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Cellular and Molecular Events in the Delayed-Onset Hypersensitivities

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List of Abbreviations

ABA	p-Azobenzenearsonyl
ABA-tyr	p-Azobenzenearsonate-N-acetyl-L-tyrosine
ADCC	Antibody-dependent, cellular cytolysis
B cell	Bone marrow-derived lymphocyte
C3	Third component of complement
CBH	Cutaneous basophil hypersensitivity
CFA	Complete Freund's adjuvant
CL	Cytotoxic lymphocyte
DH	Delayed hypersensitivity
DNP	2,4-Dinitrophenyl
DNP-D-GL	Dinitrophenylated mixed polypeptides of D-glutamic acid and D-lysine
DNP-Ficoll	ϵ-2,4-Dinitrophenyllysine-cyanuric-Ficoll
GA	Random copolymer of L-glutamic acid and L-alanine
GT	Random copolymer of L-glutamic acid and L-tyrosine
H	Histocompatibility
IFA	Incomplete Freund's adjuvant
Ig	Immunoglubulin
Ir	Immune response
KLH	Keyhole limpet hemocyanin
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte response
MIF	Migration inhibition factor
PPD	Tuberculin, purified protein derivative
RBC	Red blood cell
T cell	Thymus-derived lymphocyte
T-CL	Cytotoxic T lymphocyte
TF	Transfer factor
TNP	2,4,6-Trinitrophenyl

I. Introduction

Von *Pirquet* (1906) coined the term "allergy" to describe the state of altered reactivity that occurs after exposure of a human being or animal to an antigenic stimulus. This altered reactivity can manifest itself as either a hypersensitivity to antigen (whereby the allergic response leads to tissue distruction) or a hyposensitivity such as specific tolerance (reviewed by *Habicht* et al., 1973) or cell mediated suppression (reviewed by *Gershon,* 1974; *Asherson* and *Zembala,* 1975).

The initial interaction of antigen (allergen) and animal (host) causes the induction of the allergic state: an allergic host possessing actively allergized cells capable of recognizing the antigen and responding to it on subsequent, eliciting exposures. As might be guessed, contact with antigen under the usual clinical or experimental circumstances induces both hyper- and hyposensitivity; the observed allergy represents a balance between these two states (*Katz* et al., 1974).

Many factors affect the exact type of allergy induced after antigenic contact. Delayed-onset skin hypersensitivities have been most extensively studied in man, rabbit and guinea pig because of the ease with which these hypersensitivities can be induced and elicited in these hosts (*Turk,* 1975). It has proved much more difficult to demonstrate these skin hypersensitivities in rats and mice (*Crowle,* 1959, 1975; *Flax* and *Waksman,* 1962; *Asherson* and *Zembala,* 1970) by the techniques used for man and guinea pigs, but changes in methodology have permitted exploration of delayed-onset sensitivities in these species as well.

The nature of the antigen also plays a role in the type of allergy induced. For example, reactive small molecules such as many alkylating agents can, after topical application, induce both contact and anaphylactic hypersensitivity (*Landsteiner* and *Chase,* 1937; *Askenase,* 1977) or hyposensititivity (*Friedlaender* and *Baer,* 1972). Protein antigens such as bovine serum albumin which normally induce anaphylactic and other types of antibodies and little delayed response when injected in saline can be modified by conjugation with n-dodecanyl residues to induce marked delayed hypersensitivities and little antibody response (*Coon* and *Hunter,* 1973).

Finally, circumstances surrounding the administration of antigen can affect the type of allergy induced. Antigen administration in mycobacteria-containing adjuvants in an appropriate dosage schedule favors the induction of delayed hypersensitivities to proteins while administration in alum favors the production of antibodies (*Chase,* 1967, 1976). Immunization with water-in-oil adjuvants induces both hypersensitization and hyposensitization (*Katz* et al., 1974b), a situation that can be analyzed by immunizing cyclophosphamide-treated animals. The drug treatment blocks

induction of hyposensitivity and permits investigation of the hypersensitivity induced. Another circumstance affecting the type of allergy induced is the route of administration of antigen. Inhaled pollen is associated with anaphylactic allergy. Injected saline extracts of the same pollen are used to induce a state of hyposensitivity. A further example is that of certain reactive small molecules (such as dinitrochlorobenzene) where topical exposure induces anaphylactic and delayed skin reactivity but intravenous injection leads to hyposensitivity (*deWeck* and *Frey*, 1966).

The delayed-onset hypersensitivities are a group of allergic responses which have in common the fact that skin tests do not reach their maximum until 12–24 or more hours after their elicitation by antigen. In using the term "delayed-onset hypersensitivity" therefore, we describe a phenomenon without indicating any hypothesis as to mechanism, since recent observations have indicated that various etiologies can be associated with immune reactions with delayed-onset kinetics. Despite the differences in underlying mechanisms involved in the initiation of these allergies, the occurrence of observed tissue injury may require the presence of cell-types such as granulocytes, monocyte-macrophages and lymphocytes as a final common pathway. The assembly of these cells in suitable concentrations in the tissues from the circulation takes time and is presumably the reason for delayed-onset kinetics. Further discussion of the several delayed-onset hypersensitivities will be facilitated by a classification according to their mechanism of initiation.

II. Classification of Delayed-Onset Hypersensitivities by Initiating Mechanisms

Coombs and *Gell* (1975) proposed the classification of hypersensitivities into four reaction types depending on the underlying mechanisms of initiation (see Table 1). We present a modification of this scheme so as to accomodate recent observations of the initiating mechanisms of delayed-onset hypersensitivities. The major change we propose is to divide Type I reactions into two classes: 1) Type IA for anaphylactic responses caused by the rapid release of pharmacologic mediators from passively sensitized fixed tissue cells and 2) Type IB for delayed-onset responses mediated by circulating cells passively sensitized with antibody.

As can be seen from the classification, hypersensitivities of Types I, II or III can be passively transferred to non-immunized animals by serum from hypersensitive animals, whereas Type IV can be passively transferred only by living lymphocytes. While only Type IB reactions are initiated by lymphocytes acting without free antibody, all four types are associated with various cells in their ultimate development. For this reason, we prefer

Table 1. Classification of hypersensitivities by mechanism

Type I Reaction

Initiated by allergen or antigen reacting with circulating or fixed tissue cells passively sensitized by antibody produced elsewhere, leading to the release of active substances.

A. Anaphylactic
B. Delayed-onset
   1. Basophil-associated
   2. Macrophage-associated

Type II Reaction (Cytotoxic or Cell-Stimulating)

Initiated by antibody reacting with either (a) an antigenic component of a cell or tissue element or (b) an antigen or hapten which has become intimately associated with these; damage may then occur in the presence of complement or of certain kinds of mononuclear cells. Stimulation of secretory organs (e.g., thyroid) may also occur.

Type III Reaction (Damage by Antigen-Antibody Complexes)

Initiated when antigen reacts in the tissue spaces with potentially precipitating antibody, forming microprecipitates in and around the small vessels causing damage to cells secondarily, or being precipitated in and interfering with the function of membranes, or when antigen in excess reacts in the blood stream with potentially precipitating antibody to form soluble circulating complexes which are deposited in the blood-vessel walls or in the basement membrane and cause local inflammation or massive complement activation.

Type IV Reaction (Delayed)

Initiated by actively sensitized thymus-derived lymphocytes, responding specifically to allergen by the release of lymphokines and/or the development of specific cytotoxicity, without the participation of free antibody. Locally it is manifested by the infiltration of cells at the site where the antigen is injected.
(Modified from *Coombs* and *Gell*, 1975)

not to use the term "cell-mediated" in describing Type IV reactions, but reserve the term "delayed hypersensitivity" (DH) for them instead.

Antibody-dependent, cellular cytolysis (ADCC), a Type II delayed-onset hypersensitivity, has been reviewed (*Perlmann* et al., 1972b; *Hersey* et al., 1973) and will be only briefly discussed in this review. Target cells coated by small amounts of specific antibody can be destroyed by mononuclear cells having receptors for the F_c portion of immunoglobulin (Ig) (*Dennert* and *Lennox*, 1972; *Perlmann* et al., 1972a), so-called K or killer cells. These K cells are neither T cells (*van Boxel* et al., 1972), B cells with surface Ig (*Perlmann* et al., 1972b) nor phagocytic, glass-adherent cells (*Greenberg* et al., 1973a,b); the nature of the effector cell in ADCC remains in doubt. It has also been shown (*Evans* and *Alexander*, 1970) that macrophages can act as effector cells in killing antibody-coated targets.

The role of these reactions and of ADCC in vivo is problematic. However, the observation that cytotoxic monocyte-macrophages and cytotoxic non-T lymphocytes specific for graft antigens can be isolated from allografts undergoing rejection in man and mouse may indicate that this group of reactions is involved in the in vivo rejection process (*Strom* et al., 1975; *Roberts* and *Häyry*, 1977).

Pure Arthus reactions (Type III reactions), characterized by hyperemia, edema, hemorrhage and necrosis (*Cochrane* and *Janoff*, 1974) are usually fully established by 2–4 h after elicitation by antigen and are therefore not to be considered as delayed-onset hypersensitivities. Under conditions where the amount of antigen is in great excess relative to that of antibody, the maximal response can be delayed until 18–24 h after elicitation, although obvious reactions are present within a few hours. For example, in rabbits with circulating antibody to ovalbumin, Arthus reactions to 1 mg doses reached their maximum considerably later than those to 10 μg doses (*Butler* and *Lewis*, 1976). Other workers have commented on the protracted time course of intense Arthus reactions (*Oliveira* et al., 1970) and of Arthus components of mixed hypersensitivities (*Gell* and *Benacerraf*, 1961). A delay in the onset of the Arthus reaction might occur if the animal's antibody titer were rising rapidly at the time of antigen injection (*Coombs* and *Gell*, 1975). Finally, since the development of the Arthus depends on the presence of complement (*Ward* and *Cochrane*, 1965) and neutrophils (*Stetson*, 1951) as well as the initiating antigen-antibody complexes, insufficient amounts of these factors could lead to a delay in the course of the reaction.

These points having been mentioned, we now turn to a fuller discussion of delayed-onset hypersensitivities of reaction Types IB and IV which include both those initiated by antibody and those initiated by sensitized thymus-derived lymphocytes (T cells).

III. Antibody-Dependent, Basophil-Associated Hypersensitivity

A. Experimental Manifestations

Guinea pigs are immunized with hapten-protein conjugates (100 μg) in either complete Freund's adjuvant (CFA, containing mycobacteria) or incomplete Freund's adjuvant (IFA, without mycobacteria) and 8 days later skin-tested by applying a solution of the reactive molecule (e.g., 2-phenyl-4-ethoxymethylene oxazolone and its conjugate with keyhole limpet hemocyanin, KLH). The animals develop a delayed-onset 'contact' reaction, not present at 4–6 h after testing and maximal at 24 h (*Askenase*, 1973). These reactions are transferrable by serum from sensitized donors

and are associated with high concentrations of basophils in the skin reaction. Similar delayed-onset reactions to KLH after intradermal administration in saline are also transferrable by sera (*Askenase* et al., 1975b).

B. Specificity and Nature of Receptors

Benacerraf and *Gell* (1959a), *Gell* and *Benacerraf* (1961a) showed that when guinea pigs were immunized with conjugates of hapten with a foreign (immunogenic) carrier such as human serum albumin, the antibodies elicited were hapten-specific (i.e., combined with hapten bound to another carrier) while the delayed-onset responses were for the most part carrier-specific (i.e., could only be elicited by the immunizing conjugate or were directed at the unmodified protein). These workers did find that weak delayed reactions could be elicited by contact testing with a reactive small molecule related to the hapten or by intracutaneous testing with mg doses of conjugates containing high concentrations of hapten on another carrier and suggested that they might be anibody-associated (*Benacerraf* and *Gell*, 1959b; *Gell* and *Benacerraf*, 1961b).

Askenase found that delayed-onset ear swelling skin tests one week after immunization of guinea pigs with hapten-protein conjugates in CFA were hapten-specific and could be regularly elicited by contact testing or by hapten-conjugated proteins unrelated to the immunizing conjugate (*Askenase* et al., 1976).

This observation and the ability to transfer the reaction by intravenous injection of serum from sensitized animals pointed to an antibody-associated mechanism for the reaction. It was possible to isolate a hapten-specific 7S IgG_1 antibody from sensitized guinea pig serum which transferred delayed-onset hypersensitivity when injected intravenously; such sera also systemically transferred passive cutaneous anaphylaxis. [When transferred locally, however, these same sera transferred only a local passive cutaneous anaphylaxis reaction (*Askenase* et al., 1976).]

C. Histopathology

Basophils were found to localize to the upper apapillary dermis (*Askenase*, 1973) as reported for other reactions with a basophil component (*Dvorak* et al., 1970). They represented 25%–45% of the infiltrating cells in reactions transferred with serum. They were seen emigrating from blood vessels as early as 3–6 h after testing (*Askenase*, personal communication) at a time when no reaction was detectable at the ear skin site. Their numbers at the test site increased over the next 1–3 days, decreasing over the course

of a week despite the waning of the macroscopically observed skin reaction after 24–48 h (*Askenase*, personal communication; *Dvorak*, 1976). Few basophils showed anaphylactic type degranulation (*Dvorak* et al., 1970). *Askenase* et al. (1976) reported that eosinophils were regularly present in antibody-mediated basophil-associated hypersensitivity, while neutrophils occurred sporadically. Mononuclear cells, some resembling lymphocytes, were present in all reactions, both active and passive.

These skin reactions have also been found to have considerable deposits of fibrin (*Askenase* and *Dvorak*, personal communication).

D. Relationship to "Classic" Delayed Hypersensitivity

Since both this reaction and Type IV DH reactions in which basophols are often prominent show similar kinetics and histopathology, it would be difficult to distinguish the two on these bases alone. Antibody-mediated basophil-associated hypersensitivity may well account for some of the reported hapten-specificity of delayed-onset reactions to hapten-protein conjugates (discussed above) early after sensitization. *Dvorak* and co-workers (*Dvorak* et al., 1975) found that about a third of the basophils teased from delayed-onset reactions occurring "late" (6 weeks) after sensitization with sheep erythrocytes in IFA could specifically bind antigen, presumably by way of adsorbed antibody. The existence of Type IB delayed-onset hypersensitivities may also account for previous reports for transfer of delayed-onset hypersensitivity by serum (e.g., *Pick* and *Feldman*, 1968; *Dupuy* et al., 1969).

One week after immunization with hapten-protein conjugates, animals showed hapten-specific and carrier-specific delayed-onset skin reactions (*Askenase* et al., 1976). While the hapten-specific responses were basophil-rich whether the animals had been immunized with IFA or CFA, the carrier-specific (presumably Type IV reactions) were basophil-rich only in animals immunized with IFA. *Askenase* et al. (1976) speculate that a basophil-rich carrier-specific response in CFA immunized guinea pigs might be suppressed. (See Sect. V.B.6 for further discussion of the relation of antibody-mediated, basophil-associated hypersensitivity and "classic" DH.)

Antibody-mediated, basophil-associated, delayed-onset hypersensitivity resembles responses described by *Kay* (1970) and by *Baer* et al. (1976) and may also be related to the "late cutaneous allergic responses" seen in human subjects with Type IA hypersensitivity to pollen and mould spores (*Solley* et al., 1976; *Umemoto* et al., 1976).

IV. Macrophage Cytophilic Antibody and Antibody-Dependent, Macrophage-Associated Hypersensitivity

A. Experimental Manifestations

Mice sensitized by topical application of reactive chemicals can be shown to develop delayed-onset cutaneous hypersensitivities to the sensitizer within a week. Delayed-onset skin hypersensitivity can be transferred by peritoneal macrophages or serum from sensitized mice (*Asherson* and *Zembala,* 1970; *Zembala* and *Asherson,* 1970). Normal macrophages incubated with immune serum will also transfer a delayed-onset reaction. In contrast to hypersensitivity transferred by peritoneal lymphocytes, which reaches its maximum at 24 h after testing, macrophage associated hypersensitivity is maximal by 12 h.

B. Specificity and Nature of Receptors

Zembala and *Asherson* (1970) showed that macrophages incubated for short periods at 37°C lost the ability to transfer delayed-onset hypersensitivity while the culture fluid in which they were incubated would confer this on normal macrophages. The transfer of macrophage-associated hypersensitivity was blocked if the macrophages were incubated with rabbit antimouse Ig prior to injection. These reactions were specific for the immunizing chemical; the roles of carrier and hapten specific sensitivity could not be investigated since in contact sensitivity the exact (host) protein which serves as carrier is uncertain (*Landsteiner,* 1945). Under other circumstances the specificity of cytophilic antibody elicited by immunization with hapten-protein conjugates has been shown to be hapten-specific (*Cohen* et al., 1973b,c), the kind of specificity generally observed for antibodies to hapten-protein conjugates (*Gell* and *Benacerraf,* 1961a,b).

The concept of a circulating antibody cytophilic for macrophages was first introduced by *Boyden* and *Sorkin* (1960). This antibody was postulated to be involved in DH to sheep red cells since its presence correlated with the presence of DH in immunized guinea pigs (*Boyden,* 1963, 1964). It was then shown by *Berken* and *Benacerraf* (1966) that cytophilic antibody was present in guinea pigs lacking delayed-onset hypersensitivity, that it was of the IgG_2 class and that it bound reversibly to the macrophages through the F_c piece. Subsequent experiments by *Hulliger* et al. (1968) indicated that normal guinea pig macrophages, sensitized by serum from guinea pigs immunized to sheep red cells in CFA could transfer a local delayed-onset reaction if injected intracutaneously with sheep red cell extract. The reaction was maximal at 24 h. Injection of this serum and

sheep red cell antigen without macrophages produced delayed-onset reactions which reached their maxima between 4 and 12 h, a later time than the passive Arthus response seen if the sera came from donors immunized with IFA.

Askenase and *Hayden* (1974) showed that macrophages from contact sensitized mice had high levels of hapten-specific 7S IgG_{2a} and/or 7S IgM antibodies which were easily eluted by washing. High titers of these same antibodies were present in serum and could be shown to bind to a trypsin-sensitive receptor on macrophages from nonimmune mice. It was also found that primary contact sensitization and boosting led uniquely to high titers of these hapten-specific cytophilic antibodies. Despite the ease of removal of the cytophilic antibodies from the macrophage, it was concluded that they were associated with an aspect of delayed-onset hypersensitivity in mice.

C. Histopathology

Asherson (personal communication) states the histopathology of macrophage-associated delayed-onset reactions is indistinguishable from that of lymphocyte-mediated reactions with perivascular and dermal mononuclear cells being present in large numbers in the reaction.

D. Relationship to "Classic" Delayed Hypersensitivity

Antibody-mediated, macrophage-associated hypersensitivity may represent a mechanism whereby specifically sensitized macrophages can be mobilized to the tissue to deal with antigenic challenge more rapidly than can macrophages associated with "classic" DH (*Rabinovitch,* 1970). This hypothesis is consistent with the observation by *Wilkinson* (1976) that guinea pig peritoneal exudate macrophages from animals immunized to human serum albumin are specifically chemotactic for this antigen. It is also consistent with the observation that passively transferred macrophage-associated aspects of mouse contact sensitivity occur somewhat more rapidly than the corresponding lymphocyte-mediated reactions. The exact interaction between antibody-mediated, basophil-associated hypersensitivity, antibody-mediated, macrophage-associated hypersensitivity and "classic" DH in the clinically observed delayed-onset hypersensitivities is complex, because the tissue damage in Type IV DH is also associated with macrophages and basophils.

The presence of cytophilic antibody immune complexes on macrophages has been shown to enhance their uptake of antigen (*Steinman* and

Cohen, 1972). As will be discussed in Sect. V.B.5 the presentation of antigens by macrophages is essential for T lymphocyte responses to occur (*Rosenthal* and *Shevach,* 1976). It might be supposed that macrophages coated with cytophilic antibody would have a definite advantage over uncoated macrophages in presenting limiting concentrations of antigen to sensitized T cells and in fact, *Cohen* et al. (1973b, c) were able to show just such an effect. In addition to influencing the macrophage's ability to present antigen to other cells, cytophilic antibody has been shown to be able to mediate certain macrophage responses to antigen such as the macrophage disappearance reaction (*Nelson,* 1966) and the inhibition of macrophage migration (*Amos* et al., 1967). These macrophage responses can also be mediated by products of antigen-stimulated immune T cells (*David* and *David,* 1972) and correlate well with Type IV DH in vivo. Hence it may not always be possible to distinguish Type I and Type IV responses, apart from the transferability of the former by serum and its marked hapten-specificity, given a similarity in time course and histopathology.

V. "Classic" Delayed Hypersensitivity

A. Experimental Manifestations

The induction of DH depends on multiple factors including the dose and immunogenicity of the sensitizing antigen, the use of adjuvants and the dose of mycobacteria if water-in-oil adjuvants are used, the degree of conjugation of hapten-protein conjugates, the route of immunization. In addition, the degree of DH elicited depends on the time permitted to elapse between sensitization and testing and the dose of antigen used to elicit the response (*Gell* and *Benacerraf,* 1961a).

The addition of strongly immunogenic antigens to CFA or the topical application of highly reactive contact sensitizers usually induces a concurrent circulating antibody response, which made the study of DH reactions and their differentiation from Arthus reactions difficult. Techniques have been developed which permit the induction of a state of "pure" DH in guinea pigs at times when antibodies are not readily demonstrable by sensitive assays. The states of "pure" DH may be transient, lasting 2–10 days and waning with the appearance of circulating antibodies (*Salvin,* 1958; *Raffel* and *Newel,* 1958) or long-lasting, persisting for many weeks (*Uhr* et al., 1957; *Benacerraf* and *Gell,* 1959a; *Gell* and *Benacerraf,* 1961a).

The transient types of DH have been called "Jones-Mote sensitivity" in analogy to similar states seen during immunization of human beings with foreign proteins (*Jones* and *Mote,* 1934). This type of DH is induced

by intracutaneous injection of μg amounts of antigen-antibody complexes in antibody excess in IFA and elicited by skin testing 5–7 days later with large (0.5–1.0 mg) doses of antigen (*Raffel* and *Newel*, 1958). Alternatively, it can be induced by intracutaneous or intravenous injections of very small doses of antigen in saline (0.03–3 μg protein or 1×10^5 –2×10^5 erythrocytes) and elicited by μg doses of protein antigen (or 1×10^7 – 10×10^7 erythrocytes) 4–8 days later (*Salvin*, 1958; *Lagrange* et al., 1974; *Crowle* et al., 1977; *Askenase* et al., 1977).

Uhr et al. (1957) produced long-lasting states of "pure" DH in guinea pigs by intracutaneous injection of 2.5 μg antigen as antigen-antibody complexes (in antibody excess) in saline, IFA, or CFA. Delayed responses were elicited 5–12 days later using 0.03–3 μg antigen. Circulating antibodies were not detectable at this time. Long-lasting states of "pure" DH were also produced by *Benacerraf* and *Gell* (1959a), with 0.1–1 μg doses of trinitrophenylated proteins in CFA. DH to the immunizing conjugate or to unconjugated carrier protein alone was elicited by low doses of antigen 5–12 days after sensitization. By immunizing guinea pigs with highly conjugated carrier proteins of low immunogenicity it was possible to induce high levels of "pure" DH to the carrier protein which lasted for up to 17 weeks (*Benacerraf* and *Gell*, 1959a,b; *Gell* and *Benacerraf*, 1961a,b).

The cutaneous reaction of guinea pigs sensitized with soluble protein antigens was investigated extensively by *Nelson* and *Boyden* (1964). They distinguished two types of DH: 1) a tuberculin-type, characterized by delayed-onset induration and erythema (first appearance after 6 h) reaching its maximum at 24–30 h and still marked after 48–72 h, and 2) a Jones-Mote type, characterized by a delayed onset (after 6 h) of slight thickening and extensive erythema, maximal at 24 h and all but gone by 48 h. Tuberculin-type DH persisted after sensitization for 4 weeks or more, while Jones-Mote DH was transient and could only be demonstrated for the first 5–10 days after sensitization. The nature of Jones-Mote hypersensitivity has been a matter of controversy for a number of years, but the recent demonstration that it can be mediated by T lymphocytes (*Stadecker* and *Leskowitz*, 1976) suggests that it is, in some instances at least, a Type IV hypersensitivity. Furthermore, it was observed that guinea pigs pretreated with cyclophosphamide before immunizations of a nature usually leading to Jones-Mote DH, developed tuberculin-type DH reactions (*Turk* and *Parker*, 1973). This suggested that Jones-Mote reactions differ from tuberculin-type reactions as a result of modulation by a cyclophosphamide sensitive process such as antibody production or suppressor T cells (*Askenase* et al., 1975a). The distinction between Jones-Mote and tuberculin-type DH will be discussed further below.

Contact sensitivity to chemical agents in both man and guinea pig can be induced by the topical application of 1–20 mg cutaneous sensitizer;

sensitization is assisted by local irritation of the skin (*Turk,* 1975; *Chase,* 1976). Contact sensitization can also be induced by intracutaneous injections of 20–1000 μg sensitizer in various solvents such as alscohol-saline, corn oil, mineral oil, or in CFA. Contact reactions to topical application of the sensitizing agent can be first elicited within 5–8 days and sensitivity persists for many weeks.

DH to cell-associated histocompatibility (H) antigens can be induced by injections of histoincompatible cells or by grafts of histoincompatible tissues. *Brent* et al. (1958, 1962) showed that guinea pigs which had rejected a skin graft developed DH reactions to living cells or cell extracts of lymph node or spleen cells from the same donor. These DH reactions resembled tuberculin reactions grossly and microscopically. Similar observations were reported by *Merrill* et al. (1961).

B. Nature and Interactions of Cells Involved in Delayed Hypersensitivity

DH reactions in the tissues are associated with a complex mixture of cells including monocyte-macrophages, lymphocytes and granulocytes. While histologic studies of these reactions provided the initial understanding of the cells involved (*Gell* and *Hinde,* 1951), such studies are hampered by difficulties in distinguishing among the various mononuclear cell types on purely morphologic grounds (*McCluskey* and *Werdelin,* 1971), as well as by technical problems in preserving certain cells such as basophils in the tissues (reviewed by *Askenase,* 1977). A strictly morphologic approach has been supplemented by studies employing labeled cell populations in order to trace cell distribution in DH reactions of actively and passively sensitized animals (*Turk,* 1975), and by the use of animals of genetically defined backgrounds (*Paul* and *Benacerraf,* 1977). The inherent limitation of studies of histologic sections is that they provide a static picture of what is basically a dynamic process.

