter in some way the biochemical characters of the components of interest. The other approach, to separate subcellular components by noncentrifugal methods, has probably not yet received the attention it deserves, and a more vigorous search should be made for alternative physical separation techniques compatible with preservation of the integrity of subcellular structures and functions. Examples that have already led to successful applications are phase partition (101), free-flow electrophoresis (102), differential filtration through membranes of graded pore size (103, 104), gel filtration (55), and immunoadsorption (93). With these and other tools still to be developed, tissue fractionation will continue for a long time to provide an essential bridge between morphology and biochemistry, as it has done so successfully in the past.

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VII. Index