Much recent progress in elucidating the nature of the cells involved in DH and their interactions has come from the study of in vitro models of DH (*Bloom* and *Glade,* 1971). These models permit both a systematic manipulation of the various cells present and a biochemical analysis of the products released under more controlled conditions than are possible in the intact animal. These studies underlie the currently accepted mechanisms of DH in vivo (*Paul* and *Benacerraf,* 1977): small numbers of specifically sensitized thymus-derived (T) lymphocytes interact with antigen associated with macrophages (*Rosenthal* and *Shevach,* 1976) and release antigen-specific and nonspecific factors which activate or inactivate other lymphoid cells, affect macrophage and granulocyte movement and lead to the typical histologic picture of vascular-inflammatory DH. In addition,

certain cell-associated antigens such as H antigens, viruses and contact sensitizers induce the generation of cytolytic T lymphocytes (T-CL) which specifically recognize and lyse cells bearing these antigens and which mediate cytolytic DH.

1. Component Cells in Delayed Hypersensitivity Reactions

a) Monocytes-Macrophages. Mononuclear phagocytes are the major mononuclear cells at sites of nonspecific inflammatory reactions as well as at the sites of DH reactions at 24 h and beyond (*Gell* and *Hinde,* 1951; *Waksman,* 1960). These cells are derived from radiosensitive rapidly dividing precursor cells in the bone marrow (*Volkman* and *Gowans,* 1965a,b; *Lubaroff* and *Waksman,* 1967) which, after a brief maturation in the bone marrow are released into the circulation as monocytes (*Cohn,* 1968). Blood monocytes constantly leave the circulation and emigrate into the tissues at sites of inflammation where they differentiate into macrophages, large mononuclear phagocytes (10–15 μm) with numerous organelles including lysosomes (*Cohn,* 1968).

Macrophages have surface antigens and receptors which are important in their responses in DH (*Unanue,* 1972: *Oppenheim* and *Seeger,* 1976). In addition to macrophage-specific antigens detected by anti-macrophage sera, macrophages display antigens encoded by the major histocompatibility complex (MHC). These include classical H antigens and products of the I region, the region associated with immune responsiveness (*Shreffler* and *David,* 1975). Macrophages also carry receptors for the F_c region of IgG (*Rabinovitch,* 1968), the precise subclass of the IgG varying with species; for antibody-activated third component of complement (C3) (*Lay* and *Nussenzweig,* 1968); for products of activated lymphocytes (*David* and *David,* 1972; *Fox* et al., 1974); and for T and B (bone marrow-derived) lymphocyte antigens (*Rosenthal* and *Shevach,* 1973).

It is difficult ot distinghuish between macrophages and lymphocytes in histologic sections of DH on morphologic grounds alone (*Turk,* 1975). Macrophages have been identified in DH reactions on the basis of lysosomal enzyme activities (*Diengdoh* and *Turk,* 1965), ingested antigenic material (*Goldberg* et al., 1962), or the presence of surface membrane receptors for C3 (*Edelson* et al., 1973).

Macrophages can be isolated from mixtures of lymphoid cells because they adhere tightly to glass and plastic surfaces (*Mosier,* 1967) while lymphocytes do not. These isolated adherent cells stain with neutral red and are actively phagocytic (*Pierce* et al., 1974), have the morphologic characteristics of monocytes and macrophages and contain characteristic macrophage enzymes (*Gordon* and *Cohn,* 1973; *Li* et al., 1973; *Lipsky* and *Rosenthal,* 1975a). The function of these isolated macrophages in vitro is

affected by treatments that block reticuloendothelial function in vivo (*Unanue,* 1972) but is unaffected by ionizing radiation, suggesting that cell division is not required (*Osoba,* 1970).

b) Lymphocytes. Lymphocytes form a variable proportion of the infiltrating mononuclear cells in DH reactions, They range from less than 20% in tuberculin reactions in rats at 12–48 h (*Wiener* et al., 1965) to over 70% in guinea pig DH reactions to human gamma globulin at 48 h (*Turk* et al., 1966a) and in rat autoallergic adrenalitis reactions (*Werdelin* and *McCluskey,* 1971). Lymphocytes are small (5–7 μm), round, mononuclear cells with a prominent nucleus and little cytoplasm. The cytoplasm typically contains only scattered ribosomes and mitochondria, with few other organells (*Gowans* and *MacGregor,* 1965). It has become clear that morphologically defined lymphocytes represent a heterogeneous group of cells with respect to ontogeny, lifespan, and function (*Gowans* and *MacGregor,* 1965; *McCluskey* and *Werdelin,* 1971).

α) Lymphocyte Populations. All lymphocytes ultimately derive from precursor cells in the bone marrow, but some immature marrow lymphocytes migrate to the thymus where they develop into immunologically competent, thymus-dependent (T) lymphocytes while other immature marrow lymphocytes develop into immunologically competent thymus-independent (B) lymphocytes (*Roitt* et al., 1969). T lymphocytes released from the thymus localize in the thymus-dependent areas of the lymph nodes and spleen (*Parrott* et al., 1966) while B lymphocytes occur mainly in the thymus-independent areas of these organs (*Shevach* et al., 1973). The thymus-dependent areas of the lymph nodes are the paracortical regions: the comparable region of the spleen in the periarteriolar zone of the white pulp. T lymphocytes in the thymus-dependent areas of the lymph node transform into lymphoblasts (large pyroninophilic cells with numerous ribosomes but scanty or no endoplasmic reticular) under antigenic stimuli which lead to contact sensitivity or transplantation immunity (*Turk,* 1967). These cells have been shown to undergo further transformation back to small lymphocytes (*Gowans* et al., 1962) to yield both long-lived, recirculating and short-lived, nonrecirculating lymphocytes (*Tigelaar* and *Asofsky,* 1972). B lymphocytes transform into plasma cells under antigenic stimuli which lead to antibody production; plasma cells contain conspicuous and extensive endoplasmic reticulum, reflecting Ig synthesis for export.

β) Antigenic and Other Markers of Lymphocyte Populations. Because T and B lymphocytes are indistinguishable morphologically, lymphocyte populations have been identified on the basis of various markers such as

allo-antigens (differentiation antigens), heteroantigens, surface immunoglobulin and surface receptors other than receptors for antigen (*McConnell*, 1975). Some lymphocyte allo- or heteroantigens (i.e., antigens detectable by alloantisera or heteroantisera) are expressed on only T or B cells. Antisera raised against these antigens and made specific by absorption, can be assayed by cytotoxicity or immunofluorescence.

Thy-1, a surface glycoprotein present on mouse lymphocytes and brain (*Reif* and *Allan*, 1963, 1964; *Trowbridge* and *Mazaukas*, 1976) is an archtypal T lymphocyte marker: normally present on about 90% of thymocytes but only 50%–70% of lymph node lymphocytes and 30%–50% of splenic lymphocytes, with these percentages being reduced in thymectomized mice (*Raff*, 1971). *Ly-1*, *2*, and *3* (*Boyse* et al., 1968) are another series of mouse alloantigens found on 90% of thymocytes but on only a proportion of peripheral T cells (*Cantor* and *Boyse*, 1975a). Typically about 50% of peripheral T lymphocytes in the mouse are Ly-1$^+$2$^+$3$^+$, 30% are Ly-1$^+$ and 7% are Ly-2$^+$3$^+$. T lymphocytes have also been distinguished by suitably absorbed heteroantisera to brain or thymus cells (*Golub*, 1971) or to peripheral T cells (*Evans* et al., 1977). The former heteroantisera resemble anti-Thy-1 antisera, while the latter are similar to anti-Ly antisera.

Although B lymphocytes have been shown to possess specific heteroantigens (*Raff*, 1971; *McConnell*, 1975), they are generally detected by the presence of newly synthesized Ig on their surfaces using immunofluorescent or radioautographic techniques. Resynthesis of membrane Ig following its enzymatic removal distinguishes endogenous Ig from passively acquired Ig (*Pernis* et al., 1974). T cells are not thought to have endogenously synthesized surface Ig (*Vitetta* and *Uhr*, 1975) although this point remains controversal (*Marchalonis*, 1975).

T lymphocyte populations defined by the Thy-1 antigen, T lymphocyte subclasses as defined by Ly antigens and B lymphocytes defined by endogenously synthesized surface Ig represent stable lines of cells: T cells do not transform to B cells, nor do Ly-1$^+$ T cells become Ly-2$^+$3$^+$ T cells. These conclusions have been drawn from studies involving repopulation of lethally irradiated mice with purified cell populations (*Miller* and *Mitchell*, 1968; *Huber* et al., 1976a).

Lymphocytes also possess surface receptors for a variety of molecules in addition to surface receptors for antigens. Some of these receptors are restricted to one of the subpopulations and can be demonstrated by rosetting techniques with erythrocytes or by immunofluorescence techniques (*Shevach* et al., 1973). T cells of serveral species form nonimmune spontaneous rosettes with heterologous red blood cells (RBC); for example, sheep RBC with human T cells (*Coombs* et al., 1970), rabbit RBC with guinea pig T cells (*Wilson* and *Coombs*, 1973; *Stadecker* et al., 1973).

Receptors for antibody-activated C3, are found on B lymphocytes, as well as on polymorphs, macrophages, platelets, and human RBC (*Bianco* et al., 1970; *Shevach* et al., 1973). A receptor that binds the F_c part of Ig is present on B cells (*Basten* et al., 1972) and on some T cells (*Yoshida* and *Anderson,* 1972; *Gyöngyössy* et al., 1975; *Rubin* et al., 1975; *Basten* et al., 1975; *Stout* and *Herzenberg,* 1975; *Moretta* et al., 1977).

Antigens coded for by the major histocompatibility complex (MHC) are found on both B and T lymphocytes, although the amounts of the products of I region genes may be diminished on the T lymphocytes of some species (*Frelinger* et al., 1974; *Hämmerling,* 1976).

γ) Isolation of Lymphocyte Populations. Several methods have been used to isolate lymphocyte subpopulations. Cytotoxic antisera and complement can be used to delete a specific subpopulation such as Thy-1 or Ly-1 bearing cells (*Raff,* 1971; *Golub,* 1971; *Cantor* and *Boyse,* 1975a). Alternatively, antibodies specific for a cell-surface marker can be insolubilized, and the lymphocytes bearing this marker isolated by affinity chromotography (*Chess* et al., 1974); this method has been used to separate B cells bearing surface Ig from T cells. Lymphocyte-RBC rosettes can be isolated from nonrosetted cells by gradient centrifugation techniques (*Bφyum,* 1968) as was done by *Yoshida* et al. (1973) to isolate T lymphocytes and by *Moretta* et al. (1977) to isolate F_c receptor bearing T lymphocytes. A fluorescence-activated cell sorter has been developed which separates individual lymphocytes on the basis of bound fluorescent antibodies specific for cell surface components (*Bonner* et al., 1972). Other methods of isolation of lymphocyte populations have included density gradient separation (investigated by *Haskill,* 1967, and *Raidt* et al., 1968, among others), filtration thorugh glass bead columns (*Shortman,* 1966), filtration through rayon or nylon wool (*Rosenstreich* et al., 1971; *Julius* et al., 1973) and binding to antibody coated plates (*Barker* et al., 1975; *Nash,* 1976). This is by no means an exhaustive list and many techniques have been and are evolving to effect better separations of lymphocyte subpopulations.

c) Granulocytes. Neutrophilic granulocytes (polymorphonuclear leukocytes) are prominent during the first hours of DH responses to intradermally injected antigens in the guinea pig (*Turk* et al., 1966a), and rabbit (*Gell* and *Hinde,* 1951) and are also present at sites of nonspecific inflammation. In both cases, they become proportionally less prominent during the evolution of the response. Polymorphonuclear leukocytes are relatively scarce in contact DH reactions in guinea pigs unless secondary tissue destruction occurs (*Turk,* 1975); they are also uncommon in human DH reactions generally.

Basophilic granulocytes are found in varying proportions in all types of DH reactions of guinea pigs and man (*Wolf-Jürgensen,* 1966; *Turk, 1975; Dvorak,* 1976; *Askenase,* 1977). At various times after sensitization, they can form up to 90% of the subepidermal infiltrate in contact reactions and even as many as 30% of the cellular infiltrate in tuberculin reactions (*Turk,* 1975). Because the water-soluble granules of the basophil are not preserved during the usual formalin fixation and staining procedures, their presence in DH reactions was not noted until relatively recently.

Eosinophilic granulocytes are rarely prominent in DH reactions except in certain circumstances. At the site of repeated skin tests in a guinea pig with DH, the DH reaction evolves more rapidly than at sites tested for the first time. The cellular infiltrate of this retest reaction is composed largely of eosinophils (*Arnason* and *Waksman,* 1963).

Isolated neutrophils and their granules have been studied extensively because of the ease of preparation of these cells from peripheral blood (*Cline,* 1975), but comparable studies with basophils have been limited by the paucity of these cells in the circulation (*Dvorak* et al., 1974b). However, a circulating basophilia occurs in guinea pigs following intensive immunization with heterologous erythrocytes and *Dvorak* et al. (1974b) have developed a procedure to isolate basophils from blood. Basophils have also been isolated directly by teasing them from basophil-containing reactions in the tissues (*Dvorak* et al., 1975). Studies with isolated neutrophils and basophils in vitro have suggested that their entrance to the sites of DH reactions in vivo is mediated by a variety of lymphocyte-dependent and lymphocyte-independent mechanisms (*Snyderman* et al., 1970, 1971; *Ward* et al., 1975). (See Sect. V.B.6 and Sect. V.D for further discussion of granulocytes in DH reactions.)

2. Number of Antigen-Sensitive Cells Involved in Delayed Hypersensitivity Reactions

a) **Responses to Protein Antigens and Contact Allergens in vivo.** Although it might be supposed that the majority of the cells in an in vivo DH reaction are specifically responsive to antigen, this is not the case. Specifically sensitized cells form only a very small percentage, usually less than 1%, of the infiltrating cells in the lesion. It is still controversial whether it is possible to demonstrate the specific localization of sensitized cells in the DH lesion. It was demonstrated by *Najarian* and *Feldman* (1963a,b; *Feldman* and *Najarian,* 1963), using labeled lymphnode cells from guinea pigs with DH to contact sensitizers or to purified protein derivative (PPD). The cells were labeled by repeatedly injecting the donor animals with tritiated thymidine, a technique which labeled only those cells undergoing division. These labeled cells from highly sensitive donors were transferred to non-

sensitized recipients, the recipients skin tested, and the number of labeled cells in specific and nonspecific adoptive reactions and in nonspecific inflammatory reactions determined by radioautography. While the initial report of *Najarian* and *Feldman* (1963a) concluded that as many as 4% of the infiltrating cells in the adoptive DH reactions were of donor origin (and therefore sensitive to antigen), subsequent studies indicated that at most 0.8% of the cells in the specific adoptive lesions were of donor origin (*Najarian* and *Feldman,* 1963b). It was demonstrated that less than 0.3% of transferred lymph node cells or 0.2% of transferred spleen cells (*Feldman* and *Najarian,* 1963) were sufficient to induce an adoptive response in the recipient. These workers concluded that more than 80% of the cells in the adoptive DH response were of recipient origin and therefore not antigen-sensitive. The labeled cells of donor origin were chiefly lymphocytes.

Other workers, using similar techniques (*Turk* and *Oort,* 1963; *McCluskey* et al., 1963) were unable to demonstrate specific localization of labeled donor cells in sites of adoptive DH reactions. However, *McCluskey* et al. did show that 90% or more of the infiltrating mononuclear cells in adoptive DH reactions to protein antigens were of recipient origin, by using recipient guinea pigs injected with [^{3}H]-thymidine to label rapidly dividing cells. Since radiolabeled cells were found only at reaction sites and not in normal skin, they concluded that the cells in the tissue at DH reaction sites came from the circulation and were not produced by proliferation of precursors in the skin. *Werdelin* and *McCluskey* (1971) were also unable to demonstrate specific localization of antigen-sensitive cells in lesions of autoimmune adrenalitis, but were able to show that the majority of mononuclear cells in the lesion were not specifically sensitive to antigen.

b) Transplantation Reactions in vivo. Rejection of foreign tissue or organ grafts is as much a form of DH as DH skin reactions to soluble antigens or contact sensitizers (*Turk,* 1975). Animals can be passively sensitized for accelerated rejection only by the transfer of living lymph node cells from sensitized animals and not by sera from these animals (*Mitchison* 1953; *Billingham* et al., 1954). In addition, animals or human beings sensitized to foreign H or tumour-associated antigens develop vascular-inflammatory DH reactions to these antigens if they are injected intracutaneously (*Brent* et al., 1958; 1962; *Merrill* et al., 1961; *Oettgen* et al., 1968; *Hoy* and *Nelson,* 1969).

The rejection of foreign tissue or tumours is associated with the generation of specifically cytotoxic cells against cell-associated H antigens. Cytotoxic cells can also be associated with DH to viruses and contact sensitizers, and it is therefore necessary to discuss transplantation reactions in some detail.

The antigens controlling acceptance or rejection of grafts and appearing on the surface membrane of body cells are H antigens (*Klein*, 1975). Differences of those H antigens specified by genes located in the MHC are associated with rapid tissue rejection and strong immune responses. The MHC spans a small segment of DNA [less than 0.5 recombination units in the mouse and only twice this size in man (*Munro* and *Bright*, 1976)] and consists of several regions defined mainly on the basis of genetic recombinants. The low recombination frequency between the different MHC loci means that offspring usually inherit from each parent all the alleles found on one of the parental chromosomes; such a set being known as a haplotype.

It proved as difficult to demonstrate the specific localization of cells sensitized to H antigens in allografts as it was to demonstrate specific localization of cells sensitized to protein antigens in DH reactions in the skin (*Cerottini* and *Brunner*, 1974). One of the few demonstrations of specific localization of sensitized cells in allografts was that of *Lance* and *Cooper* (1972). Spleen and draining lymph-node cells were taken from animals having histoincompatible grafts; they were labeled in vivo by injecting the donor aminals with ^{125}I-labeled deoxyuridine or in vitro with ^{51}Cr. After passive transfer into recipients bearing skin allografts with either the same H antigens as the sensitized graft or with different H antigens, as many as 0.9% of the cells labeled with ^{125}I localized in graft to which they had been sensitized as compared with 0.3% in other control allografts. Specific localization was not demonstrated with ^{51}Cr-labeled cells. This small difference in localization was significant, and of the same order of magnitude as that seen with cells sensitive to contact sensitizers (*Najarian* and *Feldman*, 1963b). As in the case of DH protein antigens, few workers were able to demonstrate any specific localization of cells sensitive to histocompatibility antigens (reviewed by *Cerottini* and *Brunner*, 1974); the conclusion was reached that specifically sensitized cells represented only a minority of the infiltrating cells at sites of allograft rejection (*McCluskey* and *Werdelin*, 1971).

c) Antigen Sensitive Cells in in vitro Responses.

α) Soluble Proteins and Contact Allergens. One of the first in vitro models of in vivo DH was the migration inhibition assay (*George* and *Vaughan*, 1962). Capillary tubes containing peritoneal exudate cells were cultured in the presence and absence of antigen and the outgrowth of migration measured. The migration of peritoneal exudate cells from animals with DH to a soluble antigen was specifically inhibited in the presence of that antigen while the migration of cells from animals without DH but producing antibodies was not (*David* et al., 1964a). This in vitro reaction could not be transferred to nonsensitive cells by sera containing specific antibodies,

while the antigen specificity of the migration inhibition response for hapten-protein conjugates showed a carrier specificity similar to that seen in DH responses in vivo (antigen specificity of DH is further discussed in Sect. V.C). This latter observation was a further confirmation of the ability of this in vitro model to serve as a model for DH (*David* et al., 1964b,c).

David et al. (1964b) found that as few as 2.5% sensitive cells in the presence of antigen led to migration inhibition. The nature of the antigen-sensitive cells in the system was discovered by *Bloom* and *Bennett* (1966) who showed that the migration of purified macrophages (less than 0.5% lymphocytes) prepared from peritoneal exudate cells of sensitized animals was not inhibited in the presence of antigen. The addition of as few as 0.6% lymphocytes from a peritoneal exudate of a sensitized animal to macrophages from either sensitized or unsensitized guinea pigs permitted antigen-induced migration inhibition to occur. Furthermore, *Bloom* and *Bennett* (1966) as well as *David* (1966) demonstrated that sensitized lymphocytes cultured with specific antigen released a "factor" into the culture medium which inhibited migration of unsensitized macrophages from capillary tubes. Migration inhibition factor (MIF) was the first described lymphokine (*Dumonde* et al., 1969) a generic term used to describe the various non-antibody mediators generated by activated lymphocytes. (Lymphokines and their role in DH are further discussed in Sect. V.E.) In addition to MIF, other lymphokine activities have been reported such as mitogenic factor (*Wolstencroft* and *Dumonde*, 1970), macrophage activation factor (*Nathan* et al., 1971), macrophage agglutination factor (*Lolekha* et al., 1970), lymphotoxin (*Granger* and *Kolb*, 1968), interferon (*Green* et al., 1969) and various chemotactic factors (*Ward* et al., 1969). The results of in vitro studies of DH model systems confirm those of in vivo studies and indicate a mechanism whereby interaction of antigen with very small numbers of sensitized lymphocytes can lead to DH reactions in the tissues. *Bloom* et al. (1970) have further attempted to quantitative the number of antigen-responsive cells in lymph node cells from animals with DH to tuberculin. Antigen-activated lymphocytes were found to be capable of supporting viral replication by several viruses and the number of activated lymphocytes could then be determined by measuring the number of virus-infected cells. Fewer than 0.01%–0.1% of unstimulated lymph-node cells supported viral replication. Within 24 h after antigen activation by tuberculin, 0.1% of lymph-node cells were activated. Longer periods of exposure of sensitive lymph-node cells to antigen led to larger numbers of activated cells being detected, the increase with time proving to be linear, suggesting that the antigen-activated cells were not dividing. This conclusion was supported by the observation that agents which inhibited mitosis did not affect the observed numbers of activated cells at 48 h or at 96 h (*Bloom* et al., 1970).

The numbers of antigen-reactive cells in lymph nodes of guinea pigs with contact sensitivity to reactive sensitizers has been estimated directly by *Godfrey* and *Gell* (1976). Lymph-node cells from animals with contact sensititivty to 2,4-dinitrophenyl (DNP) were isolated from the bulk of lymph-node cells on DNP-substituted polyacrylamide beads. About 0.15% of the sensitive lymph-node cells applied to the column were retained and these could be specifically eluted with DNP glycine. Affinity chromatography removed those cells capable of producing MIF and macrophage aggregation factor in response to reactive DNP sensitizers; the small numbers of DNP-reactive cells recovered after specific elution were nearly as reactive as the original lymph-node cells and represented a marked purification. A small percentage of lymph-node cells from nonsensitized donors were bound and specifically eluted from these affinity columns; these cells did not respond to antigen exposure.

β) Cell-Associated Antigens. The study of cytotoxic lymphocytes (CL) has been used as an in vitro model for the study of transplantation types of DH in vivo. There is good correlation between the induction of CL and graft rejection; lymphocytes from individuals immunized against normal or tumour allografts are cytotoxic in vitro for target cells bearing H antigens to which the donors were sensitized (*Govaerts,* 1960; *Oettgen* et al., 1968; *Hoy* and *Nelson,* 1969; reviewed in detail by *Perlman* and *Holm,* 1969). Although various forms of cellular cytotoxicity can be mediated by antibody (see Sect. I),,the type of cytotoxicity considered here does not depend on serum antibody (*Cerottini* and *Brunner,* 1974). CL are also generated in response to other types of cell-associated antigen, such as so-called "weak" H antigens (H antigens not coded for by the MHC), e.g., the male antigen in the mouse (*Bevan,* 1975; *Gordon* et al., 1975), viruses (*Doherty* and *Zinkernagel,* 1975; *Zinkernagel* and *Doherty,* 1975), or reactive contact sensitizers (*Shearer* et al., 1975). (In addition to causing the generation of CL effector cells, sensitization with cell-associated antigens can also lead to the generation of sensitized lymphocytes which release lymphokines on reexposure to antigen, see Sect. B.3.b.β.)

Indirect and direct estimates of the percentage of cytolytically active effector cells in sensitized lymph-node cell populations vary from as few as 0.05% to 2% (*Wilson,* 1965; *Bonavida* et al., 1976; *Lindahl* and *Wilson,* 1977a) with estimates of CL specific for haptenic groups less than 1% and estimates of CL specific for antigens encoded for by the MHC in the1%–2% range. While relative numbers of CL (which are effector cells) directed against specific antigen may be somewhat larger than the percentage of lymphokine producing lymphocytes (also effector cells), they still represent a small percentage of the total number of cells infiltrating a graft.

d) Comment. The results discussed in these sections indicate that in all types of DH, the overwhelming number of cells present are not specifically sensitive to antigen and that it is difficult to demonstrate any preferential accumulation of specifically sensitized cells at the site of a specific DH reaction.

3. Functional Association of Delayed Hypersensitivity with Specific Lymphocyte Populations

Lymphocytes have been subdivided into several subpopulations on the basis of surface antigenic markers mentioned above. Much recent work has shown that specific lymphocyte subpopulations, isolated on the basis of these antigenic markers, are functionally specialized and are associated with specific DH effector functions (*Paul* and *Beneceraff*, 1977).

a) Role of Thymus. Among the first indications of the thymus' role in DH came from the study of immune deficiency diseases in man (*Cooper* et al., 1967). It was observed that patients with agammaglobulinemia had normal DH responses despite an extreme deficiency in all immunoglobulin classes and that their thymus, circulating lymphocytes and thymus-dependent areas in peripheral lymphoid tissues were normal. Conversely, children with congenital thymic aplasia showed a consistent absence of DH responses but appeared able to produce specific antibodies when immunized.

The earliest experimental evidence for the involvement of T lymphocytes in DH reactions derived from studies employing neonatal thymectomy in birds (*Warner* et al., 1962) and mammals (*Miller*, 1962; *Waksman* et al., 1962; *Cooper* et al., 1967). Neonatal thymectomy was shown to prevent the development of DH responses to protein antigens and to inhibit graft rejection (reviewed by *Miller* and *Osoba*, 1967). In inbred thymectomized rats, DH reactivity to tuberculin could subsequently be restored by grafts of semiallogeneic thymus (*Williams* and *Waksman*, 1969); it was possible to demonstrate that many of the donor thymus cells preferentially entered the DH reaction in its early stages.

b) Role of Specific T Cell Populations. Further evidence for the role of T lymphocytes in the initiation of DH reactions both to cell-associated and to soluble antigens in mice was obtained by studies employing the passive transfer of activated T cells (*Sprent* and *Miller*, 1971, 1972a,b). Splenic and lymph node lymphocyte populations rich in T cells were obtained by injecting thymocytes into lethally irradiated recipients bearing suitable allografts (*Sprent* and *Miller*, 1971, 1972a,b), or injected with soluble antigens (*Cooper*, 1972); populations rich in B lymphocytes were

obtained by injecting bone-marrow cells into comparable recipients. These workers showed that specific DH reactions, as evidenced by graft rejection or foot-pad swelling, could be transferred by lymphoid cell populations rich in T cells but not by populations rich in B cells or by serum from sensitized animals.

α) Delayed Hypersensitivity to Soluble Proteins and Contact Allergens. Another line of evidence for T cell involvement in the initiation of DH reactions includes experiments dealing with the isolation or deletion of specific lymphocyte subpopulations and observing the effects of these manipulations on the subsequent passive transfer of the DH response in question. T-lymphocyte-specific allo- or heteroantisera and complement have been used to delete T lymphocytes and suitable anti-Ig antisera and complement used to delete B lymphocytes (*Raff*, 1971; *Golub*, 1971). Other workers have injected specific anti-T cell sera into actively sensitized animals and have observed the effect of this procedure on DH responses (*Stadecker* and *Leskowitz*, 1976). Since T-cell-specific antisera used in this manner may cause nonspecific toxic reactions or may contain antibodies in addition to those cytotoxic to T lymphocytes which interfere with the reaction under study (e.g., antiplatelet antibodies) this latter method of demonstrating T lymphocyte dependence of DH reactions is not commonly used.

Removal of T cells from mouse lymphocyte populations with specific cytotoxic antisera and complement inhibits passive transfer of DH reactions to soluble proteins, heterologous erythrocytes and contact sensitizers (*Cooper* and *Ada*, 1972; *Asherson* et al., 1974; *Miller* et al., 1975a). Removal of B cells from lymphocyte populations by treatment with anti-Ig sera and complement (*Cooper* and *Ada*, 1972) did not affect the ability of these populations to transfer DH to soluble proteins suggesting that B cells were not required.

Similar results have been obtained in guinea pigs. *Jaffer* et al. (1973) showed that removal of phagocytic macrophages and B lymphocytes from lymph node cell populations did not affect passive transfer of local DH reactions to PPD or hapten-protein conjugates. These experiments also suggest that F_c receptor bearing T or B cells are not required for DH expression. Using a specific rabbit anti-guinea pig T cell sera, *Godfrey* (1976) demonstrated that the lymphocytes transferring DH reactions to contact sensitizers were as sensitive to its effects as were the cells transferring reactions to PPD. This was significant since it showed that basophil-associated DH reactions to contact sensitizers and DH reactions to PPD had very similar underlying mechanisms, a point confirmed by *Askenase* (1976). *Askenase* isolated guinea pig T cells nonspecifically (using nylon wool to remove adherent B cells) and showed that small numbers of these lympho-

cytes were highly active in transferring both basophil-associated DH to hapten-protein conjugates and DH to PPD. *Askenase* also demonstrated that whether DH skin reactions in the donor animals were basophil-associated (hapten-protein conjugates) or not (PPD), all passively transferred responses were. This latter observation was a further indication that the mechanisms behind basophil-associated and non-basophil-associated DH were not as different from one another as originally postulated by *Dvorak* and his co-workers (*Dvorak* et al., 1970; *Richerson* et al., 1970; *Dvorak* et al., 1971; reviewed by *Dvorak*, 1976). T lymphocyte dependence of basophil-associated DH reactions in actively sensitized guinea pigs was demonstrated by *Stadecker* and *Leskowitz* (1976), who demonstrated that injections of specific rabbit anti-T-cell serum would suppress equally both types of DH reaction.

The role of T lymphocytes in in vitro models of DH has been explored. Isolated populations of T cells in several species have been known to secrete lymphokines such as MIF, macrophage chemotactic factor, mitogenic factor and interferon in response to stimulation with low concentration of soluble antigens (*Yoshida* et al., 1973; *Rocklin* et al., 1974; *Epstein* et al., 1974). These populations were isolated on the basis of various cell surface markers including surface Ig, C3 receptor, and the ability to form non-immune rosettes with heterologous erythrocytes. Deletion experiments using specific anti-T-cell sera have confirmed the production of lymphokines by T cells (*Clinton* et al., 1974; *Gorczynski*, 1974; *Bloom* and *Shevach*, 1975; *Godfrey*, unpublished observations).

It has recently been demonstrated that the Ly alloantigens in mice, present on thymus cells and a proportion of peripheral lymphocytes, define functionally important T lymphocyte subpopulations which exist prior to antigen stimulation (*Cantor* and *Boyse*, 1975a), and which represent independent differentiation pathways of T cells (*Huber* et al., 1976a). T lymphocytes active in passively transferring DH to soluble proteins and heterologous erythrocytes in mice have been shown to belong to the Ly-1$^+$ subclass (*Huber* et al., 1976b; *Vadas* et al., 1976). This subclass also contains T cells which serve as helper cell in antibody formation by B cells (*Cantor* et al., 1976; *Vadas* et al., 1976) and T cells triggered by specific antigen to secrete MIF and other lymphokines (*Pickel* et al., 1976; *Newman* et al., 1978).

Human peripheral T lymphocytes have also been found to consist of functionally and antigenically heterogeneous cells (*Woody* et al., 1975; *Brouet* and *Toben*, 1976; *Evans* et al., 1977). Human T cell subpopulations have been delineated using antisera specific for antigens present on thymocytes and on restricted subpopulations of peripheral T cells. For example, *Evans* et al. (1977) were able to show that peripheral T cells proliferating in response to specific antigen (which represented 50% of

peripheral blood T cells) were antigenically distinct from T cells elaborating the lymphokines mitogenic factor and MIF (*R. Rocklin,* personal communication) in response to specific antigen. The occurrence of distinct subpopulations of differentiated peripheral T cells precommitted in their functional response prior to antigen exposure would therefore appear to represent a general biologic phenomenon (*Paul* and *Benacerraf,* 1977).

β) Delayed Hypersensitivity to Cell-Associated Antigens. Freedman et al. (1972) showed that the passive transfer of sensitivity to allogeneic tumour cells in mice was inhibited by treating the cells to be transferred with specific anti-T-lymphocyte serum and complement, observations which confirmed the T cell dependence of allograft rejection. *Allison* (1972) and *Rouse* et al. (1972) also demonstrated that the transfer of tumour immunity to either virus-induced or to plasma cell tumours was inhibited by pretreatment of sensitized lymphoid cells with anti-Thy-1 serum and complement. Even before the effect of anti-Thy-1 serum and complement on the in vivo transfer of DH to cell associated antigens had been demonstrated, evidence had been obtained that cell-mediated cytotoxicity in immune allogeneic sensitization was caused by specifically sensitized T cells independent of antibody, B cells, antibody-producing cells or macrophages (*Cerottini* et al., 1970; *Lonai* et al., 1971; reviewed in *Cerottini* and *Brunner,* 1974). Elimination of T lymphocytes from antibody-producing spleen cell populations with specific antisera and complement abrograted specific in vitro cellular cytotoxicity without affecting antibody formation to the same antigen. Treatment of the same immune spleen cells with antisera to B cells or plasma cells had no effect on cytotoxicity but completely inhibited antibody formation (*Cerottini* and *Brunner,* 1974).

Cytotoxic T lymphocytes specific for differences in H antigens recognize H antigens coded for by the K, I, and D regions of the mouse MHC (*Bach* et al., 1972a) and corresponding genetic regions of other species (*Bach* et al., 1972b, 1976). In mice. these T cells beong to the Ly-2^+3^+ subpopulation (*Cantor* and *Boyse,* 1975a; *Vadas* et al., 1976; *Beverley* et al., 1976; *Woody* et al., 1977). In some mouse strains and against certain antigens such as syngeneic tumour cells, T-CL may bear Ly-1 antigens as well (*Shiku* et al., 1975). Recent reports indicate that T-CL also carry Ly-5 and Ly-6 markers (*Woody* et al., 1977). The Ly-2^+3^+ T cell subpopulation also contains cells mediating suppressor functions (*Huber* et al., 1976b; *Vadas* et al., 1976) as well as cells able to secrete lymphokines in response to specific stimulation with cell-associated antigens (*Newman* et al., 1978).

γ) Proliferative Responses to Histocompatibility Antigens in Unprimed Animals. Two primary responses cognate to DH to cell-associated H antigens

are the graft vs. host response in vivo and the mixed lymphocyte response (MLR) in vitro. These responses differ from DH in that unprimed lymphocytes confronted with allogeneic H antigens for the first time make a vigorous proliferative response (*Simonsen,* 1967; *Sørensen,* 1972). These responses are T cell responses as evidenced by experiments with chromosome markers (*Johnston* and *Wilson,* 1970) and observation of immune deficiency diseases in man (*Cooper* et al., 1967; *Bach* et al., 1968). Further evidence for T cell dependency of these responses is that separated T cells but no B cells are active as responders in MLR (*Häyry* et al., 1972), while the addition of bone marrow or B cells to thymocytes does not enhance graft vs. host activity (*Stutman* and *Good,* 1969; *Cantor,* 1972).

T lymphocytes responding in MLR belong to a different subpopulation of T lymphocytes from T-CL in DH to H antigens encoded by the MHC; cytolytically active cells can be adsorbed by appropriate monolayers without affecting subsequent activity of the remaining cells in MLR (*Zoschke* and *Bach,* 1971). In mice, *Cantor* and *Boyse* (1975a) have shown that T lymphocytes active in MLR belong to the Ly-1$^+$ subpopulation, the same subpopulation to which cells active in DH to protein antigens belong. In man, *Evans* et al. (1977) have found a somewhat similar situation: the same T lymphocyte subpopulation contains cells which proliferate in MLR and cells which secrete the lymphokines MIF and mitogenic factor in response to stimulation wiht protein antigens.

δ) Additional Surface Antigens Defining T Lymphocyte Subpopulations Active in Delayed Hypersensitivity. Several other surface antigenic markers have been shown to be associated with functional subpopulations of T cells. In mice, *Ia* antigens encoded by the I region of the MHC are reported to be found on T cells which regulate the magnitude of the immune response, but not on those mediating DH to soluble proteins or heterologous erythrocytes or cellular cytotoxicity (*Vadas* et al., 1975; *Woody* et al., 1977). In guinea pigs, however, *Yamashita* and *Shevach* (1977a) found that T cells sensitized to protein antigens and active in antigen-induced proliferation and production of MIF were Ia$^+$, while primed helper T cells and T cells proliferating in MLR were Ia$^-$. Several groups have found that regulatory T cells in mice and man carry a receptor for the F_c of activated Ig of various classes (*Stout* et al., 1976; *Rubin* et al., 1976; *Moretta* et al., 1977; *Fridman* et al., 1977). T-CL themselves also have been reported to carry this receptor (*Stout* et al., 1976; *Fridman* et al., 1977).

Thy-1 and Ly markers are differentiation antigens which represent lymphocyte sublines preprogrammed to respond functionally in a fixed way after exposure to antigen: i.e., Thy-1$^+$ T cells do not differentiate into Thy-1$^-$ B cells (*Raff,* 1971) nor do Ly-1$^+$ T cells differentiate to Ly-2$^+$3$^+$ T cells (*Huber* et al., 1976a). It is not known whether Ia antigens are

differentiation antigens similar to Ly and Thy-1 or whether their appearance follows antigen exposure as is the case with the F_c receptor of mouse T cells (*Stout* et al., 1976). In the latter case, it was found that while the precursor cell for the cytotoxic T cells lacked the F_c receptor, such a receptor was present on the effector cell.

c) Role of B Lymphocytes. While T lymphocytes mediate the bulk of lymphokine-associated DH reactions to soluble proteins and contact sensitizers, recent evidence has indicated that B lymphocytes may also be associated with DH reactions in a limited way. Treatment of sensitized lymphocytes with specific anti-T-cell sera does not always inhibit antigen-induced lymphokine production (*Bloom* et al., 1975; *Godfrey,* unpublished observations). Isolated populations of guinea pig or human lymphocytes release MIF and other lymphokines in response to stimulation with B cell mitogens (*Yoshida* et al., 1973; *Rocklin* et al., 1974; *Wahl* et al., 1974; *Wilton* et al., 1975). Isolated B lymphocytes have also been shown to release lymphokines in response to specific antigenic stimulation with T dependent antigens (*Rocklin* et al., 1974; *Bloom* and *Shevach,* 1975; *Wahl* and *Rosenstreich,* 1976). B lymphocytes on their own appear able to release lymphokines after mitogenic stimulation (*Wahl* et al., 1975), but it is not yet settled whether they require T lymphocyte factors to release lymphokines in response to T-dependent antigens. *Rocklin* et al. (1974) reported that human B cells produced MIF in response to antigen while *Bloom* and *Shevach* (1975) and *Wahl* and *Rosenstreich* (1976) observed that T cells or a factor produced by T cells after specific antigenic stimulation were needed for B lymphokine response. Although both B cells and T cells could produce lymphokines affecting macrophage migration or monocyte chemotaxis, only T cells released mitogenic factor and proliferated after antigenic stimulation (*Rocklin* et al., 1974; *Wahl* et al., 1974; *Littman* et al., 1976; *Rosenstreich, Wahl* and *McMaster,* unpublished observations). Agents which trigger B cells to release lymphokines do not induce their proliferation (*Wahl* et al., 1974; *Littman* et al., 1976; *Rosenstreich, Wahl* and *McMaster,* personal communication). B cells have also been shown to release MIF in vivo in response to specific antigen, but only if T cells are present 10 days before challenge; however, once primed in the presence of T cells, B cells could release MIF after antigenic challenge even in the presence of anti-Thy-1 serum (*Salvin* et al., 1977).

In many cases in vivo, B lymphocytes may not release lymphokines after specific antigenic stimulation because of the simultaneous release of inhibitory factors by T lymphocytes (*Cohen* and *Yoshida,* 1977). On the other hand, some aspects of DH, especially hapten-specific DH or DH to carbohydrates may be mediated by B lymphocytes. *McMaster* et al. (1977b) have recently shown that the thymus-independent antigen

ε-DNP-lysine-cyanuric-Ficoll (DNP-Ficoll) elicits typical hapten-specific DH reactions in guinea pigs immunized with DNP-Ficoll in CFA or with large amounts of DNP-keyhole limped hemocyanin (DNP-KLH) in CFA. These DH reactions were transferred by living cells, not by serum and were characterized by mononuclear cell infiltrates with few basophils. Earlier demonstrations of hapten-specific DH indicated that it could be elicited by large doses of hapten-protein conjugates in guinea pigs immunized with large quantities of hapten coupled to a different carrier protein (*Benacerraf* and Gell, 1959a,b; *Gell* and *Benaceraff*, 1961a,b). Hapten-specific DH to DNP is also demonstrable in vitro: DNP-Ficoll causes MIF release from lymph-node cells of guinea pigs sensitized with DNP-KLH (*McMaster* et al., 1977a). Further studies in this system by *Rosenstreich, Wahl* and *McMaster* (personal communication) have shown that DNP-Ficoll induces hapten-specific lymphokine release only in B lymphocytes from lymph nodes draining sites of sensitization and not from lymph node T cells or spleen cells. DNP-Ficoll does not induce hapten-specific proliferation in vitro of lymph-node cells or peritoneal exudate lymphocytes from DNP-KLH-sensitized animals. Since it has been shown that DH to a polysaccharide (as measured by skin test reactivity and MIF production) can be induced by injecting the polysaccharide into an unrelated DH reaction (*Brunda* and *Raffel*, 1977; *Crowle* et al., 1977), and since DH reactions in vitro to polysaccharides and certain other antigens are associated with MIF release without proliferation (*Godfrey* et al., 1969; *Chaparas* et al., 1970; *Gerety* et al., 1970; *Spitler* et al., 1970; *Senyk* et al., 1971), it is tempting to speculate that this entire class of in vivo DH reactions is B cell mediated although T cells are required for its induction. B-cell-mediated DH may also be present in contact-sensitive guinea pigs immunized with reactive chemicals in CFA. Using a specific anti-T-cell serum, *Godfrey* (1976a) showed that passively transferred contact reactions and DH sensitivity to PPD were mediated by T lymphocytes inhibited by a high dilution of this serum while delayed-onset sensitivity to a related hapten-protein conjugate was unaffected by this treatment. The delayed reactions to this hapten-protein conjugate could represent a B lymphocyte mediated aspect of contact sensitization. Antigen-specific lymphokine release from lymph node cells of these animals showed a similar pattern of sensitivity to specific anti-T cell serum and complement in vitro (*Godfrey*, unpublished observations): responses to the reactive chemical or PPD were readily inhibited by high dilutions of specific antisera while responses to hapten-protein conjugates were not. A conclusive judgment on the role of B lymphocytes in DH is not possible at this time because of the paucity of direct evidence.

4. Interaction of Lymphocyte Subpopulations in Delayed Hypersensitivity

It has become clear that the regulation of several types of DH reactions is associated with interactions between T lymphocyte subpopulations (*Cantor* and *Weissman,* 1976). Specific interactions between subpopulations of T cells are important in the production of T-CL in vitro and presumably in vivo (*Cantor* and *Boyse,* 1975b); i.e., synergistic interactions between T cell subpopulations serve to increase this response. Interactions between T lymphocyte subpopulations may, on the other hand, serve to suppress or decrease the observed DH response (*Asherson* and *Zembala,* 1975). The role of T-T lymphocyte interactions has been extensively reviewed (*Gershon,* 1974; *Asherson* and *Zembala,* 1975, 1976; *Cantor* and *Weissman,* 1976).

a) T Lymphocyte Synergy in the Generation of Cytotoxic Lymphocytes. The generation of T-CL against allogeneic H antigens encoded in the K or D regions of the mouse MHC has been shown to involve cooperative interactions between T cells recognizing H-2 antigen differences and those recognizing differences in Ia antigens (*Alter* and *Bach,* 1974; *Cantor* and *Boyse,* 1971a,b; *Stout* et al., 1976). Lymphocyte mixtures of cells with only I region differences at the MHC (different Ia antigens) undergo proliferation (MLR) without generating T-CL, whereas cells differing only in K or D regions neither proliferate nor produce T-CL (*Alter* and *Bach,* 1974). *Cantor* and *Boyse* (1975a,b) showed that the generation of T-CL against K or D H-2 antigens required the interaction of Ly-1$^+$ amplifier T cells which recognized differences in Ia antigens by proliferating and Ly-2$^+$3$^+$ precursor cells which responded to differences in K or D H-2 antigens to generate T-CL effector cells. *Stout* et al. found that this synergistic reaction required Ly-1$^+$ cells bearing an IgG F_c receptor and Ly-2$^+$3$^+$ T cells lacking this receptor. The differentiation of precursor cells to T-CL effector cells was associated with the appearance of the F_c receptor. It might be speculated that the amplifier function of Ly-1$^+$ cells is mediated by a mitogenic lymphokine (*Geczy,* 1977).

It is not unlikely that synergy between T cell subpopulations is a general phenomenon in the generation of cytolytic DH to cell-associated antigens: *Pang* et al. (1976) noted cooperation between Ly-1$^+$ and Ly-2$^+$3$^+$ T cells in the generation of T-CL specific for a mouse poxvirus.

b) Regulation of Delayed Hypersensitivity by T Lymphocytes. The experiments of *Gershon* and his associates (reviewed by *Gershon,* 1974) indicate that interactions between T lymphocyte subpopulations can suppress or regulate DH responses. One mechanism of this suppression might be that antigen stimulation of one T cell subclass causes the production of substances

(regulatory lymphokines) which, alone or in concert with antigen, reduce the responsiveness of other T lymphocyte subclasses. For example, *Asherson* and *Zembala* (1975, 1976) showed that such a regulatory mechanism is operative in the suppression of contact sensitivity in mice to trinitrochlobenzene by previous intraveneous injections of trinitrobenzene sulfonic acid. They isolated a regulatory lymphokine, which mediated suppression, from antigenically stimulated suppressor T cells. This soluble factor was found to be a stable, antigen-specific molecule of 50,000 daltons molecular weight containing Ia antigenic determinants (*Zembala* et al., 1975, 1977; *Greene* et al., 1977b). Another antigen-specific soluble factor derived from T cells which regulated an aspect of DH was described by *Greene* et al. (1977a). This lymphokine, which suppressed resistance to transplanted tumors, was also 50,000 daltons molecular weight and contained antigenic determinants coded for by the MHC. (See Sect. V.E. for further discussion of regulatory lymphokines.)

T cell regulation of T cell mediated functions in mice by short-lived, rapidly proliferating cyclophosphamide-sensitive T cells has been shown to occur in DH responses to sheep red cells, soluble proteins and cell-associated antigens (*Cantor* and *Simpson*, 1975; *Simpson* and *Cantor*, 1975; *Askenase* et al., 1975a, 1977; *Mitsuoka* et al., 1976; *Zembala* and *Asherson*, 1976; *Morikawa* et al., 1977; *Röllinghoff* et al., 1977). These suppressor cells have been shown to bear receptors for the F_c protein of IgG (*Stout* and *Herzenberg*, 1975) and have a surface antigen phenotype Ly-$2^+3^+Ia^+$ (*Vadas* et al., 1976; *Pickel* and *Hoffman*, 1977; *Woody* et al., 1977). In man, suppressor T cells also have receptors for the F_c portion of IgG (*Moretta* et al., 1977). Regulatory cells may also possess receptors for histamine since cell chromatography over histamine-containing substrates removed a T cell population capable of regulating lymphokine release (*Rocklin* and *Melmon*, personal communication).

c) Regulation of Delayed Hypersensitivity by B Cells and Antibody. Specific antibody can regulate the degree of DH induced by antigen (*Uhr* and *Möller*, 1968). There is clear evidence (*Parish* and *Liew*, 1972; reviewed by *Parish*, 1972a) that antibody production to modified flagellin in mice bears a reciprocal relationship to the degree of DH induced by the antigen. It might be speculated that this inhibition of DH responses by antibody resulted from the interaction of the antibody with F_c receptors found on suppressor T cells.

In guinea pigs, DH reactions to soluble proteins or to contact sensitizers appear to be regulated by suppressor B cells (*Katz* et al., 1974b; *Turk* et al., 1976; *Zembala* et al., 1976). These regulatory B cells can be depleted by high doses (300 mg/kg) of cyclophosphamide given before sensitization. [This dose would also affect suppressor T cells as well, since

they are inhibited by lower doses of drug than are B cells (*Askenase* et al., 1975a; *Zembala* and *Asherson*, 1976; *Mitsuoka* et al., 1976; *Röllinghoff* et al., 1977)]. The resulting DH to contact sensitizers or to protein antigens in IFA is prolonged and increased to the level of reactions produced after immunization with antigens in CFA (*Turk* and *Parker*, 1973). Animals treated with cyclophosphamide showed partial or complete inhibition of production of specific IgG antibodies. Transfer of splenic B cells from animals immunized without cyclophosphamide treatment to cyclophosphamide-treated, immunized recipients suppressed the delayed reactions in the latter (*Katz* et al., 1974b, 1975; *Turk* et al., 1976). However there is a possibility that the method of isolation of guinea pig B lymphocytes using rabbit anti-guinea pig Ig by *Turk* and co-workers might have also isolated F_c-receptor-bearing T cells. The presumptive B lymphocytes were not treated with a cytotoxic, specific anti-T-cell serum before transfer, nor were control columns with other specific rabbit antibodies used. On the other hand, the regulatory B cells isolated by *Zembala* et al. (1976) were shown to lack Thy-1 antigen and could not therefore be claimed to represent a T cell subpopulation.

Neta and *Salvin* (1974, 1976) have also suggested the presence of suppressor B cells in guinea pigs immunized to soluble proteins in CFA. Transfer of isolated splenic B cells from immunized animals could suppress the expression of DH in already sensitized recipients (*Neta* and *Salvin*, 1974). These workers subsequently found that antigen-induced proliferation of draining lymph-node cells in vitro could be reduced by the addition of isolated splenic B cells or by isolated lymph node B cells from immunized donors (*Neta* and *Salvin*, 1976). The mechanism by which suppressor B cells regulate DH is uncertain. Again, one might speculate that F_c receptors on T cells and specific antibody secretion were involved.

5. Macrophage Participation in Delayed Hypersensitivity

Macrophages have a dual role in DH. They are main effector cell in DH reactions and are acted upon by various lymphokines released by T (and B) lymphocytes after contact with specific antigen. In addition, their presence has been shown to be necessary for the induction of DH in vivo (*Oppenheim* and *Seeger*, 1976) as well as for the triggering of T lymphocyte proliferation and mediator production in vitro (*Rosenthal* and *Shevach*, 1976).

a) Role of Macrophages in the Induction of Delayed Hypersensitivity in vivo. Indirect evidence for the importance of macrophages in the induction of immune responses in vivo is that agents which interfere with macrophage function such as colloidal carbon, carageenan, or heterologous

antimacrophage serum, reduce antigen uptake by macrophages and reduce the immune response (*Schwartz* and *Leskowitz*, 1969; *Unanue*, 1972). Direct evidence for the importance of macrophage-associated antigen in the induction of immune responses has been obtained by passive transfer experiments in which peritoneal exudate macrophages have been exposed to antigen in vitro, washed to remove free antigen and injected into normal syngeneic recipients (*Mitchison*, 1969; *Unanue* and *Askonas*, 1968; *Unanue* and *Feldman*, 1971; reviewed by *Unanue*, 1972). These recipients develop normal antibody and DH responses to the macrophage-associated antigens which are considerably more immunogenic than the same doses of free antigen.

In guinea pigs, low doses of macrophage-associated antigen induce DH but no antibody response at 3 weeks; comparable doses of soluble antigen induce only antibody response (*Seeger* and *Oppenheim*, 1972a). The duration of exposure of antigen to macrophages also has an important effect on the immunogenicity of macrophage-associated antigen. The injection of sheep red cells exposed to macrophages for 1 h into guinea pigs led only to the production of antibodies while animals injected with sheep red cells incubated with macrophages for 24 h developed DH (*Pearson* and *Raffel*, 1971). Macrophage-associated antigen was found to be more immunogenic for sensitization for DH than antigen associated with lymphocytes, thymocytes or hepatoma cells when equal doses of cell-associated antigen were injected into syngeneic guinea pigs (*Seeger* and *Oppenheim*, 1972b). The induction of DH by macrophage-associated antigen required living macrophages (*Pearson* and *Raffel*, 1971; *Seeger* and *Oppenheim*, 1972a), suggesting that the enhancement of immunogenicity by macrophages requires some metabolid processing of the antigen.

b) Role of Macrophages in Activation of Lymphocytes in vitro. Macrophages are required for the in vitro activation of antigen-induced T and B lymphocyte proliferation (*Oppenheim* et al., 1968; *Waldron* et al., 1973; *Rosenstreich* and *Rosenthal*, 1973; *Thomas* et al., 1977a) and for lymphokine production (*Ben-Sasson* et al., 1974; *Nelson* and *Leu*, 1975; *Wahl* et al., 1975). Macrophages have been also shown to be required for stimulation of T lymphocytes by mitogens (*Oppenheim* et al., 1968; *Lohrmann* et al., 1974; *Rosenstreich* et al., 1976), by alloantigens in some species (*Oppenheim* et al., 1968; *Rode* and *Gordon*, 1974; *Greineder* and *Rosenthal*, 1975b), and by oxidizing chemicals (*Greineder* and *Rosenthal*, 1975a). On the other hand, the activation of B lymphocytes by thymus-independent antigens and B cell mitogens requires far fewer macrophages than do other types of lymphocyte activation and may be macrophage-independent (*Rosenstreich* et al., 1971; *Yoshinaga* et al., 1972; *Rosenstreich* et al., 1976). This point remains unsettled. Studies showing a macrophage requirement

for lymphocyte activation have generally demonstrated that depletion of a radioresistant, adherent, phagocytic cell with morphologic features of the mononuclear phagocyte or macrophage inhibits the in vitro assay under study and that readdition of macrophages but not other cell types such as fibroblasts, restores the activity in question.

Macrophages have several functions in lymphocyte culture systems. They are needed to maintain lymphocyte viability in culture (*Mosier* and *Pierce,* 1972; *Chen* and *Hirsch,* 1972; *Bevan* et al., 1974), a function which can be replaced by reducing agents such as 2-mercaptoethanol. A second function of the macrophage in vitro is to secrete soluble activating factors which stimulate T cells which have bound mitogen, been chemically oxidized, or which have been exposed to allogeneic H antigens in MLR (*Novogrodsky* and *Gery,* 1972; *Rode* and *Gordon,* 1974; *Calderon* and *Unanue,* 1975; *Rosenstreich* et al., 1976). This activating function of macrophages can also be carried out by isolated macromolecular factors secreted by nonstimulated macrophages. A third function of macrophages in vitro systems lies in antigen presentation to lymphocytes.

The initial antigen binding cell in lymphoid cell populations in guinea pigs is a macrophage (*Waldron* et al., 1973; *Rosenstreich* and *Rosenthal,* 1974). Brief exposure of macrophages from sensitized or normal animals to soluble protein antigens, followed by washing to remove loosely associated antigen, produces macrophage-associated antigens which can stimulate sensitized T lymphocytes to proliferate or to secrete lymphokines. Lymphocyte stimulation occurs in response to doses of antigen too low to stimulate a response if present continuously in the culture (*Rosenstreich* and *Rosenthal,* 1973). Lymphocyte cultures lacking macrophages do not proliferate after pulse exposure to antigen. Subsequent addition of macrophages to these cultures after removal of antigen is not associated with lymphocyte stimulation and suggests that the macrophages are not functioning in this case by secreting nonspecific factors required for lymphocyte viability or activation. These nonresponding, antigen-pulsed lymphocytes have not been rendered tolerant by their brief antigen exposure since they can be triggered by a subsequent exposure to antigen-pulsed macrophages (*Rosenstreich* and *Rosenthal,* 1974). The macrophage is also important in presenting and/or processing contact sensitizers to suitably sensitized T lymphocytes (*Thomas* et al., 1977a,b). T lymphocytes from guinea pigs with contact sensitivity to 2,4,6-trinitrophenyl (TNP) can be stimulated in vitro with TNP-conjugated macrophages, but not with conjugated thymocytes, lymph-node lymphocytes or erythrocytes. It should also be mentioned that macrophages function as stimulator cells in the guinea pig MLR, presenting allogeneic H antigens to T lymphocytes; macrophages are 10–80 times more stimulatory to T cell proliferation than purified lymphocytes (*Rode* and *Gordon,* 1974; *Greineder* and

Rosenthal, 1975b). The role of H antigens in the presentation of antigen to T cells by macrophages and in macrophage-lymphocyte interactions generally will be discussed as an aspect of the antigen-specificity of T lymphocytes (see Sect. V.C.4).

Studies on the ability of macrophage-associated antigens such as 2,4-dinitrophenyl(DNP)-guinea pig albumin (*Ellner* and *Rosenthal,* 1975) to trigger T lymphocyte proliferation have shown that preliminary culture of antigen-pulsed macrophages results in a linear decrease in the ability of the macrophages to stimulate T cells which reflects neither the rapid catabolic phase of antigen handling nor the ensuing period of antigen stability. In this system trypsin treatment of the macrophages removes surface-associated antigen but does not affect the subsequent immune response. This observation suggests that the stable surface pool of antigen is not the critical site of antigen retention. After treatment of macrophages with trypsin there is no restoration of surface antigen (*Unanue* et al., 1969; *Ellner* and *Rosenthal,* 1975) and the manner in which intracellular antigen causes T lymphocyte stimulation is unclear.

c) Physical Interaction Between Macrophages and Lymphocytes in vitro. Clustering of lymphocytes around blast-like cells and macrophages has been observed during lymphoid cell culture of many species (*McFarland* et al., 1966; *Mosier,* 1969; *Salvin* et al., 1971; *Sulitzeanu* et al., 1971; *Werdelin* et al., 1974). In guinea pigs, lymphocytes and macrophages bind to each other in the absence of antigen (*Lipsky* and *Rosenthal,* 1973, 1975b). This binding requires active macrophage metabolism and viable macrophages, the role of the lymphocyte being passive. It is abolished by treatment of the macrophages with trypsin; treatment of the lymphocytes with trypsin is without effect. It is temperature dependent, being most active at 37°C, dependent on divalent cations, is not selective to T or B lymphocytes, nor does it distinguish between syngeneic or allogeneic lymphocytes. The binding of lymphocytes by macrophages is not inhibited by excess Ig and is a dynamic reversible process. It is likely therefore, that the antigen-independent binding of lymphocytes is mediated by a distinct receptor mechanism. Similar effects may occur in cultures of human monocytes and lymphocytes (*Braendstrup,* personal communication).

The formation of clusters of immune lymphocytes and macrophages in the presence of antigen has been studied by *Lipsky* and *Rosenthal* (1975a) and by *Werdelin* and co-workers (*Werdelin* et al., 1974; *Nielsen* et al., 1974; *Braendstrup* et al., 1976, 1977; *Petri* et al., 1978). *Lipsky* and *Rosenthal* found that the degree of macrophage-lymphocyte interaction after 1 h was independent of the presence of antigen. In the absence of antigen there was a steady decline in clusters while in the presence of

antigen cluster formation increased beginning at 8 h and was maximal at 20 h. Using somewhat different techniques to measure lymphocyte-macrophage interaction, *Werdelin's* group found little interaction after 2 h of culture in the presence or absence of antigen but lymphocyte-macrophage clusters after 8 h; the number of these clusters with more than seven lymphocytes per macrophage did not increase during the ensuing 12 h. Cluster formation in culture was independent of DNA synthesis but required active RNA and/or protein synthesis as well as intact microtubule and microfilament systems. Once formed, clusters required continuous protein synthesis for their maintenance, but intact microtubule and microfilament systems were not necessary (*Braendstrup* et al., 1977). These newer observations confirm and extend those of *Rosenthal* et al. (1975) and *Ben-Sasson* and *Rosenthal* (1975).

The formation of clusters was shown to be specific for the sensitizing antigen (*Werdelin* et al., 1974). Cells from animals sensitized with hapten-protein conjugates formed clusters only in the presence of the sensitizing conjugate and not in the presence of other conjugates containing the same hapten (*Rosenthal* et al., 1976). This type of antigen specificity is characteristic of T cells and T-cell-mediated reactions (*Paul* and *Benacerraf*, 1977). Since the number of clusters increased when T lymphocytes rather than lymph-node lymphocytes were used, *Werdelin* and his associates concluded that the lymphocytes in the cluster were T cells. Direct staining of the clusters for membrane Ig showed that B cells were to a large extent excluded from the clusters (*Petri* et al., 1978). Both *Rosenthal's* and *Werdelin's* groups found that cluster formation occurred when antigen-pulsed macrophages were used and no free antigen was present in the culture. Antigen-dependent cluster formation between sensitized lymphocytes and macrophages occurred only between histocompatible cells; the formation of clusters by histoincompatible cells was unaffected by the presence of antigen (*Rosenthal* et al., 1976). The observed histocompatibility constraints are likely to derive from conditions associated with the initial sensitization of antigen-responsive lymphocytes (*Paul* and *Benacerraf*, 1977).

Antigen-specific clusters have been found to consist of a central, larger T lymphocyte (presumably antigen-committed) attached to the macrophage with smaller peripheral lymphocytes (90% T, 10% B) attached to the central lymphocyte (*Nielsen* et al., 1974; *Petri* et al., 1978). Examination of clusters during the first 20 h of culture revealed that the central lymphocyte appeared to be undergoing early blast transformation (larger nucleus, presence of one or two nucleoli, size, increase in polyribosomes, large Golgi apparatus), and could be shown to be the only DNA-synthesizing lymphocyte in the cluster (*Nielsen* et al., 1974; *Braendstrup* et al., 1976). At later time periods, other cells in the cluster entered DNA synthesis. These studies taken as a whole suggest that physical interaction

between macrophages and lymphocytes is important in antigen-induced T lymphocyte proliferation and that such interaction may proceed from antigen-independent reversible binding to antigen-stabilized specific interactions.

6. *Role of Basophils*

The role of the basophil in delayed-onset hypersensitivities is the subject of an extensive recent review (*Askenase,* 1977). Basophils are prominent in those flat, evanescent DH reactions occurring after immunization with soluble proteins in saline or in IFA (Jones-Mote reactions) as well as in long-lasting sensitivities to contact allergens or tissue grafts in both guinea pigs and man (*Richerson* et al., 1970; *Dvorak* et al., 1970, 1971; *Dvorak* et al., 1971, 1976).

These basophil-rich reactions were originally described by *Richerson* et al. (1970) as examples of *cutaneous basophil hypersensitivity* (CBH) to distinguish them from basophil-poor DH reactions to tuberculin. *Askenase* (1973) subsequently demonstrated that some basophil-associated, delayed-onset reactions in the guinea pig were actually mediated by 7S IgG_1 antibody (*Askenase* et al., 1976). Additional experiments have indicated that the presence or absence of basophils in a DH reaction is a locally determined phenomenon: animals immunized with a contact allergen in CFA have basophil-associated DH (CBH) to the contact allergen and DH to PPD (*Dvorak* et al., 1971; *Godfrey* and *Askenase,* unpublished observations). Finally, DH reactions to PPD in man contain few basophils (*Dvorak* et al., 1974a; *Askenase* and *Atwood,* 1976) making the original distinction between CBH and DH so unclear that the term CBH probably should be replaced by basophil-associated DH reaction (or in the case of serum-mediated reactions, basophil-associated serum-mediated hypersensitivity).

Since over 90% of the basophils teased from basophil-associated DH reactions 6–7 days after sensitization and two-thirds of the basophils from combined cutaneous reactions 6 weeks after sensitization showed no specificity for antigen (*Dvorak* et al., 1975), the basophil in DH reactions is, like the macrophage, not a primary antigen-specific cell. (Basophil antigenic specificity derives from adsorbed Ig; see Sect. III.B for futher discussion.) The presence of basophils in DH reactions is under the control of several factors including a specific lymphocyte-produced chemotactic factor (*Ward* et al., 1975), regulatory B cells (*Katz* et al., 1974b, 1975b) and T cells (*Askenase,* 1976). Active regulation of basophil infiltrates in DH reactions has been inferred from the observation that DH reactions to tuberculin passively transferred by isolated T lymphocytes showed heavy basophil infiltrates, although the DH reactions in the actively sensitized donors contained fewer than 1% basophils in the cellular infiltrate (*Askenase,*

1976). In other words, guinea pigs actively sensitized to PPD had T cells capable of mediating basophil-associated DH to this antigen whose activity was evident only when transferred to nonimmune recipients lacking antigen-induced regulatory or suppressor functions present in actively sensitized donors.

Several roles for the basophil in DH have been proposed. Basophils have plasminogen activator on their surfaces and in their granules (*Orenstein* and *Dvorak*, personal communication) and may be involved in removing fibrin deposits from sites of DH reactions (*Colvin* et al., 1973; *Colvin* and *Dvorak*, 1975).

Once basophils arrive at DH sites, further local administration of antigen causes immediate release of vasoactive mediators from basophil granules, a process that could be involved in reactions to metazoan parasites (*Askenase*, 1977). Further hypotheses on the role basophils play in DH reactions (reviewed by *Askenase*, 1977) are based on the known effects of histamine on various T cell regulator and effector subpopulations, and on vascular permeability to fluids and to cells.

C. Nature and Specificity of Antigen Receptors

Evidence from a wide variety of experiments supports the hypothesis that the antigen receptor of the B lymphocyte is aurface-bound Ig (*Ada*, 1970; *Paul*, 1970; *Vitetta* and *Uhr*, 1975). B cells have been shown to bear Ig of a single specificity which is identical to that of the antibody they produce upon activation (*Raff* et al., 1973; *Wigzell*, 1973). The chemical nature and precise specificity of T cell receptors are not known mainly because the specific antigen receptors are cell bound and not secreted as antibodies are. For these reasons, studies of the "elusive" T cell receptor (*Crone* et al., 1972) have had to rely on indirect approaches. These indirect approaches have included the use of various specific antisera to block the function of T cells active in DH, studies of the detailed antigen specificity of T cell responses, and comparison of these T cell responses with the antigen specificity of the B cell receptor (often studies of serum antibodies rather than of B lymphocytes as such).

1. Criteria for T Lymphocyte Antigen Receptors

The concept of cellular receptors, cell-bound materials whose combination with ligands leads to changes in cellular metabolism, is a general one in physiology and biochemistry. The explanation of T cell responses as a result of the combination of antigen and specific cell-bound receptors is not very different from the explanation of other types of cellular response

to drugs or hormones. In dealing with antigen receptors, however, a distinction should be made between primary ones, synthesized by the T cell itself, and secondary receptors, synthesized by other cells and passively adsorbed to the T cell. Regulatory lymphokines (*Asherson* and *Zembala,* 1976) and Ig are examples of antigen-specific substances which may act as secondary T lymphocyte antigen receptors. Any substance proposed as a primary T cell antigen receptor would have to meet certain criteria based on the known properties and antigen specificity of T-lymphocyte-mediated reactions before it would be generally accepted as such. Such criteria would include the following (*Wigzell,* 1973; *Marchalonis,* 1975):

a) It should be synthesized by the cells themselves and not be passively adsorbed from plasma or other tissue fluids.

b) It should be detectable on the (external) plasma membrane of specifically reactive T lymphocytes.

c) It should possess specific binding capacity for antigens which reproduces the functional specificity of in vivo and in vitro T lymphocyte responses.

d) The combination of antigen with surface T cell antigen receptor should initiate specific processes of immune response.

e) Antibodies to the T cell antigen receptor should block T lymphocyte response by a mechanism which does not involve their direct combination with antigen.

With these points in mind, we can continue the discussion of the nature of T cell antigen receptors.

2. *Role of Surface Immunoglobulin*

Surface Ig is readily demonstrable on B cells (reviewed by *Marchalonis,* 1975); the majority of workers have had difficulty demonstrating it on T cells (*Crone* et al., 1972; *Wigzell,* 1973). In vitro, after removal of surface Ig on B cells by complexing with antigen, it is regenerated during culture (*Pernis,* et al., 1971). When comparable experiments were performed using activated thoracic-duct lymphocytes from irradiated mice (consisting almost exclusively of T lymphocytes), surface Ig was removed but not regenerated during an 18 h culture period in vitro (*Pernis* et al., 1974). This observation suggested that T cells were able to bind Ig passively by its F_c piece to a cell-surface receptor. Similar conclusions were reached by *Jensenius* (1976) who showed that the amount of antigen-specific surface Ig was markedly reduced on T lymphocytes from bursectomized chickens in comparison with that present on T lymphocytes of normal chickens. Several groups have reported that subpopulations of T cells can passively bind IgG or IgM to their cell surface by means of F_c receptors (*Stout* et al., 1976; *Rubin* et al., 1976; *Moretta* et al., 1977). It is on the basis of

this evidence that many workers in this field believe that most T cell Ig is passively adsorbed rather than actively synthesized (*Vitetta* and *Uhr, 1975).*

A second means of establishing the presence of Ig on lymphocytes is by determining whether antisera to Ig will block antigen binding or other T lymphocyte functions. The fact that antiserum to a particular cell membrane component will inhibit lymphocyte functions does not necessarily show that the molecule in question is the antigen receptor; *Bluestein* (1974) was able to show that Fab monomers of anti-H antibodies combined with T lymphocytes but did not block antigen-induced blast transformation. On the other hand, $F(ab)_2$ fragments or complete Ig did block proliferation, in accord with the observations of *Shevach* and *Rosenthal* (1973). This result suggested that the process of inhibition was associated with aggregation of specific surface molecules rather than with inhibition of antigen binding. *Bluestein* also found that $F(ab)_2$ dimers inhibited this T cell dependent response even when added to the cultures up to 3 h after antigen, again suggesting that the observed inhibition did not reflect interference with T cell antigen receptors. Most reports of inhibitory effects of anti-Ig on T cell antigen-binding or function have not been investigated to the degree reported by *Bluestein* and the exact mechanism of the observed inhibition is open to doubt. Not all anti-Ig sera are effective in inhibiting reactions even in the hands of workers who have demonstrated inhibition (*Hogg* and *Greaves,* 1972). Despite numerous reports of inhibition of T-cell-mediated reactions by anti-Ig (mostly anti-κ light chain sera) (cited by *Marchalonis,* 1975), these observations remain controversial, especially in view of the observations that a) some delayed-onset reactions are mediated by antibodies (see Sections III and IV) and b) certain aspects of in vitro models of non-antibody-mediated delayed-onset hypersensitivities may be associated with B cells (see Sect. V.5.3.c). Many workers have been unable to demonstrate any effect of anti-Ig sera on T cell functions (e.g., *Crone* et al., 1972; *Sprent* and *Miller,* 1972a; *Vitetta* and *Uhr,* 1975; *Godfrey,* 1976a) and clearly, rigid criteria for the specificity of the Ig sera need to be applied, since the antigens used to raise these sera may contain cell products not known about, not tested for, and not absorbed against (*Wettstein* et al., 1976).

3. Antigen Specificity of T Lymphocyte Responses

One of the reasons for doubt of the role of Ig as a primary T lymphocyte receptor stems from the markedly different specificities for antigen of B cell receptors (i.e., serum antibodies) and T cell receptors, as manifested in various T cell responses in vivo and in vitro. Guinea pigs, following immunization with hapten-protein conjugates in CFA, develop antibodies

capable of recognizing haptens conjugated to other proteins (hapten-specific antibodies) and DH specific for the immunizing conjugate and for the unmodified carrier protein (carrier specific DH) (*Gell* and *Benacerraf*, 1961a). The animals also develop hapten-specific, antibody-mediated delayed-onset hypersensitivity (*Askenase*, 1973). *Gell* and *Benacerraf*'s results were consistent with the hypothesis that the receptors of cells mediating DH recognized larger determinants than did those of B cells, though they also suggested that recognition might depend on the successive responses of two or more kinds of cell.

Schlossman and his associates (reviewed by *Schlossman*, 1972) investigated the ability of a related series of DNP-polylysines to induce and elicit B and T cell mediated responses. They found that ϵ-DNP-trilysine and longer DNP-polylysines could combine with ϵ-DNP-lysine specific antibodies to mediate passive cutaneous anaphylaxis but could not induce production of these antibodies; i.e., these small molecules were not immunogenic. The shortest material that elicited specific antibodies was the heptamer, ϵ-DNP $(lys)_7$. This material was also active in the T-cell-mediated reactions of antigen-induced blast transformation and elicited the production of the lymphokine, MIF. *Schlossman* concluded that DNP-specific antibodies recognized smaller portions of the eliciting antigen than did T lymphocyte antigen receptors. He also calculated that the difference in the energy of binding between antibody and DNP-hexalysine or DNP-heptalysine was only 9% greater for the latter than the former, and that factors other than binding energy must account fot the marked difference in biologic activity of the two materials. Further evidence of the difference in specificity of serum antibodies and T cell-mediated reactions was found when it was shown that immunization with α-DNP-$(lys)_7$ and ϵ-DNP-$(lys)_7$ led to the production of DNP-specific antibodies in both cases which were completely cross-reactive, while the DH reactions in vivo and in vitro showed specificity for the immunizing antigen and could distinguish between α-DNP and ϵ-DNP. These results again indicate that the T cell antigen receptor of DH must recognize part of the carrier as well as the hapten. Further evidence that the T lymphocyte receptor recognizes a portion of the carrier molecule as well as the hapten was obtained by *Janeway* and *Paul* (1973). They immunized guinea pigs with hapten-conjugated mycobacteria, a method developed by *Benacerraf* and *Gell* (1959a,b) to study DH which could be elicited by hapten on various carrier proteins. *Janeway* and coworkers showed this type of DH to be not truly hapten-specific since it could only be elicited by haptens on certain carriers. This DH was not elicited by dinitrophenylated mixed polymers of glutamic acid and lysine nor was it elicited by protein conjugates containing DNP-ala-$(gly)_2$. Both of these conjugates combined readily with anti-DNP antibodies and inhibited the binding of ϵ-DNP-lysine to them competitively in low concentrations

(*Janeway,* 1976). Guinea pigs immunized with DNP-mycobacteria did have T cells which recognized ε-DNP-lysyl and DNP-O-tyrosyl since DNP conjugates of mixed polymers of L-glutamic acid, L-alanine and L-lysine or L-glutamic acid, L-alanine and L-tyrosine elicited DH in these animals and the response to the tyrosine-containing antigen was reversibly lost after treatment which selectively removed DNP from tyrosine (*Janeway* et al., 1975, 1976a). Unconjugated mixed polymers did not elicit DH, an indication that the T cell receptor recognized the hapten as well as the carrier. The ability of hapten-conjugates other than the one used for sensitization to elicit T-cell-mediated reactions may not therefore be sufficient to call a response hapten-specific, since conjugation may generate antigenic determinants (epitopes) larger than the hapten itself (*Rubin* and *Aasted,* 1973; *Rubin,* 1974). It is important to distinguish true specificity to the carrier (i.e., to an epitope present on the carrier as a native molecule) from this type of carrier-involved specificity.

Janeway and *Paul* (1976) found that cells recognizing ε-DNP-lysyl and DNP-O-tyrosyl groupings were independent, distinct lymphocyte subpopulations. Peritoneal lymphocytes from inbred guinea pigs sensitized with DNP-mycobacteria were cultured with or without antigen for 3 days, 5-bromodeoxyuridine added on day 2 and the cultures exposed to light 24 h later. This procedure prevents further DNA synthesis by cells actively synthesizing DNA (*Zoschke* and *Bach,* 1970). The cells were then washed and recultured with or without antigen. It was possible to show by this method that the cells responding to PPD were different from those responding to DNP conjugates, and those recognizing ε-DNP-lysyl-conjugates were different from those recognizing DNP-O-tyrosyl.

An additional difference in the specificity of B and T cell receptors is their differential response to dinitrophenylated mixed polypeptides of D-glutamic acid and D-lysine (DNP-D-GL). *Davie* et al. (1972) showed that repeated injections of this material led to long-lasting central suppression of antibody response. *Cohen* et al. (1973a) then demonstrated that DH to DNP-GPA was not affected by previous injections of DNP-D-GL which suppressed the DNP-specific antibody response. *Benacerraf* and *Katz* (1974) similarly showed that DNP-D-GL had no effect on the development or elicitation of contact sensitivity to dinitrochlorobenzene and *Janeway* (1976) confirmed that DNP-D-GL inhibits antibody response to DNP conjugates without affecting T-lymphocyte-mediated responses. These results may again suggest that the T lymphocyte receptor does not recognize only the hapten of hapten-conjugates. However, since conjugates of D-GL and haptens are generally tolerogenic only for B cells, these results may imply that tolerance induction in T and B cells occurs by two different mechanisms (*Bullock* et al., 1975).

The question of the existence of hapten-specific T cells is not completely resolved. *Rubin* and *Wigzell* (1973) were able to deplete helper T cells using cell chromatography over a hapten-protein conjugate different from the one used for immunization. *Doughty* and *Klinman* (1973) also found low numbers of hapten-specific T cells in mice immunized with hapten-protein conjugates. Studies using p-azobenzenearsonate-N-acetyl-L-tyrosine (ABA-tyr) suggested that hapten-specific T cells were generated in response to immunization with this chemical (*Leskowitz* et al., 1967; *Alkan* et al., 1972; *Hanna* and *Leskowitz,* 1973). However, the DH generated was not to p-azobenzenearsonyl (ABA) alone as primed cells responded to ABA only on certain carriers (*Collotti* and *Leskowitz,* 1970). After immunization of guinea pigs with a reactive DNP sensitizer in CFA, under conditions where little DNP antibody was induced, *Godfrey* (1976b, and unplublished observations) showed that hapten-dependent T cells mediating lymphokine release were generated. Lymphokine release by these cells in response to DNP-containing materials was blocked by DNP-glycine. *Godfrey* and *Gell* (1976) were subseqeuntly able to isolate these hapten-dependent cells on DNP-polyacrylamide beads and elute them with DNP glycine; TNP-polyacrylamide beads did not bind lymphokine-releasing cells but did bind DNP-rosette-forming cells. These observations were consistent with the conclusion that these hapten-dependent T cells were hapten-specific. On the other hand, DNP-rosette formation could be inhibited by an anti-guinea pig κ chain antiserum (*Gell* and *Godfrey,* 1974; *Godfrey,* 1976a) as well as by DNP glycine. It was concluded that the hapten-specific receptors of these DNP-rosette-forming cells were similar to serum antibodies. Many workers have been able to bind T cells to small haptenic molecules (*Rutishauser* and *Edelman,* 1972; *Möller,* 1974; *Roelants* and *Ryden,* 1974; *Roelants* et al., 1974) but no functional studies have been carried out on these cells. Recently *Burakoff* et al. (1976) have described hapten-specific T-cell-mediated cytolytic activity in mouse cells sensitized to TNP-conjugated cells of one H-2 type in vitro and tested with TNP-conjugated, histoincompatible cells. However, using a similar cytolytic system, *Rehn* et al. (1976) found the specificity of receptors to be against larger antigenic determinants than the haptenic molecule alone. In general, it has been difficult to document the existence of T cells recognizing only the hapten and no part of the carrier of hapten-carrier conjugates (*Janeway* et al., 1976a).

T cell responses to related haptens such as TNP and DNP can often be more specific than serum antibodies to these compounds (*Benacerraf* and *Gell,* 1959a,b; *Little* and *Eisen,* 1969; *Fleischman* and *Eisen,* 1975; *Godfrey,* 1976a,b). In clear-cut T-lymphocyte-mediated responses such as contact sensitivity (*Asherson* and *Zembala,* 1974; *Godfrey,* 1976a,b; *Claman,* 1976) or cell-mediated cytolysis (*Forman,* 1977; *Wagner,* personal com-

munication), TNP- and DNP-dependent DH reactions show strict specificity and few cross-reactions between these two haptenic groupings are found. These reports indicate that T cell antigen receptors are as able as B cell antigen receptors to distinguish subtle differences between antigens.

Certain observations interpreted as indicating that antigen recognition by T cells is less specific than antigen recognition by B cells may be open to other interpretations. For example, *Janeway* and *Paul* (1976) found that sensitization of guinea pigs with DNP-mycobacteria induced sensitized cells that were equally stimulated by DNP- and TNP-containing conjugates but it is not clear that the same populations of cells recognized both haptens. A somewhat similar situation occurs when guinea pigs are immunized with reactive DNP compounds in CFA (*Godfrey* and *Baer*, 1971) or with DNP conjugates (*Gell* and *Benacerraf*, 1961b; *Fleischman* and *Eisen*, 1975). These animals develop delayed-onset hypersensitivities to both DNP and TNP conjugates. In this case, responses to TNP conjugates may represent a non-T-cell response to antigen, mediated either by cytophilic antibodies (*Askenase*, 1973) or by B cells with Ig antigen receptors (*McMaster* et al., 1977b) since the antigen specificity of the response resembles that of serum antibodies raised against DNP which show considerable cross reactivity for TNP (*Little* and *Eisen*, 1969). A third type of experiment purporting to show the lesser specificity of T cell antigen recognition as compared to B cell specificity has involved studies on the immune response to mammalian erythrocytes (*Hoffman* and *Kappler*, 1972; *Falkoff* and *Kettman*, 1972; *Parish*, 1972b) or *Salmonella* flagellins (*Parish*, 1972a; *Parish* and *Liew*, 1972). Rather than indicating that antigen recognition by T cells is less specific than that of B cells, the observed differences in T and B cell specificity to these complex antigens may be due to T and B cells recognizing completely different epitopes on the molecule (*Langman*, 1972).

As an example of the last point, *Senyk* et al. (1971) have analyzed the immune response to a simple natural antigen, bovine glucagon. Immunization of guinea pigs with bovine glucagon and synthetic and tryptic peptides related to it showed that most of the antibody raised against this 29 amino acid peptide was directed against the amino-terminal part of the molecule, while most of the DH was directed against the carboxy-terminal part of the antigen. This difference in recognition of epitopes between B and T cells may be due to factors such as mode of presentation or route of administration, and not solely to differences in the determinant B and T cells are able to recognize. For example, in the case of bovine glucagon injected in CFA, small amounts of antibodies were found with specificity for the carboxy-terminal portion of the molecule and a small amount of DH was directed against its amino-terminal end. If the molecule was conjugated to a carrier and injected in CFA, antibodies to both determinants were elicited but no DH was found (*Senyk* et al., 1972).

Studies of guinea pigs immunized with simple chemicals such as ABA-tyr and L-tyrosine-p-azophenyl-trimethylammonium chloride have shown that immunization causes clonal expansion of both B and T cells specific for a given determinant but that the mode of administration of the determinant activates preferentially one or the other class (*Prange* et al., 1977a). These conclusions are similar to those derived from the analysis of the immune response to glucagon. Injection of the simple chemicals themselves in CFA induced DH, no serum antibody, but caused increases in the numbers of B and T cells which reacted with them. Injection of conjugates of the simple chemicals with proteins induced serum antibodies as well as DH; expansion of specific clones of B and T cells was only two-fold larger than that observed after immunization with the simple chemicals alone. It is clear that while B cell and T cell receptors can recognize the antigen itself, expression of this recognition depends on other conditions associated with induction of the immune response.

Further evidence for similarities of antigen recognition sites between B and T cells was obtained by *Rajewsky* and co-workers (*Rajewsky* and *Pohlit,* 1971; *Rajewsky* and *Mohr,* 1974) after studying the cross-induction and cross-tolerance of various albumins at the cellular level. They concluded that both B and T cells could recognize the same epitopes with the same refined specificity, a conclusion also reached by others (*Weinbaum* et al., 1974).

To summarize, T cells and B cells appear to recognize antigens somewhat differently. The data indicate that the antigen receptors of T lymphocytes are able to discriminate between closely related epitopes as well as can those of B lymphocytes. Furthermore, T cells and B cells can recognize the same determinants, i.e., they have similar "dictionaries" of antigens to which they are able to respond (*Roelants* et al., 1974). T cells may recognize larger antigenic groupings than do B cells and this ability is reflected in the specificity of their antigen receptors. Alternatively, T and B cells may recognize large epitopes equally well, but B cells can also recognize smaller ones.

4. Role of the Major Histocompatibility Complex in Antigen Recognition by T Lymphocytes Involved in Delayed Hypersensitivity

a) **Regulation of Immune Responses by Genes Located in the Major Histocompatibility Complex.** Studies of the induction of DH in genetically defined animal strains to structurally simple antigens such as synthetic polypeptides have shown that certain strains fail to respond to a specific antigen. For example, guinea pigs of the Sewell-Wright strain 2 respond to random copolymers of glutamic acid and alanine (GA) but not to copolymers of glutamic acid and tyrosine (GT) while strain 13 guinea pigs respond

to GT and not to GA (*Bluestein* et al., 1971; reviewed by *Benacerraf* and *McDevitt,* 1972). Similar deficiencies in immune response are seen in mice (*Benacerraf,* 1973) and have been observed in response to more than 40 antigens. The ability to respond to a specific antigen is genetically dominant to the inability to respond and is ascribed to the presence of specific immune response (Ir) genes in responding strains; the Ir genes map in the I region of the MHC (*Benacerraf,* 1973). Recent studies in mice have indicated that in addition to Ir genes, I regions contain other genes which regulate specific suppressive responses (e.g., *Debre* et al., 1975). Ir genes have been found in all the higher vertebrates where they have been sought and their presence is inferred in man (*Munro* and *Bright,* 1976).

The H antigen encoded in the I region are termed *Ia* antigens. In guinea pigs and mice, it has been shown that an intimate association exists between Ir gene product function and Ia antigens, as response to antigens controlled by Ir genes is specifically blocked by alloantisera to Ia antigens (*Shevach* et al., 1972; *Schwartz* et al., 1976). These studies have been recently extended to show that associations exist between specific Ir genes and individual Ia specificities in the guinea pig (*Shevach* et al., 1977).

b) Recognition of Major Histocompatibility Antigens by T Lymphocyte Subpopulations. T cells which proliferate in MLR in mice respond to differences in Ia antigens; a comparable situation exists in man (*Bach* et al., 1972a,b; *Schwartz* et al., 1976). In mice, these T lymphocytes belong to the Ly-1$^+$ subpopulation, a T cell subpopulation containing cells active in DH to proteins or heterologous erythrocytes, cells able to secrete lymphokines in response to antigenic stimulation, and helper or amplifier regulatory cells (*Cantor* and *Boyse,* 1975a; *Jandinski* et al., 1976; *Newman* et al., 1978). There is evidence that some of these functions may be represented by distinct Ly-1$^+$ subgroups (*Vadas* et al., 1976). MLR in man has similarly been associated with a specific T cell population (*Evans* et al., 1977).

Cytotoxic T lymphocytes recognize different H antigenic determinants from those recognized by Ly-1$^+$ lymphocytes, since adsorption of lymphoid cells on HLA-bearing monolayers abolishes subsequent cytotoxic activity without affecting subsequent MLR activity (*Zoschke* and *Bach,* 1971). Cytotoxic T lymphocytes respond to differences in H antigens encoded in the K and D regions of the MHC. These T cells belong to the Ly-2$^+$3$^+$ subpopulation, a subpopulation which also contains suppressor regulatory cells and cells able to secrete lymphokines in response to antigenic stimulation (*Cantor* and *Boyse,* 1975a; *Huber* et al., 1976b; *Newman* et al., 1978).

c) Participation of Major Histocompatibility in T Lymphocyte Antigen Recognition. Several lines of evidence indicate that as many as 4%–12% of unprimed lymphocytes are specifically reactive to allogeneic antigens

encoded by the MHC (*Simonsen,* 1967; *Wilson* et al., 1968; *Atkins* and *Ford,* 1975; *Ford* et al., 1975). The frequency of T lymphocytes responding to cell-surface alloantigens encoded by the MHC is 100–1000 times greater than the frequency of T cells responding to other antigens. For example, only 0.003%–0.12% of the cells in an unstimulated mouse lymph-node cell population were precursors to CL recognizing TNP-conjugated target cells (*Lindahl* and *Wilson,* 1977b), a number similar to that obtained for cells from unstimulated animals able to bind radioactive TNP (*Roelants* and *Ryden,* 1974; *Roelants* et al., 1974). The same authors estimated that 0.4%–1% of unprimed lymph-node cells were precursor cells to CL with specificity for an H antigen encoded by the MHC; this fraction represented a substantial proportion of the precursor cells present, given the diversity of allogeneic H antigens. Solely on the basis of the available numbers of T lymphocytes, it has been suggested that the same clones of T cells that recognize MHC alloantigens also recognized conventional antigens (*Simonsen,* 1967; *Heber-Katz* and *Wilson,* 1976; *Wilson* et al., 1977).

Recent experimental evidence has confirmed that T cell recognition of "conventional" (non-MHC) antigens is linked to MHC gene products. Mouse T-CL derived by conventional in vivo primary procedures and nominally specific for target cells either modified by virus infection (*Doherty* and *Zinkernagel,* 1975; *Zinkernagel* and *Doherty,* 1975; *Koszinowski* and *Thomssen,* 1975), reactive chemicals (*Shearer* et al., 1975; reviewed by *Shearer* et al., 1976) or differing in minor histocompatibility antigens (*Bevan,* 1975; *Gordon* et al., 1976) expressed their lytic activity only when effector and target cells had the same H2K or H2D MHC antigens. Similarly, cytolysis of tumor cells by syngeneic T-CL required recognition of MHC antigens as well as tumor-specific antigens (*Germain* et al., 1975; *Schrader* and *Edelman,* 1975). This association between recognition of antigen and MHC-encoded antigens might derive from the necessity for antigens recognized in the secondary, eliciting phase of the immune response to be the same as those recognized in the primary, inducing phase. Evidence for this hypothesis was obtained by in vitro sensitization of T lymphocytes from irradiated F_1 mice reconstituted with bone marrow from one of the parental strains (*Pfizenmaier* et al., 1976; *Zinkernagel,* 1976; *von Boehmer* and *Hass,* 1976). As a result of development in the F_1 environment, the parental cells were tolerant of the H antigens of the other parent and could be sensitized to virally infected or chemically modified cells of the tolerated allogeneic antigens. T-CL generated after such sensitization preferentially lysed suitably modified allogeneic rather than syngeneic cells. The cell whose MHC encoded antigens are chemically modified may be a macrophage since conjugated peritoneal macrophages but no peritoneal lymphocytes could elicit in vitro responses to contact sensitizers (*Thomas* et al., 1977a).

A similar association between that recognition of conventional antigens and MHC encoded antigens was found by *Miller* et al. (1975b, 1976, 1977) in their studies on the transfer of DH between different strains of mice. DH to protein or polypeptide antigens could be transferred only between mouse strains syngeneic for the I region of the MHC while the transfer of DH to contact sensitizers required identity at either I, K, or D regions. These MHC constraints presumably arose from the MHC context of primary sensitization of T cells: if F_1 thymocytes were sensitized in nude mice of one parental strain, they could passively transfer DH only to recipient mice of that parental strain and not to recipient mice of the other strain. Control experiments made it unlikely the inability to transfer DH was due to rejection of the transferred cells, loss into areas such as the spleen or recruitment into MLR (*Vadas* et al., 1977). The MHC constraints are likely to function at the level of interaction between antigen-presenting macrophages of the recipient at the test site and antigen-sensitive donor T lymphocytes. This interpretation is consistent with the observation that F_1 mice heterozygous for a given Ir gene could transfer DH only to recipients with the responder parental genotype (*Vadas* et al., 1977) and not to recipients of nonresponder parental genotype. If the Ir gene functioned at the T cell antigen receptor level, one would expect that F_1 animals would have receptors capable of recognizing antigens in association with nonresponder MHC antigens. Similar conclusions were reached by *Shevach* (1976) using an in vitro assay of T cell activation.

There is further evidence that MHC restriction in secondary DH responses arises from the requirement of antigen presentation to T lymphocytes by macrophages. *Paul* et al. (1977) have found that there are independent populations of antigen-reactive T lymphocytes that can be activated to undergo proliferation in response to antigen-pulsed parental macrophages. Since MHC antigens of both parental types occur on F_1 macrophages, these results suggest that each T lymphocyte population was primed by macrophage-presented antigen associated with MHC coded antigens of one or the other parental type. Secondary response of such T cells occurs only with macrophage-presented antigen associated with the same parental histocompatibility type as was seen in the primary response. Additional support for this concept comes from the work of *Thomas* and *Shevach* (1977) who showed that T lymphocytes of inbred guinea pigs could be primed in vitro by allogeneic TNP-macrophages, if T lymphocytes reactive to MHC differences were deleted; these primed cells subsequently responded only to TNP-allogeneic macrophages with the same MHC type used for priming and not to TNP-conjugated syngeneic macrophages.

Using this same experimental protocol, *Thomas* et al. (1977b) found that antisera directed against macrophage Ia antigens eliminated the response of the primed T cells to TNP-allogeneic macrophages, while antisera

directed against responder T cell Ia antigens were without effect. These findings suggested that macrophage Ia antigens were more important for efficient T cell-macrophage interaction than T cell Ia antigens. In fact, inactivation of Ia^+ T cells with the appropriate antisera and complement either before or after priming had no effect on the ability of the remaining T lymphocytes to be primed or restimulated with TNP-conjugated macrophages. *Yamashita* and *Shevach* (1977b) subsequently reported that a subpopulation of peritoneal exudate macrophages which displayed Ia antigens were of critical importance in presenting antigens to T lymphocytes: after removal of this subpopulation with anti-Ia sera and complement, the remaining macrophages, though actively phagocytic, were markedly deficient in their ability to present antigen to sensitized T lymphocytes and to function as stimulator cells in MLR. This observation again emphasized the critical importance of macrophage Ia antigens in the activation of T cells to protein and cell-associated antigens.

T lymphocyte antigen recognition therefore seems to involve both recognition of antigenic determinants and the MHC antigens in which context these other antigens are originally presented to the T lymphocyte by the macrophage.

5. *Chemical Nature of T Lymphocyte Antigen Receptor*

Since T lymphocytes recognize conventional antigens in the context of MHC gene products, it might be supposed that the T lymphocyte antigen receptor consists of two genetically separate receptor molecules rather than a single one (*Wilson* et al., 1977; *Janeway* et al., 1976b). Evidence as to the number of antigen-recognition structures on the T lymphocyte surface is incomplete and the question remains unresolved at present. However, some studies with T-CL recognizing TNP-modified cells suggest a single receptor may be sufficient for cellular cytolysis to occur (*Zinkernagel,* 1976; *Pfizenmaier* et al., 1976).

It is now generally accepted that the antigen receptor of T lymphocytes is not an Ig molecule with identifiable κ or λ light chains and that T lymphocytes do not bear endogenously secreted surface Ig (*Binz* and *Wigzell,* 1976). On the other hand, several laboratories have demonstrated that serum antibodies and T lymphocyte receptors directed against the same antigen are coded for by at least one of the same variable gene subsets (*Binz* and *Wigzell,* 1975a,b,c; *Binz* et al., 1975, 1976; *Eichmann* and *Rajewsky,* 1975; *Black* et al., 1976; *Hämmerling* et al., 1976; *Binz* and *Wigzell,* 1976; *A.F. Geczy* et al., 1976; *Krawinkel* et al., 1977a,b; *Prange* et al., 1977b). These experiments have usually utilized anti-idiotype antibodies directed against the unique determinants of a particular V region of an antibody molecule, presumably those at or near the antigen binding

site (*Hopper* and *Nisonoff*, 1971), but antibodies against other V region antigens have also been used successfully.

Binz and *Wigzell* (1975a) showed that rat IgG anti-idiotype antibodies raised against rat antibodies specific for a rat alloantigen, bound to T lymphocytes, and blocked GVH reactions in vivo and MLR in vitro. These results suggested the presence on T lymphocytes of receptor molecules with V regions similar to those on Ig of the same specificity. *A.F. Geczy* et al. (1976) reached similar conclusions using strain-specific, guinea pig anti-idiotype antibodies raised against antibodies to hapten-protein conjugates; such anti-idiotype antibodies were absorbed by T lymphocytes from hapten-sensitized guinea pigs and blocked antigen-induced proliferation by T lymphocytes. The results of *Eichmann* and *Rajewsky* (1975) also showed similarity of V regions between mouse serum antibodies and T cell antigen receptors but in this case guinea pig IgG_1 anti-idiotype antibodies were found to induce sensitivity to a streptococcal antigen rather than suppress it. *Binz* and *Wigzell* (1976) have found that cultured normal T cells shed molecules which bind to immunoabsorbants made with the same anti-idiotypic antibodies against rat alloantigen antibodies which inhibited MLR; these T-lymphocyte-derived molecules also bind specifically to rat alloantigens. This antigen binding material was found to contain three molecular species on gel electrophoresis, of molecular weights 150,000, 70,000 and 30,000–40,000. The largest species consisted of disulfide-bonded dimers of the 70,000 dalton species, while the smallest material appeared to represent degradation products of the 70,000 dalton species. The only detectable antigenic markers on the 150,000 dalton molecules were variable-region Ig (anti-idiotypic); immunoabsorbants with antibodies to conventional polyvalent Ig antigens, or κ, λ, or Ia antigens did not bind these molecules. *Rajewsky* and co-workers (*Krawinkel* et al., 1977a,b) also found that isolated shed antigen receptors from T lymphocytes did not show antigenic markers for conventional Ig, light chains or H antigens.

6. Comment

We can summarize this section by stating that the T lymphocytes of DH are able to recognize a wide variety of protein antigens and H antigens with similar subtlety of discrimination as B lymphocytes. The primary antigen receptor of these T cells is not Ig but is coded for by the same variable region genes used by B cells for Ig. T lymphocytes initially recognize "conventional" antigens in association with H antigens; on subsequent antigen exposure T cells will respond to these antigens only if they are presented in the same context of H antigens as initially. This linkage of recognition of H antigens and "conventional" antigens by T cells is in contrast to antigen recognition by B cells which does not depend on any associated recognition of H antigens.

D. Histopathology of Delayed Hypersensitivity Reactions

The histopathology of DH reactions is somewhat variable, both from species to species and from tissue to tissue within a given species, according to the techniques used. Typical cutaneous lesions of DH in guinea pigs and man 24 h after testing are composed predominantly of perivascular infiltrates of mononuclear cells in the deep dermis, often with more macrophages than lymphocytes (*Gell*, 1959; *Turk*, 1975; *Cohen*, 1977). In mice, however, the cellular infiltrate of DH contains many polymorphonuclear leucocytes (*Crowle*, 1975). Lymphocytes are the predominant infiltrating cell in DH reactions associated with experimental autoimmune throiditis or adrenalitis (*Werdelin* and *McCluskey*, 1971). Basophils, though prominent in cutaneous DH reactions to contact sensitizers in guinea pigs and man (*Dvorak* et al., 1971; *Dvorak* et al., 1974a), are found to a much smaller extent in other organs (*Dvorak* et al., 1977).

Common to all DH reactions, however, is the local interaction of a small number of sensitized lymphocytes with specific antigen which leads to the generation of an inflammatory response at the reaction site. In the following sections we will touch on some of the more prominent histopathologic characteristics of cutaneous DH reactions in guinea pigs and man.

1. Cellular Infiltrates in the Dermis

Cutaneous DH reactions in guinea pig or man, whether elicited by contact allergens or intracutaneously injected antigens have been described as beginning with perivascular infiltrates of polymorphs and mononuclear cells 4–8 h after testing (*Gell* and *Hinde*, 1951; *Waksman*, 1960; *Boughton* and *Spector*, 1963; *Turk* et al., 1966a; *Dvorak* et al., 1974a), leading over the next few hours to mononuclear predominance, presumably owing to the retention of mononuclear cells. The distribution of these initial infiltrates depends on antigen distribution (*Waksman*, 1960; *A.M. Dvorak* et al. 1976). In man, intracutaneously injected antigens are associated with more intense reactions in the deep dermis while contact allergens are associated with an initial perivenular and perivenous cuffing of mononuclear cells in the superficial dermis (*Dvorak* et al., 1974a). The vessels of the superficial dermis which are involved in DH reactions belong to the superficial venular plexus, a vascular system oriented parallel to the skin surface and located at the juncture of the (upper) capillary dermis and (lower) reticular dermis (*A.M. Dvorak* et al., 1976). Perivascular infiltrates increase progressively in intensity and extent, reaching their maxima at 12–24 h in the guinea pig and at 2 days for intradermal antigens or 3–6 days for contact allergens in man (*Dvorak* et al., 1974a; *Turk*, 1975). In both guinea pig and man, macrophages are prominent in the mononuclear cell

infiltrate in response to intracutaneous antigen while small and medium lymphocytes predominate after contact allergen exposure. In guinea pig contact reactions, the infiltrating mononuclear cells tend to form a diffuse band in the upper dermis immediately under the epidermis, rather than remaining localized to the perivenular areas as in man. Although it is difficult to separate mononuclear cells into lymphocytes and macrophages in skin sections on purely morphologic grounds in light microscopy, such differentiation is possible if special histochemical techniques or electron microscopy are used. With these techniques, it was found that perivascular mononuclear cell infiltrates in guinea pig DH reactions contained many more macrophages than did comparable infiltrates in man (*Goldberg* et al., 1962; *Turk* et al., 1966a,b; *Dvorak* et al., 1974a).

The localization of intervascular mononuclear infiltrates depends on antigen localization, intracutaneous antigen being associated with infiltrates extending into the deep dermis and subcutis while contact allergens are associated with heavy infiltrates in the superficial dermis and epidermis (*Dvorak* et al., 1974a; *A.M. Dvorak* et al., 1976; *H.F. Dvorak* et al., 1976).

In man and guinea pig, basophilic leukocytes are also often present in the dermal cellular infiltrate of DH reactions as early as 8 h, initially localized to the perivascular cuff. They are commonly present at 24 h and reach a maximum at 72 h (*Richerson* et al., 1970; *Dvorak* et al., 1970, 1971, 1974a; *Dvorak* and *Mihm,* 1972; *Askenase,* 1977). In the guinea pig, basophils are commonly seen in DH reactions occurring after immunizations with low doses of antigen in saline or in IFA, in contact sensitivity and in rejection of tumors and tissue grafts, while they are less common in DH reactions elicited by microbial antigens such as PPD. Basophilic infiltrates in guinea pig DH reactions are localized to the upper dermis where they may account for 90% of the infiltrating cells (*Askenase,* 1977). These infiltrates do not correspond exactly to the distribution of antigen in the skin (*Stadecker* and *Leskowitz,* 1973). In man, in contrast to the guinea pig, no more than 15% of the infiltrating cells of contact reactions are basophils, basophils are frequently present in DH reactions to microbial antigens and are randomly distributed in the intervascular dermis (*Dvorak* et al., 1974a; *Askenase* and *Atwood,* 1976). Clearly, the significant differences in the frequency and localization of basophils in the cellular infiltrates of DH both in man and guinea pigs suggests that classifications of DH based on tissue basophilia may be of limited usefulness (see also Sect. V.B.6 for further discussion of basophils in DH reactions). Limited electron microscopic observations suggest a slow degranulation of basophils in human contact dermatitis; 40% of basophils were found fully granulated in 24 h reactions and less than 10% degranulated while these frequencies had more than reversed in 72 h reactions (*H.F. Dvorak* et al., 1976).

2. *Cellular Infiltrates in the Epidermis and Epidermal Cell Alterations*

In DH reactions to intracutaneously injected antigens, there are fewer infiltrating cells in the epidermis than in the dermis. *Dienes* and *Mallory* (1932) found evidence of cell death in the basal layer between 7 and 12 h after testing sensitized guinea pigs and by 24 h small numbers of mononuclear celsl had infiltrated the epidermis. Polymorphonuclear leukocyte infiltration was most intense near areas of degenerating basal cells. *Turk* (1975) mentions a marked thickening of the epidermis at the site of strong DH reactions to tuberculin. In man, *Dvorak* et al. (1974a) found relatively mild epidermal changes during DH reactions to injected antigens in comparison to events in the dermis: patchy zones of intercellular edema were common as were small mononuclear cell infiltrates, while focal zones of vesiculation and granulocyte infiltrates were not; 30%–50% of the reactions, showed epidermal thickening at the site of the reaction. Epidermal thickening has been thought to result from epidermal and dermal damage (*Turk,* 1975).

During DH reactions to contact sensitizers there are striking alterations in the epidermis. There are focal zones of intercellular edema 6–8 h after contact testing in both guinea pig and human reactions involving primarily the Malphigian layer and to a lesser extent the basal layer of the epidermis; in man changes in hair follicle epithelium and sweat ducts occur earlier and are more severe than in other parts of the epidermis (*Dvorak* et al., 1974a; *Turk,* 1975). At about this time (6–8 h), mononuclear cells appear in the epidermis of guinea pig and man; in both species, the mononuclear cell infiltrates are composed primarily of lymphocytes. Mononuclear cell infiltration of the epidermis is maximal at about 24 h in the guinea pig and at 72 h in man (*Dvorak* et al., 1974a; *Turk,* 1975). Basophils are sometimes present in the epidermal infiltrate and can comprise up to 20% of the infiltrating cells but polymorphs are rare in the absence of significant epidermal necrosis. Human contact reactions usually show grossly visible vesicle formation, guinea pig reactions microscopic vesicle formation (*Fisher* and *Cooke,* 1958; *H.F. Dvorak* et al., 1976). The epidermis is probably a main site of hapten attachment and is certainly severely damaged as a result of the immune reaction (*Godfrey,* unpublished observations). By 48 h in the guinea pig (*Flax* and *Caulfield,* 1963) and by 72 h in man (*Dvorak* et al., 1974a), increased cell division in the basal layer is noted with the production of epidermal hyperplasia and hyperkeratosis.

3. *Microvascular Changes*

Voisin and *Toulet* (1960, 1963) showed that increases in capillary permeability to serum proteins occur at the site of DH reactions. These observations

have been repeatedly confirmed (*Willms-Kretschmer* et al., 1967; *Wiener* et al., 1967; *Morely* et al., 1972; *Colvin* and *Dvorak,* 1975). Vascular leakage attributable to specific immunologic reactivities is first detected 6–12 h after skin test in the guinea pig, becomes maximal at 18–24 h and remains increased even at 48 h. The vascular permeability changes are not affected by conventional antihistamines which block H-1 histamine receptors (*Askenase,* 1977). In DH reactions of man, *A.M. Dvorak* et al. (1976) have demonstrated the formation of gaps between the endothelial cells of the superficial capillary-venules (vessel loops in the upper dermis which connect arteriolar and venular branches of the superficial vascular plexus) as a likely cause of the increased vascular permeability. This increased permeability is likely to account for the edema seen in the upper dermis (*Black* et al., 1963; *Dvorak* et al., 1974a), as well as the extreme vascular compaction and erythrocyte extravasation noted mainly in superficial capillary-venules but also in the superficial venular plexus. Another result of the increased vascular permeabiltiy in DH is the leakage of fibrinogen into sites of DH reactions (*Colvin* and *Dvorak,* 1975). *Dvorak* and co-workers (*Dvorak* and *Mihm,* 1972; *Colvin* et al., 1973; *Dvorak* et al., 1974a; *Colvin* and *Dvorak,* 1975) established that fibrin deposition in the intervascular portion of the dermis was a regular feature of DH reactions in man and guinea pig. Fibrin was not observed in the perivascular zones of the superficial venous plexus which contained the highest density of infiltrating cells nor was it present in blood vessel walls. Its distribution was therefore quite different from the intraluminal thrombi and vessel wall fibrin deposits that have been described in antibody-mediated hypersensitivities (*Peters,* 1975). Extravascular fibrin was detectable as early as 4–8 h after testing using fluorescent antibody techniques in both guinea pig and human DH reactions and was regularly detectable at 24 h using improved light microscopic techniques. No deposition of Ig or complement components was seen in DH reactions. Fibrin deposition is likely to play an important role in DH reactions since anticoagulants are known to inhibit the expression of these reactions in animals (*Cohen* et al., 1967; *Schwartz* and *Leskowitz,* 1969). The local induration seen in DH reactions may be a result of the insertion of a meshwork of hydrophilic immobile molecules in the dermal ground substance between the collagen bundles (*Colvin* and *Dvorak,* 1975). Since DH reactions containing many basophils are grossly less indurated than other DH reactions and contain less fibrinogen (*Colvin* and *Dvorak,* 1975), it might be hypothesized that basophils contain plasminogen activators capable of activating tissue fibrinolysis; such basophil-associated plasminogen activators have been recently observed by *Orenstein* and *Dvorak* (personal communication). The mechanism by which clotting is initiated in the tissues is unknown.

During DH reactions in the skin, the endothelial cells of the vessels which are the site of perivascular cuffing by lymphocytes, become swollen and increase in height and show increase in organelles, especially ribosomes (*Gell,* 1959; *Willms-Kretschmer* et al., 1967; *Wiener* et al., 1967; *Dvorak* et al., 1976). The enlarged endothelial cells bulge into the vessel lumen, sometimes compromising it. Necrosis of endothelial cells sometimes occurs (*Waksman,* 1963). Autoradiographic study of endothelial proliferation in DH reactions in guinea pigs has documented activation of venule endothelium and synthesis of DNA within 12 h of skin testing (*Polverini* et al., 1977).

4. Comment

The histopathology of DH reactions indicates that they are more than simple infiltrations of lymphocytes and macrophages, and that basophils, the microvasculature, and the clotting system all have roles in producing the gross lesion. The local induration of DH in particular is likely to be a result of the presence of extravascular fibrin rather than of cellular infiltration.

E. Lymphokines

Lymphokines are nonimmunoglobulin effector molecules generated by activated lymphocytes (*Dumonde* et al., 1969). They are generated as a result of lymphocyte activation by mitogens, soluble protein antigens, or various cell-associated antigens (such as H antigens, tumor-specific antigens, contact sensitizers, viruses) (*David* et al., 1964a; *Ruddle* and *Waksman,* 1968; *Granger* and *Kolb,* 1968; *Kronman* et al., 1969; *Malmgren* et al., 1969; *Geczy* and *Baumgarten,* 1970; *David* and *David,* 1972; *Evans* and *Alexander,* 1972; *Godfrey,* 1976b). Recent studies have indicated that synthesis and secretion of effector molecules may be a general property of activated cells and the terms monokines (for products of activated monocytes-macrophages) and cytokines (for products of activated nonlymphoid cells) have been proposed (*Waksman* and *Namba,* 1976).

As a result of the definition of various mediator substances on the basis of their cell type of origin or on the basis of biologic activity, it is not yet certain how many chemically distinct lymphokines there may be for each activity and rigid classification is premature. However, several of these mediators have been purified, some to the degree that antibodies have been raised against them so that their role in DH in vivo is beginning to be clarified. A very general classification of lymphokines on the basis of biologic activities is, however, helpful in discussing them. On such classification and some examples of each class might be:

1) Inflammatory, including factors active in vivo (skin reactive factor) or acting on inflammatory cells in vitro (MIF, macrophage agglutination factor, macrophage activation factor, various chemotactic factors).

2) Cytotoxic (lymphotoxin).

3) Mitogenic (mitogenic factor).

4) Regulatory (various factors enhancing or suppressing antibody and DH responses).

This is not an exhaustive classification; e.g., interferon, an anti-viral factor released by lymphocytes after antigenic or mitogenic stimulation, has been omitted (*Epstein* et al., 1974). There is, however, some evidence that it may function as a regulatory lymphokine (*Johnson* et al., 1975).

1. Characterization of Lymphokines

Lymphokines are proteins, synthesized de novo after lymphocyte activation and lymphokine activities are destroyed by proteolytic enzymes (*Bloom* and *Glade*, 1971). If guinea pig lymphocytes are treated with actinomycin D (an inhibitor of DNA-dependent RNA synthesis) or puromycin (an inhibitor or protein synthesis) before antigen or mitogen stimulation, they do not then produce MIF. *Sorg* and *Geczy* (1976) have shown that a specific anti-guinea pig lymphokine antibody combined only with newly synthesized proteins present in stimulated lymphocyte culture fluids.

Lymphokines with the same biologic activity often differ chemically and physically from species to species. For example, guinea pig and mouse MIF are glycoproteins as judged by isopycnic centrifugation while human MIF is not (*Remold* et al., 1970; *Rocklin* et al., 1972; *Kühner* and *David*, 1976). Furthermore, guinea pig and mouse MIF activity is localized to molecules of 65,000 daltons and/or of 45,000 daltons (*Sorg* and *Geczy*, 1976; *Kühner* and *David*, 1976; *Remold* and *Mednis*, 1977) while human MIF is associated with a single molecular species of 25,000 daltons (*Rocklin* et al., 1972). The activity of lymphotoxin, a cytotoxic lymphokine, is associated with a protein of about 43,000 daltons in mouse and guinea pig preparations (*Goguel* and *Nauciel*, 1974; *Gately* and *Mayer*, 1974; *Trivers* et al., 1976); in human material, such activity is associated with molecules of 80,000–90,000, 50,000 and 10,000–15,000 daltons (*Hiserodt* et al., 1976). A similar diversity in estimated molecular weights has been found in molecules having activity as macrophage chemotactic factors (discussed in *Leonard* and *Meltzer*, 1976); in this case activity in murine preparations is associated with molecules of about 40,000 daltons, while activity in human and guinea pig preparations is associated with molecules of 13,000 daltons.

Despite this variation in the chemical and molecular properties of lymphokines from species to species, within a given species a given activity

usually has the same molecular attributes independent of the inducing stimulus. Human monocyte chemotactic factor had similar physicochemical characteristics whether it was derived from B cells or T cells (*Altman* et al., 1975). Antisera prepared against antigen-induced guinea pig MIF adsorbed mitogen-induced guinea pig MIF (*Geczy* et al., 1975). *C.L. Gately* et al. (1975) have also observed that antigen-induced and mitogen-induced lymphotoxin were physically as well as antigenically identical. On the other hand, *C.L. Geczy* (1977) found that antigen-induced mitogenic factor in guinea pigs was physically and antigenically distinct from mitogenic factor generated during MLR.

2. Antigens Expressed on Lymphokines

Lymphokines from different species with similar biologic activities are usually antigenically different. *Sorg* and *Geczy* (1976) found that rabbit anti-guinea pig lymphokine antibody did not cross-react with products of activated human or murine lymphocytes. *M.K. Gately* et al. (1975) showed that antibody to guinea pig lymphotoxin did not neutralize mouse lymphotoxin. In contrast to the species specificity of rodent lymphokines, human MIF can be assayed using guinea pig macrophages (*Thor* et al., 1968), monkey MIF released by virus infected cells has been reported to cross-react with guinea pig MIF (*Yoshida* et al., 1975) and human lymphotoxin affects cells of human and rodent origin (*Granger* and *Jeffes*, 1976). As a result of improved isolation techniques and the availability of specific antisera, the various lymphokine activities of lymphocyte culture supernatants have been separated from each other. Guinea pig macrophage chemotactic factor, MLR-mitogenic factor, antigen-induced mitogenic factor, lymphotoxin, MIF and macrophage agglutination factor are antigenically distinct molecules with molecular weights ranging from 13,000 to over 100,000 (*Gately* and *Mayer*, 1974; *C.L. Gately* et al., 1975, 1976; *M.K. Gately* et al., 1975; *G.L. Geczy* et al., 1975, 1976a,b; *Postlethwaite* and *Kang*, 1976; *Kurasuji* et al., 1976; *Geczy*, 1977; *Godfrey* and *Geczy*, unpublished observations). In other species, however, isolation procedures have indicated that multiple lymphokine activities may be associated with the same molecule. This, human basophil and monocyte chemotactic factor activity are not separable on gel filtration or ion exchange chromatography (*Lett-Brown* et al., 1976) and are thought to be activities of the same molecule. Studies of human and rat lymphotoxin (*Jeffes* and *Granger*, 1976; *Namba* and *Waksman*, 1976) have suggested that lymphotoxin, cloning inhibitory factor and proliferation inhibitory factor are functionally similar and associated with the same molecules. In the mouse, it has not been possible to separate MIF activity from a soluble factor which suppresses the immune response in vitro (*Tadakuma* et al., 1976), while

in the guinea pig *Nathan* et al. (1973) were unable to separate MIF activity from macrophage activation activity.

Regulatory lymphokines in mice (soluble factors associated with the regulation of various immune responses) have been shown to bear histocompatibility determinants coded for by the I region of the MHC. They can be adsorbed to immunoabsorbants containing the appropriate anti-Ia antibodies. Such lymphokines include a specific T cell factor which suppresses contact sensitivity (*Zembala* et al., 1977; *Greene* et al., 1977b), several specific T cell factors which suppress IgE or IgG antibody formation (*Okumura* and *Tada,* 1974; *Takenori* and *Tada,* 1975; *Taniguchi* et al., 1976; *Kapp* et al., 1976; *Thèze* et al., 1977), a T-cell-replacing factor for antibody production (*Taussig* et al., 1975) and a factor which suppresses resistance to tumors (*Greene* et al., 1977a). Antigens coded for by the MHC have not been noted to be present on any of the more purified preparations of lymphokines associated with cytolysis or the inflammatory response. None of the various types of lymphokines have been found to display antigenic determinants found on Ig.

3. Antigenic Specificity of Lymphokines

The majority of inflammatory lymphokines as well as the cytotoxic lymphokine, lymphotoxin, show no specificity for antigen (*Turk,* 1975). However, several workers have reported that MIF activity is potentiated by specific antigen (*Bennett* and *Bloom,* 1967; *Švejcar* et al., 1968) or requires the presence of specific antigen (*Amos* and *Lachmann,* 1970; *Lowe* and *Lachmann,* 1974) suggesting the existence of antigen-dependent MIF. Antigen-dependent MIF has been reported to be associated with molecules of 50,000 daltons molecular weight, much smaller than IgG (*Lowe* and *Lachmann,* 1974). *Evans* and *Alexander* (1972) have described an antigen-specific factor produced by lymphocytes which arms macrophages for specific cytolysis of tumor cells. Studies by *Torisu* et al. (1973) have indicated that the lymphokine eosinophil chemotactic factor is associated with specific antigen when secreted in a reactive precursor form but that specific antigen is lost during the process of activation.

In contrast to cytolytic or inflammatory lymphokines, lymphokines associated with suppression of DH or regulation of antibody response are regularly antigen specific and have been isolated using insolubilized antigens (*Okumura* and *Tada,* 1974; *Zembala* et al., 1975, 1977; *Taussig* et al., 1975, 1976; *Takenori* and *Tada,* 1976; *Kapp* et al., 1976; *Theze* et al., 1977; *Greene* et al., 1977a). These regulatory lymphokines, 30,000–50,000 daltons molecular weight, differ from the majority of inflammatory lymphokines in two ways: they are specific for antigen and possess antigens coded for by the MHC (see also Sect. V.B.4). It is yet not known if

antigen specific lymphokines have idiotypic determinants similar to serum antibodies of the same specificity (*Taussig* et al., 1976).

4. Role of Lymphokines in vivo

Direct evidence for the contention that lymphokines mediate DH reactions in vivo has been difficult to obtain. Intradermal injection of antigen-stimulated lymphocyte culture supernates into normal recipients results in an inflammatory reaction whose histopathology resembles that of DH (*Bennett* and *Bloom,* 1968; *Pick* et al., 1969); this reaction was ascribed to an inflammatory lymphokine, skin-reactive factor.

Several lymphokines have been found to be produced in vivo after antigenic stimulation. For example, 24 h after PPD injection of sheep sensitized to BCG, a bovine mycobacterium, *Hay* et al. (1973) found strong MIF and mitogenic factor activity in the lymph draining the skin test site. Similarly, *Postlethwaite* and co-workers (*Postlethwaite* and *Synderman,* 1975; *Postlethwaite* et al., 1976) were able to demonstrate the presence of macrophage chemotactic factor and MIF in peritoneal fluids of guinea pigs with DH to horseradish peroxidase which had been challenged intraperitoneally; these lymphokines were similar in molecular weight, susceptibility to proteases and heat stability to lymphocyte-derived macrophage chemotactic factors generated in vitro. Macrophage and lymphocyte chemotactic factors have also been obtained by extraction of sites of DH to various protein antigens or to contact allergens (*Cohen* et al., 1973; *Baba* et al., 1977; *Kambara* et al., 1977). These extracts were active in vitro and caused macrophage accumulation when injected into normal guinea pigs. Surprisingly, MIF activity has not been detected in extracts of DH reaction sites, perhaps because the small amount of material produced and released in the in vivo reaction is rapidly adsorbed onto cells or tissue components or inactivated. Any of these possibilities would account for the inability to demonstrate MIF using the currently available extraction procedures.

Anti-lymphokine antisera have proved to be useful tools in exploring the relation of lymphokine activity to observed in vivo responses. For example. antisera against a guinea pig lymphokine fraction with MIF, mitogenic factor, and skin reactive factor activity neutralized MIF in vitro and inhibited DH to PPD or contact sensitizers when injected intracutaneously, intravenously or intraperitoneally (*C.L. Geczy* et al., 1975, 1976a). This anti-lymphokine antiserum caused a reduction in mononuclear cell infiltrates at the specific DH reaction site, but did not affect nonspecific inflammation evoked by intracutaneously injected turpentine. The mechanism of antilymphokine sera in inhibiting DH was not the same as that of a crude antilymphocyte serum which caused marked changes in the

T dependent areas of the lymph nodes. Similarly, an anti-guinea pig lymphokine serum which neutralized MIF, macrophage chemotactic factor and skin reactive factor was found to inhibit DH reactions when injected in vivo (*Yoshida* et al., 1974, 1975b; *Kurasuji* et al., 1976). These observations strongly implicate lymphokine involvement in the expression of DH in vivo.

Despite the implication of lymphokines in the mediation of DH reactions, it is still unknown what regulates the release of a given factor and how this release is controlled. *Cohen* (1977) has suggested that different kinds of DH reactions may result from different patterns of lymphokine activity in each. He has also pointed out that if regulatory mechanisms for lymphokine release did not exist, an initial triggering mitogenic event could cause an endless cycle of production, release and stimulation of lymphokines, since mitogenic activation can trigger lymphokine production (*C.L. Gately* et al., 1976). The study of these problems has just begun and investigation of the regulatory factors of T cells and B cells involved in DH constitutes an important area of study at present (*Asherson* and *Zembala,* 1976; *Cohen* and *Yoshida,* 1977).

F. Cytotoxic T Lymphocytes

T-CL generated as a result of sensitization with cell-associated antigens mediate cytolytic DH associated with allograft destruction and play an important role in immunity to tumors (see reviews by *Perlmann* and *Holm,* 1969; *Häyry* et al., 1972; *Henney,* 1973; *Cerottini* and *Brunner,* 1974) and viruses (*Koszinowski* and *Thomssen,* 1975; *Zinkernagel* and *Doherty,* 1975; *Zinkernagel,* 1976; *Perrin* et al., 1977). They presumably also underlie the epidermal injury seen in contact sensitivity reactions (*Shearer* et al., 1976). Specific T-CL are rapidly generated after antigenic exposure: cytotoxic activity can be isolated from allografts 1–2 days after transplantation (*Roberts,* 1977), is detectable in the draining lymph node within 2–4 days after allografting (*Roberts,* 1975) and is found in peripheral blood lymphocytes 2–4 days after infection with vaccinia virus (*Koszinowski* and *Thomssen,* 1975; *Perrin* et al., 1977).

The exact mechanism by which T-CL cause target cell death is not known (*Cerottini* and *Brunner,* 1974). It has been shown that target cell lysis requires direct contact between living, metabolically active T-CL and target cells and that T-CL activity can be reversibly inhibited by cytochalasin B (*Brunner* et al., 1968; *Perlmann* and *Holm,* 1969; *Cerottini* and *Brunner,* 1974). Cellular cytolytic activity is temperature dependent and does not occur at 4°C, nor does it depend on radiation-sensitive processes such as cell division (*Henney,* 1973). It has also been shown that T-CL

activity does not require complement (*Henney,* 1973) and is not associated with the release of the cytolytic lymphokine, lymphotoxin (*M.K. Gately* et al., 1975). *Mayer* (1977) has suggested that the mechanism of action of T-CL in causing irreversible changes in the permeability of the target cell plasma membrane (*Perlmann* and *Holm,* 1969) may bear a formal similarity to the mechanism of cell lysis mediated by antibody and complement. For example, both lytic activities are one-hit processes (i.e., interaction of a single sensitized T-CL with a target cell is sufficient to kill that cell) (*Wilson,* 1965; *Cerottini* and *Brunner,* 1974) and both lytic processes require the presence of divalent cations (*Henney,* 1973). It may prove possible to extend the analogy between these two lytic processes in the future as our knowledge of the mechanism of cell lysis by T-CL expands.

VI. Transfer Factor

A. Definition and Characterization

Transfer factor (TF) is a dialysable extract of sensitized human leucocytes which specifically transfers skin test reactivity (DH) from a positive donor to a previously negative recipient (*Lawrence,* 1955; *Lawrence* et al., 1963; *Lawrence,* 1969, 1974). It is still an unsettled point, whether material similar to TF is found in cell-free extracts of lymphoid cells of laboratory animals such as the guinea pig or rat (*Bloom* and *Chase,* 1967; *Burger* and *Jeter,* 1971; *Liburd* et al., 1972; *Dunnick* and *Bach,* 1975, 1977). On the other hand, several laboratories have found that TF-like material can be prepared from lymphocytes of nonhuman primates (*Maddison* et al., 1972; *Zanelli* and *Adler,* 1975). TF activity of human leucocyte extracts can be determined in vivo using as recipients either nonhuman primates or man (*Zanelli* and *Adler,* 1975; *Steele* et al., 1976). Various in vitro assays for TF have been proposed whereby TF activity has been measured by its ability to induce naive lymphocytes to respond to specific antigen by undergoing blast transformation (*Ascher* et al., 1974) or producing MIF (*Dunnick* and *Bach,* 1975; *Rytel* et al., 1975). *Gallin* and *Kirkpatrick* (1974) noted that activity of leucocyte lysates correlated with chemotactic activity of the lysates. It has subsequently been shown that TF is not capable of activating naive lymphocytes to undergo transformation in response to antigen, but, in fact, TF only augments precommited cells to respond to antigen (*Cohen* et al., 1976; *Burger* et al., 1976a). However, *Burger* et al. (1976b) found that dermal reactivity of TF in vivo was well correlated with augmentation activity in blast transformation assays in vitro and were able to use this assay in their fractionation of leucocyte lysates. Human TF contains many biologic activities besides the ability to

transfer DH. It has been used to reconstitute immunodificient patients (*Wybran* et al., 1973; *Valdimarsson* et al., 1974). TF also contains substances which suppress lymphocyte responses to antigens and mitogens in vitro (*Burger* et al., 1976c). The diversity of biologic activities in TF may reflect a corresponding number of distinct components or a smaller number of components with multiple activities. It may be that the activity of antigen-specific components of TF is only evident in in vivo assays using primates or human beings with essentially normal thymus function. Under other in vivo assay conditions or in vitro, the activity of antigen non-specific components present in TF preparations may predominate.

Fractionation of dialyzable TF has shown that donor-specific transfer activity is associated with molecules of less than 5000 daltons molecular weight whose activity is eliminated by pronase but not diminished by pancreatic DNAase, RNAase or typsin (*Arala-Chaves* et al., 1967; *Lawrence,* 1969, 1974; *Gottlieb* et al., 1973; *Burger* et al., 1976b; see also *Ascher* et al., 1976, for extensive discussions on fractionation of transfer factor). The highest transfer activity resides in the fraction with the highest 254 nm/280 nm ultraviolet absorption; dermal transfer activity is thought to be associated with nucleotide and peptide-containing material (*Gottlieb* et al., 1973; *Burger* et al., 1976b). *Vandenbark* et al. (1977) have found dermal transfer activity associated with 254 nm-absorbing material with an isoelectric point of 1.6, and able to enter Sephadex G-25; these properties are consitent with those of small polynucleotides. *Dunnick* and *Bach* (1977) found a guinea pig analog of TF to be a small molecule susceptible to pronase and phosphodiesterase containing amino acids and phosphate. It was not possible to state definitely if this material contained ribose or was destroyed by ribonuclease.

B. Therapeutic Use

Dialyzable TF has been used clinically to treat a variety of diseases characterized by reduced T lymphocyte reactivity. These deficits can be relatively specific, as was seen in about a third of a group of patients with chronic mucocutaneous candidiasis (*Kirkpatrick* and *Smith,* 1974) or more generalized, as seen in patients with Wiscott-Aldrich syndrome, ataxia telangiectasia or dysgammaglobulinemia (*Lawrence,* 1974). Since TF transfers only DH and does not transfer antibody induction, it has been used in the treatment of malignancies, where T cell function is often depressed (*Fudenberg,* 1976) and where induced antitumor antibodies might enhance tumor growth and thereby have an adverse effect on the patient.

The therapeutic use of TF has been most effective when it has been used as an adjunct to other forms of therapy. In patients with mucocutaneous

candidiasis who had experienced relapses after protracted antibiotic therapy, *Kirkpatrick* and *Smith* (1974) found that TF alone was not an efficacious therapeutic agent. When TF derived from donors with strong DH to Candida antigens was combined with antibiotic therapy, however, long term remissions were induced. TF has been also used as adjuvant therapy to surgery in the treatment of osteogenic sarcoma (*Ivins* et al., 1976; *Fudenberg*, 1976; *Byers* et al., 1976). *Levin* et al. (1973) reported clinical improvement only when patients received TF derived from donors shown by in vitro tests to have cytotoxic cells against osteogenic sarcoma cell lines. TF has also been used with clinical benefit in adjunctive therapy of malignant melanoma (*Spitler* et al., 1976). Because of these encouraging therapeutic results, double blind studies would appear warranted.

Dialyzable TF also been used to treat a wide range of illnesses of infectious or possible infectious etiology with some clinical improvement (*Lawrence*, 1974). These diseases include juvenile rheumatoid arthritis, Behçet's disease, miliary tuberculosis, lepromatous lerosy and multiple sclerosis. These studies have been limited to a few patients without double-blind design; it is not clear whether TF would be generally useful in the treatment of these diseases.

C. Comment

Dialyzable TF contains several biologically active materials which influence DH in vivo and in vitro by various antigen-specific and nonspecific mechanisms. The therapeutic efficacy of this material is only roughly correlated with its ability to transfer DH to antigens associated with a given disease process (*Kirkpatrick* and *Smith*, 1974; *Spitler*, 1976); it is unclear which of the many biologically active materials present in the leucocyte lysate is actually effective. The mechanism by which a small, 2000 dalton nucleotide-containing peptide can transfer specific DH is equally unclear.

VII. Conclusions and Summary

Delayed-onset hypersensitivities represent a heterogeneous group of vascular-inflammatory and cytolytic responses with various mechanisms of initiation. These reactions are associated with the same cell types – granulocytes, macrophages, lymphocytes, mast cells – whether the initiating mechanism is the interaction of antigen and (cell-bound) antibody or the interaction of antigen and small numbers of sensitized T lymphocytes as seen in "classic" Type IV DH. The initiating event is followed by the assembly of cells from the circulation in the tissues, which presumably accounts for the delayed-onset kinetics.

Some of the vascular changes seen in delayed-onset hypersensitivities may be associated with the release of vasoactive amines which affect pharmacologic receptors different from those active in anaphylactic "immediate-type" hypersensitivities (*Askenase,* 1977). Since the accumulation of fluid and serum proteins at the site of Type IV DH reactions in guinea pigs and man is not consistently inhibited by pharmacologic agents which interfere with the actions of vasoactive amines, there must be additional mechanisms in operation which remain to be elucidated.

Fibrin, derived from extravasated fibrinogen, is found in all types of delayed-onset hypersensitivity reactions to some extent. It is present in the largest amounts, however, in classic DH reactions. The hydrophilic fibrin in the tissues is able to bind and localize extravasated fluid and is thought to be the underlying cause of the typical localized induration seen in these reactions. The mechanisms controlling the conversion of fibrinogen to fibrin in the tissues and the extent of extravascular fibrin formed remain unclear.

Since Type IV DH ractions are inflammatory states, they are therefore unique to the intact organism. In vitro models of DH have permitted some analysis of the complex interactions between the various cell types present in vivo and have led to the discovery and partial characterization of various humoral factors associated with the in vivo reaction. The macrophage has a dual role in DH: it processes and presents antigen to T lymphocytes and is in turn affected by various lymphokines, nonantibody mediators generated by activated lymphocytes. Both T and B lymphocytes can release lymphokines in response to antigenic stimulation, and the release of lymphokines by both cell types is controlled by regulatory T and B cells. Functionally and antigenically distinct T cell subpopulations have been shown to exist in several species; e.g., T cells mediating vascular-inflammatory DH to proteins and specific cellular cytolysis in mice belong to different T cell subpopulations. However, the subpopulation of T cells which releases inflammatory lymphokines in response to protein antigens is likely to be the same subpopulation which releases a nonspecifically cytotoxic lymphokine, while the subpopulation of T cells to which T-CL belong can release inflammatory lymphokines in response to stimulation with cell-associated antigens (*Newman* et al., 1978). It is not yet clear whether lymphokines with the same activity released by different T cell subpopulations in a given species differ in molecular size or antigenicity. It is certain that lymphokines have a definite role in DH reactions in vivo: specific antibodies to some of them inhibit the expression of DH in sensitized animals and they can be isolated and purified from in vivo DH reactions. The precise regulation of lymphokine release is a biologic necessity in order to control the amounts of these highly active and potentially damaging materials in the tissues. Since the release of lymphokines can be

associated with a positive feedback loop, the mechanisms regulating the release of a given lymphokine at a given time and place in the organism as well as the number of lymphokines which a given cell can release are important questions which require further study.

There are still many unanswered questions as to the exact mechanism by which T cells involved in DH reactions recognize antigens. Recognition of epitopes by T cells is genetically controlled and is associated with recognition of histocompatibility antigens encoded in the genes of the major histocompatibility complex. The T cell antigen receptor is not immunoglobulin, but uses the same variable region genes for a given antigen as do antibodies against that antigen. Although B cells may be able to recognize smaller epitopes than can T cells and although T lymphocytes and B lymphocyte-produced antibodies can differ in their specificity for a given antigen, both populations are able to respond to the same antigens with similar degrees of specificity. Any observed differences in the ability of T and B lymphocytes to respond to antigen may reflect differences in presentation of antigen or not yet understood regulatory aspects of the immune responses of these cells.

The role of dialyzable transfer factor in human DH reactions and the mechanism whereby a small molecule (which by reason of its low molecular weight must contain limited genetic information) can transfer DH to complex antigens remains a mystery. Recent successes in the fractionation and characterization of this material should dispell some of these questions as well as rationalize its use in the treatment of human disease.

Despite the vast increase in our understanding of the delayed-onset hypersensitivities in recent years, many questions about the induction, manifestation and regulation of these inflammatory states remain. "When all questions are fully answered, the subject loses all scientific interest. Delayed hypersensitivity is still a very intersting subject" (*Gell*, 1967).

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DNA Replication in Eukaryotes

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Contents

1. Introduction

Replication of genetic material is one of the fundamental processes in biology and much effort has gone into elucidation of the detailed biochemical mechanism and the regulation of this reaction. However, there is still a considerable lack in our knowledge in this area of molecular biology which is mainly due to the fact that DNA synthesis takes place in an extremely fragile multicomponent complex which has so far resisted isolation in an intact form. Nevertheless, important progess has been made

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in recent years in the elucidation of the mechanism of synthesis of phage and plasmid DNA and it is hoped that the enzymology of replication of these simple genomes will be known in the near future. Combined biochemical and genetic studies have thrown some light on the composition of the multienzyme complex (the replication apparatus) within which the elongation of replication forks takes place; much less is known about how replication forks are initiated. All these studies revealed the important fact that the enzymology and the mechanism of replication even of these small genomes is surprisingly elaborate.

Progress in the elucidation of the mechanism and regulation of DNA replication in eukaryotes has been still slower. This is due to several reasons: The DNA content of eukaryotic cells is up to 1000 times higher than that of the bacterial cell and whereas chromosomes of viruses, bacteria and blue-green alga consist of single DNA molecules, the genome of eukaryotic cells is subdivided into several chromosomes where DNA is intimately associated with chromosomal proteins, particularly histones, which play an important role in the organization of the eukaryotic genome. These chromosomes have to be duplicated during each cell-division cycle. It is clear then that this process requires faithful duplication of DNA molecules, synthesis of the proper species and amounts of chromosomal proteins, and formation of chromosomes from a long DNA molecule and many different chromosomal proteins. The synthesis of DNA and of histones (and possibly also of some nonhistone chromosomal proteins) is strongly regulated and is confined to a particular period in the division cycle of eukaryotic cells that is called the S-phase. Additional mechanisms must exist which assure that the total genome is replicated once and only once in an S-phase. Replication of the eukaryotic genome and its regulation, therefore, must be expected to be considerably more complex than DNA duplication in bacteria.

A variety of systems have been used during the past few years to study the basic mechanism of DNA replication in eukaryotes. As in bacterial systems, much progress has come from studies on the replication of small genomes such as DNA viruses.

This review concentrates on problems connected with replication of eukaryotic DNA in chromatin, on synthesis of mitochondrial DNA, and on a process called gene amplification. Many aspects of the mechanism of DNA replication which are more relevant to bacterial and phage systems will be dealt with only marginally. For details on these systems the reader is refered to several excellent recent reviews (*Kornberg, A.*, 1974; *Gefter*, 1975; *Dressler*, 1975; *Edenberg* and *Huberman*, 1975; *Alberts* and *Sternglanz*, 1977).

Since replication of nuclear DNA in eukaryotic cells takes place in the S-phase of the cell cycle, the structure which is being duplicated is the interphase chromatin; I therefore want to begin this review with a summary of basic features of chromatin structure.

2. Structure of Chromatin

2.1 Constituents of Chromatin

The DNA helix inside the nuclei of eukaryotic cells is coiled in a precise, nonrandom manner in interphase chromatin and in mitotic chromosomes. The simplest form of coiling is verified in the chromatin subunit called the nucleosome (see below) and the most complicated form in metaphase chromosomes. Chromosomes and chromatin are composed of DNA and a variety of chromosomal proteins which can be divided into basic histones and nonhistone chromosomal proteins. Histones are the predominant group of proteins in chromatin. In nearly all organisms studied so far (including unicellular eukaryotes) the histone to DNA ratio is approximately 1:1. Most cells contain five different histones, some of which have a very conservative amino acid sequence (*De Lange* and *Smith,* 1971; *Elgin* and *Weintraub,* 1975).

Histones are involved in the folding of DNA in chromatin subunits and in the formation of higher order chromatin structures. The basic unit of chromatin, the nucleosome (*Oudet* et al., 1975) or nu-body (*Olins* and *Olins,* 1974), is a roughly spherical particle which contains about 140 base pairs of coiled DNA (*Hewish* and *Burgoyne,* 1973b; *Noll,* 1974a; *Axel,* 1975; *Soliner-Webb* and *Felsenfeld,* 1975; *Shaw* et al., 1976) and two each of the small, conservative histones H2A, H2B, H3, H4 (*Kornberg, R.,* 1974; *Weintraub* et al., 1976; *Kornberg, R.,* 1977) (Fig. 1). Between individual nucleosomes there is a stretch of DNA with a variable length of 20–70 base pairs which is probably also coiled (*Compton* et al., 1976; *Noll,* 1976; *Lohr* et al., 1977a). This generates a jointed flexible chain,

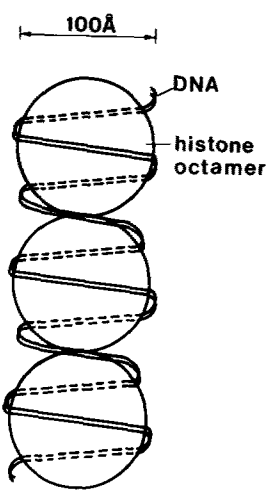

Fig. 1. Nucleosome structure of chromatin showing histone octamers around which the DNA is wound. This drawing must be considered highly schematic because neither the form of the histone octamer nor the path of the DNA are yet known in detail. In fact, recent data suggest that the histone octamer may look more like a flat cylinder rather than a simple sphere as drawn here

about 100 Å thick, of "beads on a string" (*Woodcock*, 1973; *Oudet* et al., 1975; *Woodcock* et al., 1976). Such a fiber has been seen in electron microscope studies of eukaryotic nuclei. The 100 Å chromatin fiber compacts into a 200–300 Å fiber in the presence of salt and bivalent cations (*Du Praw*, 1970; *Brasch* et al., 1971; *Finch* and *Klug*, 1976). This 200–300 Å chromatin fiber must itself be compacted further in metaphase chromosomes (*Du Praw*, 1970; *Carlson* and *Olins*, 1976; *Carpenter* et al., 1976; *Bak* et al., 1977). The role of the fifth histone, H1, is not absolutely clear yet. Biochemical and nuclear magnetic resonance studies suggested that histone H1 binds to the outside of the chromatin subunits (*Varshavsky* et al., 1976; *Puigdomenech* et al., 1976; *Whitlock* and *Simpson*, 1976a; *Noll* and *Kornberg*, 1977; *Renz* et al., 1977). Removal of histone H1 exposes 20–70 base pairs of DNA between the nucleosomes and produces a transition in the structure of chromatin fibers which leads to the appearance of "beads on a string" with the individual nucleosomes separated from each other by distances of about 100–200 Å (*Worcel* and *Benyajati*, 1977; *Thoma* and *Koller*, 1977). The involvement of histone H1 in the supercoiling of chromatin fibers is also suggested by the observation that this histone is modified at particular times in the cell cycle. Thus, it was found that specific phosphorylation reactions occur on histone H1 at the time of condensation of chromatin fibers into metaphase chromosomes (*Lake*, 1973; *Balhorn* et al., 1972. 1975; *Bradbury* et al., 1973, 1974; *Gurley* et al., 1973, 1975).

The second class of chromosomal proteins, the nonhistones, is a very heterogeneous fraction of mostly acidic proteins (*Elgin* and *Weintraub*, 1975). These proteins play structural, enzymatic, and regulatory roles in the chromatin. They have a marked tendency to aggregate. This, together with the small amounts in which most nonhistone proteins occur in chromatin, rendered their isolation rather difficult. Up to 450 different species of nonhistone proteins are observed upon analysis in sodium dodecyl sulfate (SDS) polyacrylamide gels, depending on the source of chromatin and the resolving power of the method employed. Some of these proteins occur in considerably larger quantities than average and these may have structural functions (*Douvas* et al., 1975; *Chiu* et al., 1975). Among the nonhistone proteins having enzymatic properties are the enzymes involved in transcription and replication of the genetic material and also several other enzymes catalyzing various modifications of proteins (histones and nonhistones) and DNA. A further subset of nonhistone proteins may be involved in determining the specificity of transcription and the regulation of replication (*Blüthmann* et al., 1975). Depending on the method of isolation, the total fraction of nonhistone proteins may, however, also contain various proteins from nuclear membrane material as well as proteins associated with RNA. The latter function either in the transport of mRNA

from the nucleus to the cytoplasm or they represent ribosomal proteins which bind to ribosomal RNA precursors in nucleoli (*Elgin* and *Weintraub*, 1975).

2.2 Structure of Nucleosomes

Digestion of chromatin within nuclei by an endogeneous nuclease [first observed in rat liver nuclei (*Hewish* and *Burgoyne*, 1973a)] or by exogeneously added micrococcal nuclease (*Noll*, 1974a; *Sollner-Webb* and *Felsenfeld*, 1975; *Axel*, 1975; *Shaw* et al., 1976) leads to a fragmentation of the DNA as revealed by an analysis of the digestion products in polyacrylamide gels. The DNA fragments so produced migrate initially in a broad band corresponding to a size of around 200 base pairs. This DNA apparently includes the 140 base pairs of intranucleosomal DNA plus part of the DNA linking two nucleosomes. In addition, multiples of these fragments having lengths of about 400, 600, 800, etc., base pairs are seen which arise from dimers, trimers, tetramers, etc., of nucleosomes (*Finch* et al., 1975). Upon further degradation, the size of the monomer fragment is reduced until a relatively stable fragment appears which migrates as a sharp band containing about 140 base pairs of DNA. Upon prolonged incubation with nuclease most of the material accumulates at this stage before further digestion to even smaller fragments. The chromatin particle containing 140 base pairs of DNA still contains an octamer of the four conservative histones (two each of the histones H2A, H2B, H3 and H4) but no histone H1. This particle is often termed the "core particle" and the DNA which connects one core particle to the next and which is of variable length among different cell types is termed the "linker". The basic features of the core particle are found in chromatin of all eukaryotic cells studied so far, which includes several lower eukaryotes such as the yeast, *Saccharomyces cerevisiae* (*Rogall* and *Wintersberger*, 1975; *Thomas* and *Furber*, 1976; *Lohr* et al., 1977a), *Neurospora* (*Noll*, 1976), or *Aspergillus* (*Morris*, 1976). The core particle is roughly spherical and has a diameter of about 100 Å and a sedimentation coefficient of 11–11,5 S (*Longmore* and *Wooley*, 1975; *Olins* et al., 1976; *Whitlock* and *Simpson*, 1976b; *Weintraub* et al., 1976; *Bakayev* et al., 1977; *Finch* et al., 1977). Nuclease digestion and neutron scattering data suggest that the histone octamer is on the inside of the core particle while the DNA is wrapped around the histone core (see Fig. 1). The precise arrangement of the four histones within the octamer is not yet known. It was suggested that the octamer forms by protein-protein interactions between individual histones involving the hydrophobic C-terminal halves of the histone molecules and leaving the basic N-terminal halves free to interact with the DNA on the outside

of the histone core (*Bradbury,* 1976). This model is likely to be oversimplified, however, since current experimental findings indicate much more subtle interactions between histones and DNA. It was, for instance, observed that cleavage of the basic N-termini of histones with trypsin does not cause a release of DNA from the histone core nor does it result in gross changes in the nucleosome structure (*Whitlock* and *Simpson,* 1977). So far, it is also unknown how the 140 base pair stretch of DNA is wrapped around the histone core. Clues as to the path of the DNA in the nucleosome come from analyses of the products of digestion of chromatin with deoxyribonuclease I (*Noll,* 1974b; *Lohr* et al., 1977b; *Sollner-Webb* and *Felsenfeld,* 1977). The DNA fragments produced by such digestion form a series of bands corresponding to single-strand lengths of 10, 20, 30, etc., bases. This suggests that all of the DNA in a nucleosome is accessible to the cleavage by nucleases at intervals of 10 bases along both strands. The binding sites on the histones could be oriented such that 10 base pairs are covered, making the DNA accessible to nucleases as it passes from one binding site to the next. While simple continuous bending of the DNA is, therefore, possible and in accord with the experimental findings, the formation of "kinks" at intervals of 10 base pairs in the DNA has also been suggested (*Crick* and *Klug,* 1975; *Sobell* et al., 1976). It is assumed that both the arrangement of histones and the path followed by the DNA in the nucleosome possess a twofold axis of symmetry. This view is supported by the observation that the histone octamer may be composed of two histone tetramers (containing one histone each of H2A, H2B, H3, H4) which interact isologously in the nucleosome (*Weintraub* et al., 1975). The formation of nucleosomes is probably not sequence specific with respect to DNA (*Steinmetz* et al., 1975; *Polisky* and *McCarthy,* 1975; *Ponder* and *Crawford,* 1977). Nucleosomes do not migrate along a DNA molecule at a measurable rate under physiologic conditions (*Cremisi* et al., 1976b), however, they may easily do so under certain conditions in vitro. This has been born out by studies on a model chromatin; the minichromosomes formed by papovaviral DNA (the DNA from the small tumor viruses polyoma and SV 40) and histones. DNA from SV40 or polyoma virus occurs within the infected cell and in the virus particle as a nucleoprotein containing the four conservative histones from the host cell (*Griffith,* 1975; *Bellard* et al., 1976; *Cremisi* et al., 1976a,b). There ist still uncertainly whether these minichromosomes also include histone H1 in some stages. Studies on these minichromosomes have also revealed the important fact that the superhelical turns found in circular viral DNA are due to winding of DNA around the histone core in nucleosomes. Removal of protein from chromatin leads to formation of approximately one negative superhelical turn of DNA per nucleosome, indicating that DNA is coiled around the histones in a left handed sense (*Germond* et al., 1975; *Keller,* 1975*a*

Detailed structure of the basic units of chromatin is certainly of importance in considerations of the mechanism of transcription and some aspects of the replication of DNA within the nuclei of eukaryotic cells. More specific questions concerning, for instance, the interrelations between DNA and histone synthesis and aspects of chromatin formation from these constitutents will be dealt with in later chapters.

3. Chromatin Replication

3.1 General Features of Eukaryotic Chromosome Replication

In 1968, *Huberman* and *Riggs* proposed a scheme for the topology of chromosomal DNA replication which has obtained much support in recent years. This scheme now seems to be of widespread, if not universal, validity for eukaryotic organisms and its general features are summarized in Figure 2.

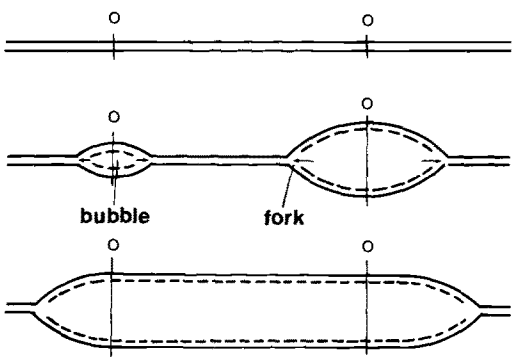

Fig. 2. Bidirectional replication of DNA in eukaryotes. Each chromosome is divided into many replication units which appear as bubbles in the electron microscope. In each bubble, replication takes place bidirectionally in two forks moving in opposite directions

For the purpose of replication, the DNA in each chromosome of eukaryotes is subdivided into many replication units or replicons of varying size. This has been proven for eukaryotes as different as mammals, insects, plants, and yeast. In each replicon, DNA replication is initiated at an "origin" and proceeds semiconservatively and bidirectionally in two forks (*Weintraub*, 1972b; *Hand* and *Tamm*, 1973; *Huberman* and *Tsai*, 1973; *Kriegstein* and *Hogness*, 1974). This mode of replication gives rise to "bubbles" which can be visualized by fiber autoradiography (*Cairns*, 1963, 1966; *Huberman* and *Riggs*, 1968) or by electron microscopy (*Blumenthal* et al., 1973; *Wolstenholme*, 1973; *Newlon* et al., 1974). Biochemical studies, such as analyses of replication intermediates in neutral or alkaline sucrose gradients, are consistent with this model.

It is not clear yet whether origins of replication are defined nucleotide sequences within chromosomal DNA although there is some evidence which suggests that this may be the case. Replicons can greatly vary in size depending on the rate at which DNA is replicated in different stages of the development of an organism. For instance, the average distance between origins of replication (and therefore the replicon size) in *Drosophila melanogaster* can vary from 2.7 μm in early embryos (S-phase = 3.4 min) to 13.6 μm in cells grown in culture (S-phase = 10 h) (*Blumenthal* et al., 1973). Similarly, replicon size in *Triturus vulgaris* varies between 40 μm in the neurula stage (S-phase = 4 h) and much more than 100 μm in the spermatocyte (S-phase = 220–440 h) (*Callan,* 1973). In contrast to the variable replicon size, the rate of the movement of the replication fork (elongation rate) is remarkably similar in fast replicating and slow replicating cells. In other words, the overall rate of DNA replication depends almost exclusively on the number of replicons that are active at any time in the S-phase. The larger this number is (the more replicons a given stretch of DNA is divided into), the faster is the replication. As the number of replication units can therefore vary quite drastically in a particular organism, the nature of replication origins cannot be explained in simple terms.

Both the size of replicons and the rate of fork movement vary among different organisms. The replicon sizes found vary from less than 10 μm to about 200 μm, the rate of the fork movement commonly lies between 0.2 and 2 μm/min (*Taylor,* 1968; *Huberman* and *Riggs,* 1968; *Painter* and *Schaefer,* 1969; *Hand* and *Tamm,* 1972; *Callan,* 1972; *Weintraub* and *Holtzer,* 1972; *Gautschi* and *Kern,* 1973; *Blumenthal* et al., 1973). It has frequently been observed that active replicons are distributed nonuniformally along DNA molecules. In other words, evidence was obtained for the existence of batteries or clusters of replicons whereby different clusters of replicons are initiated at different times during the S-phase of the cell cycle (*Huberman* and *Riggs,* 1968; *Blumenthal* et al., 1973; *Hand,* 1975a). There is also some indication that fork movement may vary slightly during the S-period of a cell (*Housman* and *Huberman,* 1975). In most higher eukaryotes, different clusters of replicons are initiated throughout the S-phase in a defined order which appears to remain the same in consecutive S-periods (*Mueller* and *Kajiwara,* 1965; *Plaut* et al., 1966; *Amaldi* et al., 1973). This also seems to hold for lower eukaryotes such as *Physarum polycephalum* (*Braun* and *Wili,* 1969) whereas in the yeast, *Saccharomyces cerevisiae* (*Newlon* et al., 1974), and in the fast growing early *Drosophila* embryos (*Blumenthal* et al., 1973) all replicons appear to be initiated during the first third of the S-period.

The fact that the number of active origins of replication is variable within a single eukaryotic organism, dependent on the particular physiological state of the cell, rises questions about the regulation of origin

initiation. One possibility is that there are different initiator-proteins that interact with different sets of origins (each set being defined by a particular nucleotide sequence). It is also possible, however, that a single set of initiator-proteins interacts with varying affinity with different sets of origins. In the second case, the timing of initiation and the differences between different cells with respect to the number of active origins would be dependent on the concentration of initiator molecules present. A further possibility is, of course, that origins are specified by a particular secondary structural feature of the DNA and not simply by a one-dimensional nucleotide sequence.

The nature of specific termini at which replication of replicons stops is even less clear than that of specific origins. Theoretic consideration and several experimental observations argue against the existence of fixed termini. In fact there is no stringent need for defined termination sequences because termination of replicons could simply occur at those points along the DNA, where two adjacent replicons meet. Evidence for this model comes from studies on the synthesis of DNA in the small tumor viruses SV40 and polyoma. These viruses contain circular, double-stranded DNA molecules of $3 \times 10^6 - 3.6 \times 10^6$ daltons MW and the DNA molecule comprises a single replicon that is initiated at a specific point (for review see *Fareed* and *Davoli*, 1977). Replication proceeds semiconservatively and bidirectionally and terminates 180° away from the origin, when the two replication forks meet (Fig. 3). In mutants containing a deletion, and thus

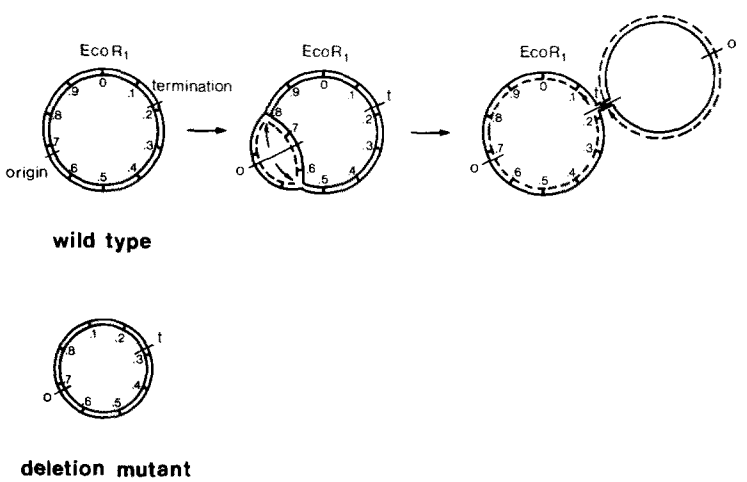

Fig. 3. Mode of replication of polyoma or SV40 DNA. Replication starts at a fixed origin and occurs bidirectionally until the two replication forks merge 180° away from the origin. In a deletion mutant this termination point is again 180° from the origin but now is a different sequence than in the wild type, providing good evidence against specific termination sequences for DNA replication

a smaller molecule of circular DNA, termination again occurs 180° away from the origin which is now a different sequence than that where termination occurred in wild type virus (*Brockman* et al., 1975; *Lai* and *Nathans*, 1975). This finding strongly argues against the existence of a specific termination sequence.

There is strong evidence that replicating DNA in bacteria is connected to cell membranes (*Gefter*, 1975) and the possibility was, therefore, considered that DNA replication in eukaryotic nuclei might likewise take place in complexes attached to the nuclear membrane. Several experimental findings support this idea but more recent results show no connection between replication sites and the nuclear membran. In my opinion the case is not yet definitely solved although more and more investigators tend to think that replication takes place at sites which are distributed throughout the nucleus and that it is not concentrated around the nuclear membrane (*Williams* and *Ockey*, 1970; *Fakan* et al., 1972; *Huberman* et al., 1973; *Wise* and *Prescott*, 1973; *Comings* and *Okada*, 1973). Accordingly, neither ongoing replication nor the initiation of replication might require the attachment of DNA to the nuclear membrane.

3.2 Intermediates of DNA Replication

All known DNA polymerases synthesize DNA in the 5′ → 3′ direction. There appeares to be no enzyme in any organism known so far which could polymerize desoxyribonucleoside triphosphates in a 3′ → 5′ direction (the same holds for RNA polymerases). As one daughter chain of each replication fork must be synthesized with an overall polarity 3′ → 5′, this presents a fundamental problem. For bacterial and phage systems a solution to this problem was found by *Okazaki* et al. (1968) who observed that short segments of DNA, generally called Okazaki fragments, are synthesized in the 5′ → 3′ direction and are afterwards linked by DNA ligase. Thus, an overall synthesis in the directions 3′ → 5′ can occur by the synthesis of small fragments in the 5′ → 3′ direction on a stretch of free, single-stranded DNA template. There is now strong evidence suggesting a similar process for eukaryotes. Such a discontinuous synthesis, however, immediately poses another problem: not one of the known DNA polymerases can initiate an oligonucleotide chain de novo. Evidence, again coming originally from bacterial systems, suggests that this problem is solved by the use of short RNA chains as primers which can then be extended by a DNA polymerase. This second mechanism also appears to be verified in eukaryotes.

Many experimental systems have been employed for detection of Okazaki fragments in eukaryotes and for measuring their size and the

kinetics of their ligation to larger replication intermediates. Important contributions to the solution of this problem come from studies on the replication of polyoma virus DNA and SV40 DNA. These experiments indicate that Okazaki fragments of a size of about 50–150 nucleotides, sedimenting around 4S are the primary synthesis products in these systems (*Magnusson*, 1973; *Magnusson* et al., 1973; *Pigiet* et al., 1973; *Salzman* et al., 1973; *Laipis* and *Levine*, 1973; *Fareed* et al., 1973; *Francke* and *Hunter*, 1974; *Hunter* and *Francke*, 1974). Analyses of the size of Okazaki fragments in nuclei of mammalian cells and *Drosophila* agree well with this estimate (*Huberman* and *Horwitz*, 1973; *Gautschi* and *Clarkson*, 1975; *Tseng* and *Goulian*, 1975; *Blumenthal* and *Clark*, 1977a). These fragments appear to be ligated giving rise to larger replication intermediates of defined lengths (*Rawles* and *Collins*, 1977; *Blumenthal* and *Clark*, 1977b).

Theoretically, there is no stringent need for discontinuous synthesis in the DNA strand which is copied in the 5′ → 3′ direction (Fig. 4). This strand is synthesized ahead of the complementary strand and is therefore often called the "leading strand" to distinguish it from the complementary "lagging strand" which is definitely synthesized in a discontinuous fashion. Synthesis of the "lagging strand" requires a stretch of single-stranded DNA of the template long enough for an RNA polymerase molecule to synthesize an initiator RNA fragment which is later extended by the DNA polymerase. It is to be expected that such single-stranded regions occur at

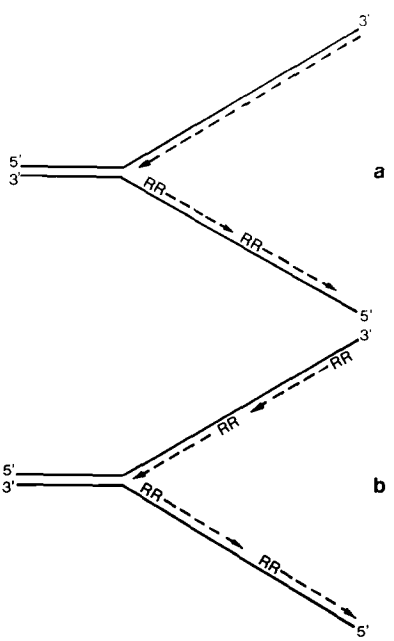

Fig. 4 a and b. Replication can occur by the synthesis of Okazaki fragments on one (a) or both (b) strands

replication forks where the initiation of Okazaki intermediates has not yet taken place. Electron microscope studies did indeed reveal such single-stranded pieces of DNA in replication forks (*Kriegstein* and *Hogness*, 1974), a finding which strongly supports the general model described above.

Answers to the question whether discontinuous synthesis occurs on both strands of the DNA are still controversial. Evidence for both, totally discontinuous or semidiscontinuous synthesis, was obtained in various systems but the techniques that have to be used to resolve this problem are delicate and not free of pitfalls. Although a clear-cut and general answer is, therefore, still lacking, there is now strong experimental support for the assumption that DNA of small DNA viruses is primarily synthesized semidiscontinously (*Hunter* et al., 1977; *Perlman* and *Huberman*, 1977).

Eukaryotic Okazaki fragments are smaller than those of bacteria and phages (*Kornberg, A.*, 1974; *Gefter*, 1975). The size of primary replication intermediates may be related to the organization of the DNA. In eukaryotes it might, therefore, be dictated by some structural feature of the organization of DNA in the nucleosome. For instance, if initiation of Okazaki fragments occurs only in DNA stretches between the nucleosomes then the size of the fragments should be between 150 and 200 nucleotides. It is not known whether the nucleosomal organization of DNA does influence the size of the primary replication intermediates but models based on this hypothesis were suggested (e.g., *Rosenberg*, 1976). The most widely used method to measure the size of Okazaki fragments, sedimentation in alkaline sucrose gradients, is not accurate enough to establish a correlation between the size of Okazaki fragments and the nucleosomal DNA in different organisms. In fact, recent determinations, using gel electrophoresis, of the size of primary replication intermediates in *Drosophila* cells gave a mean value of only 61 nucleotides (*Blumenthal* and *Clark*, 1977b) which is definitely smaller than the DNA stretch involved in the formation of nucleosomes. On the other hand, this value is rather close to the size of the DNA piece linking two nucleosomes. Whatever the answer to this problem will be, it is clear that the initiation of Okazaki fragments must be determined by some structural feature of chromatin rather than by specific sequences in the DNA. This is evident from the studies on polyoma DNA replication mentioned above as well as from the simple reasoning that specific initiation sequences of only about 10 base pairs, if they were to occur at each Okazaki fragment, would use up much of the coding capacity of the DNA and would also put a considerable constraint onto the evolution of genetic information. As an alternative explanation for the formation of primary replication intermediates one could assume that DNA polymerases themselves measure the lengths of these molecules and may thus be self-limited to synthesizing about 60 nucleotide molecules.

Despite much effort, it has only recently become clear that Okazaki fragments in eukaryotes are initiated by short (about 10 nucleotides long) RNA fragments. These RNA stretches contain all four ribonucleotides and pppA or pppG at their 5′ end. There is no unique base sequence of initiator RNA but the size of the oligoribonucleotide seems to be well defined. It was proposed that the size of the initiator RNA, rather than a specific nucleotide sequence, may signal the switch from RNA to DNA synthesis. The junction from RNA to DNA involves all 16 possible combinations of ribo- and desoxyribonucleotides; there is, therefore, no specificity with regard to nucleotide sequence at this junction. This important conclusions came originally from studies on SV40 and polyoma virus DNA replication (*Magnusson* et al., 1973; *Hunter* and *Francke,* 1974; *Pigiet* et al., 1973; *Kaufmann* et al., 1977) and have recently been confirmed and extended to Okazaki fragments in the DNA of mammalian cells (*Waqar* and *Huberman,* 1975; *Tseng* and *Goulian,* 1977).

This initiation mechanism has interesting consequences for the enzymology of DNA replication because a DNA polymerase is required which is capable of extending an RNA primer with desoxyribonucleotides and additional enzymatic activities are needed for the removal of the RNA primer and the filling of the resulting gap. Moreover, problems arise with the replication of the ends of linear DNA molecules: Removal of the priming oligoribonucleotide creates a gap which, if situated at the 5′ end of a DNA double strand, can not be repaired because of the lack of an initiator for DNA polymerase. This would result in a gradual loss of DNA sequences at the ends of linear DNA molecules unless special mechanisms existed to overcome these difficulties. Most linear bacteriophage DNA molecules therefore have terminal repeats or single-stranded complementary ends which allow formation of circular or concatemeric replication intermediates (*Watson,* 1972). Several interesting models have been proposed to explain replication of the 5′ ends of eukaryotic chromosomes and linear DNA molecules which cannot circularize. These models consider internal repeats (*Heumann,* 1976) or self-complementary hairpin loops at chromosome ends allowing the parental 3′ end to act as a primer for DNA polymerase (*Cavalier-Smith,* 1974). Rolling hairpins were proposed for the replication of parvovirus DNA (*Tattersall* and *Ward,* 1976). For the linear molecule of adenovirus DNA, hairpin-primed synthesis of the ends was excluded (*Stillman* et al., 1977). In this case a protein was found to be covalently attached to the ends of the DNA molecule (*Rekosh* et al., 1977); this protein may be involved in the replication of adenovirus DNA.

4. Enzymology of DNA Replication

4.1 DNA Polymerases

The best studied enzymes known to be involved in DNA replication are the DNA polymerases. Although first reports on DNA-dependent DNA polymerases from animal cells appeared more than 20 years ago it was not until recently that separation and characterization of the various enzymes was achieved. One reason for this slow progress in our understanding of the structure and function of eukaryotic DNA polymerases is the low concentration at which these enzymes are present in the cell. It turned out to be extremely difficult to obtain the enzymes in a state of purity sufficient for structural characterization. Modern techniques of enzyme purification have in part helped to overcome these difficulties and some basic answers to the properties, structure and specific role of eukaryotic DNA polymerases are emerging. Many properties of eukaryotic DNA polymerases are summarized in several recent reviews (*Fansler*, 1974; *Loeb*, 1974; *Bollum*, 1975; *Weissbach*, 1975; *Holmes* and *Johnston*, 1975; *Wintersberger*, 1977).

Eukaryotic cells contain three to four classes of DNA polymerases. The enzymes are localized in different cellular compartments. A nomenclature was proposed recently (*Weissbach*, 1975) and has been widely used since (Table 1). Many properties are shared by all the cellular DNA polymerases:

Table 1

DNA polymerase	α	β	γ	δ	mitochondrial
Location	nuclei cytoplasm	mainly nuclei	nuclei cytoplasm mitochondria	cytoplasm?	mitochondria; now assumed to be identical with polymerase γ
Sedimentation coefficient	6–8 S	3.4 S	6.5 S	7 S	
Exonuclease activity	no	no	no	yes	
Sensitivity to SH blocking reagents	yes	no	yes	probably no	
Preferred template primer	Activated DNA	PolydA-$(dT)_{10}$	PolyrA-$(dT)_{10}$	Polyd(A–T)	
Ability to copy ribopolymer	no	yes	yes	no	
Ability to extend oligoribonucleotide primer	yes	no	yes (PolydT-$(rA)_{12-18}$	?	

they accept "activated" DNA (i.e., DNA into which single-strand breaks and gaps were introduced by incubation with small amounts of desoxyribonuclease I) as a template primer and they generally lack the associated enzyme activities that are typical of bacterial DNA polymerases. Most mammalian DNA polymerases show no associated nuclease activity (there are, however, exceptions, see later) and fail to catalyse a pyrophosphate exchange reaction to a significant extent. The various enzyme classes differ in size, structure, primer- and template-specificity, necessity of SH groups for enzyme activity, and in their response to mitotic stimuli. The major fraction of cellular DNA polymerase activity, DNA polymerase α, is commonly found in large amounts in the cytoplasm. This observation seems to be in conflict with a possible role of the enzyme in nuclear DNA replication, except if one assumes that the enzyme efficiently leaks from nuclei during cell fractionation. Nuclei isolated by nonaqueous procedures were found to contain high concentrations of DNA polymerase α (*Foster* and *Gurney,* 1976) and recent reports indicate that the enzyme is held more strongly in nuclei actively synthesizing DNA (*Fansler* and *Loeb,* 1972; *Wintersberger* and *Wintersberger,* 1975, 1977; *Chiu* and *Baril,* 1975; *Närkhammar* and *Magnusson,* 1976; *Martini* et al., 1976). This suggests that the cytoplasmic localization of DNA polymerase α is an artifact. It cannot be excluded, however, that α polymerase is actively transported from the cytoplasm to the nucleus during the onset of DNA replication, while it is largely located in the cytoplasm in nonreplicating cells.

DNA polymerase α has been isolated, purified, and characterized from many different animal cells and from lower eukaryotes (*Loeb,* 1969; *Wintersberger* and *Wintersberger,* 1970a; *Weissbach* et al., 1971; *Momparler* et al., 1973; *Brun* et al., 1974; *Wintersberger, E.,* 1974; *Holmes* et al., 1974, 1976; *Crerar* and *Perlman,* 1974; *Craig* and *Keir,* 1975; *Sedwick* et al., 1975; *McLennan* and *Keir,* 1975a; *Banks* et al., 1976; *Loomis* et al., 1976; *Matsukage* et al., 1976; *Fisher* and *Korn,* 1977). The enzyme is usually obtained from total cell homogenates and sediments (sometimes heterogenously) with sedimentation coefficients of 6–8 S indicating a high MW. As in the case of other DNA polymerases and many unrelated nuclear proteins, sedimentation properties strongly depend on the ionic strength. At low ionic strength the enzyme forms aggregates of MW greater than 200,000. At high ionic strength, sedimentation analyses indicate a MW for DNA polymerase α of about 160,000–180,000. Electrophoreses in SDS polyacrylamid gels of highly purified DNA polymerases from human KB-cells (*Fisher* and *Korn,* 1977) yeast (*Wintersberger, E.,* 1974) and calf thymus (*Holmes* et al., 1976) give a protein band of MW between 155,000 and 175,000, as well as components having a MW approximately half of this size, i.e., 66,000–76,000 for KB-cells and 80,000 for yeast. This indicates that the active enzmye with MW of about

170,000 may by a dimer. Dissociation of the 170,000 dalton species is, however, often incomplete even after heating the solution in the presence of SDS. DNA polymerases from *Drosophila* and *Tetrahymena* also seem to have subunits of 80,000–90,000 MW (*Crerar* and *Pearlman,* 1974; *Karkas* et al., 1975).

DNA polymerase α requires sulfhydryl groups for activity: the enzyme is strongly inhibited by N-ethyl maleimide and by p-chloromercuribenzoate. It requires a polydeoxynucleotide chain as a template and a 3′-hydroxyl group of an oligodeoxynucleotide or an oligoribonucleotide as a primer (*Chang* and *Bollum,* 1972; *Keller,* 1972; *Spardari* and *Weissbach,* 1975). DNA polymerase α is inhibited by the CTP-analog, cytosine arabinoside triphosphate; this inhibition may be the cause of the effect of cytosine arabinoside on DNA synthesis in vivo. Of particular interest is the recent observation that calf thymus DNA polymerase α, but not polymerase β, is stimulated by helix destabilizing proteins isolated from calf thymus glands (*Herrick* and *Alberts,* 1976; *Herrick* et al., 1976). These proteins have properties similar to the gene-32 product of phage T4, for which genetic evidence indicates an involvement in the replication of T4 DNA and which is known specifically to stimulate the activity of T4 DNA polymerase.

The second most abundant DNA polymerase of mammalian cells, DNA polymerase β, is predominantly found in the nucleus after cell fractionation. The enzyme was isolated and purified to apparent homogeneity from a number of tissues and cells (*Chang* and *Bollum,* 1971; *Chang,* 1973a,b; *Wang* et al., 1974, 1975; *Tsuruo* et al., 1974; *Craig* and *Keir,* 1975; *Stalker* et al., 1976). In buffers of high ionic strength it sediments with a sedimentation coefficient of 3.4 S and upon electrophoreses in SDS polyacrylamid gels it gives a single band with a MW of 43,000–45,000, a value which is in agreement with the sedimentation data. Unlike DNA polymerase α, polymerase β is not inhibited by SH-group reagents. The β polymerase is also distinct from the α polymerase in some other enzymatic properties. In particular, it is capable of copying a ribohomopolymer such as poly rA:oligo dT, a template-primer not used by DNA polymerase α. Unlike the α-enzyme, DNA polymerase β cannot elongate ribonucleotide primers at the free 3′-hydroxyl group. DNA polymerases α and β are now found to be immunologically different enzymes (*Spadari* et al., 1974; *Brun* et al., 1975), disproving earlier claims of a structural (*Hecht* and *Davidson,* 1973; *Hecht,* 1973; *Lazarus* and *Kitron,* 1973) and immunologic (*Chang* and *Bollum,* 1972) relationship between the two enzymes.

Studies on yeast DNA polymerases have led to the surprising observation that polymerase β is absent from nuclei of lower eukaryotes (*Wintersberger, U.,* 1974). This was more recently confirmed and extended to a number of other lower eukaryotes, plants, and protozoa (*Chang,* 1976).

The observation is interesting with regard to the phylogeny of DNA polymerase β and with respect to speculations about the function of this enzyme.

Lower eukaryotes, such as yeast, contain enzymes which are similar in many respects to α polymerase of animal cells. The similarities include: size; sensitivity to SH blocking reagents and cytosine arabinoside triphosphate; ability to use RNA primers, and regulation during the cell cycle. In addition, yeast contains a second enzyme of high MW which is distinct from yeast polymerase α in several enzymatic properties as well as immunologically (*Wintersberger, E.*, 1974, 1978; *Chang*, 1977). Most significantly, this enzyme contains an associated $3' \rightarrow 5'$ exonuclease activity (*Helfman*, 1973; *Wintersberger, E.*, 1974, 1978; *Chang*, 1977). Similar enzymes were found in other lower eukaryotes (*McLennan* and *Keir*, 1975b; *Banks* and *Yarronton*, 1976) and they may in fact be related to DNA polymerase δ (*Byrnes* et al., 1976, 1977), a polymerase containing an associated exonuclease activity recently discovered in bone marrow cells.

A third DNA polymerase, DNA polymerase γ, was originally found in HeLa cells (*Fridlender* et al., 1972) and is now known to be present in most animal cells (*Bolden* et al., 1972; *McCaffrey* et al., 1973; *Fry* and *Weissbach*, 1973; *Lewis* et al., 1974; *Livingston* et al., 1974; *Yoshida* et al., 1974; *Knopf* et al., 1976). It represents only a minor fraction of the total DNA polymerase activity. The enzyme is found in the cytoplasm as well as in nuclei but was recently suggested to be identical with the mitochondrial DNA polymerase of animal cells (*Bolden* et al., 1977). Polymerase γ is characteristically different from polymerase α and β. It is able to copy the synthetic polymer poly rA:oligo dT at a much higher rate than desoxyribopolymers or activated DNA. Sedimentation analyses indicate a MW of about 120,000. DNA polymerase γ requires SH groups for activity and exhibits surprisingly low K_m values for desoxyribonucleoside triphosphates (0.5 μM) which are an order of magnitude lower than the values determined for DNA polymerases α and β. Because of its ability to copy a polyribonucleotide template, the properties of DNA polymerase γ were extensively compared with those of the RNA-directed DNA polymerase of RNA tumor viruses. These experiments revealed characteristic differences between the cellular polymerase and the viral enzyme. Most significantly, DNA polymerase γ is not able to copy natural RNA and is not inhibited by antibodies against the RNA-directed DNA polymerase of RNA tumor viruses.

It is interesting to note that in vitro both DNA polymerase α and β (polymerase γ has not yet been studied in this respect) catalyse DNA synthesis in a distributive and not in a processive manner (*Chang*, 1975). The polymerases easily separate from the template-primer complex after each polymerization step and reassociate with free template-primer at random,

rather than sticking to the template-primer complex, until the entire template strand is copied. A similar behaviour was found for bacterial DNA polymerases. It was speculated that additional proteins present in the cell are required to allow the polymerase to catalyse DNA synthesis in a processive manner. This idea is supported by recent experiments. It was found that DNA polymerases II and III from *Escherichia coli,* which are stimulated in their activity by *E. coli* "unwinding protein", catalyse DNA synthesis in a processive manner in the presence of unwinding protein and in a distributive manner in its absence (*Sherman* and *Gefter,* 1976). A similar behaviour may be expected for the α polymerase since the activity of this enzyme is stimulated by helix-destabilizing proteins (*Herrick* et al., 1976; *Otto* et al., 1977).

Some insight into the role of the various cellular DNA polymerases of eukaryotes has been sought by analysing the levels of activity of the various enzymes in cells moving from a nonreplicative to a replicative state or from G1 to the S-phase of the cell-cycle. In all these instances, such as in regenerating versus normal liver cells (*Baril* et al., 1973), in the cell cycle of synchronously growing cells (*Chang* et al., 1973; *Spadari* and *Weissbach,* 1974; *Chiu* and *Baril,* 1975), upon activation of nondividing cells by mitogens (*Fridlender* et al., 1974; *Bertazzoni* et al., 1976), or by infection with DNA tumor viruses (*Wintersberger* and *Wintersberger,* 1975; *Närkhammar* and *Magnusson,* 1976; *Mechali* et al., 1977) total polymerase activity in the cell increases. This increase is due to drastic elevation of the level of DNA polymerase α. In some instances also the activity of DNA polymerase γ increased. Except for a few recent reports (*Närkhammar* and *Magnusson,* 1976; *Bertazzoni* et al., 1976), DNA polymerase β was found to remain at a fairly constant level throughout the cell cycle or in dividing as compared to nondividing cells. The results of these experiments indicate that DNA polymerase α and possibly γ are involved in the replication process. They do not, however, exclude the additional participation of DNA polymerase β in this process.

Further indications as to the role in replication come from the primer requirement of the different enzymes. As outlined above there is now good evidence that eukaryotic DNA replication starts with the synthesis of fragments which are covalently linked to short RNA chains. According to this model, elongation of the RNA primer must be carried out by a DNA polymerase capable of using such initiators. The only DNA polymerase known so far that can catalyse such a reaction is DNA polymerase α (*Keller,* 1972; *Chang* and *Bollum,* 1972; *Spadari* and *Weissbach,* 1975). This, as well as the activation of α polymerase by helix-destabilizing proteins again suggest that DNA polymerase α is involved in the replication process. Each of the enzymes, however, could in addition function in DNA repair and/or recombination. DNA polymerase δ from bone

marrow cells is the only enzyme of mammalian origin known so far to contain an associated 3′ → 5′ exonuclease activity but its role is still unclear as it has only recently been detected (*Byrnes* et al., 1976). However, an enzyme containing exonuclease activity is probably required for proofreading and repair functions as well as for recombination. As indicated above, such enzymes are frequently found in lower eukaryotes and it is quite possible that they are also present in most or all mammalian cells.

4.2 Other Enzymes Involved in DNA Replication

DNA ligase is required for DNA replication in prokaryotes (*Kornberg, A.*, 1974) The fact that the joining of Okazaki fragments is also essential for replication of DNA in eukaryotes suggests the need for a similar enzyme in higher organisms. In fact, DNA ligases have been detected, isolated, and characterized from several mammalian species. The enzymes were found to require ATP as a cofactor (*Lindahl* and *Edelman*, 1968; *Beard*, 1972; *Pedralinoy* et al., 1973; *Söderhall* and *Lindahl*, 1973).

Other proteins which are involved in DNA replication are helix-destabilizing proteins like the gene-32 product of bacteriophage T4 (*Alberts* and *Fry*, 1970). Such proteins were recently isolated from calf thymus (*Herrick* and *Alberts*, 1976); their effect on DNA polymerases was described above.

Enzymes may also be required for the unwinding of superhelical turns in DNA. Prototypes of such enzymes are the ω protein of *E. coli* (*Wang*, 1971) and similar proteins isolated from rodent (*Champoux* and *Dulbecco*, 1972; *Champoux* and *McConaughy*, 1976), human (*Keller*, 1975b) and *Drosophila* (*Basse* and *Wang*, 1974) cells. Desoxyribonucleases are probably necessary for replication in order to provide swivel points for the unwinding of the DNA double helix. Several mammalian desoxyribonucleases have been found and purified (*Lindahl* et al., 1969; *Churchill* et al., 1973; *Hewish* and *Burgoyne*, 1973a), but no such enzyme has so far been proven to be essential for DNA replication.

Current evidence suggests that RNA synthesis is required to provide the initiator RNA for the synthesis of new strands during the formation of Okazaki fragments (see above). Although several RNA polymerases are known to occur in mammalian cells (*Chambon*, 1975) none of these has yet been proven to be involved in DNA replication. Replication of polyoma DNA was found to be insensitive to low or high concentrations of α amanitin, excluding a participation of RNA polymerases II and III (or B and C) in this process. A possible involvement of the nucleolar RNA polymerase I (or A) in DNA replication has not yet been excluded.

As already mentioned above, the olicoribonucleotides must be removed later in the replication process and replaced by deoxyribonucleotides. Whereas the later reaction is catalysed by one of the cellular DNA polymeases, specific enzymes are required to digest the RNA moiety of the DNA-RNA hybrid. In bacteria this reaction may be catalysed by the exonuclease activity of a DNA polymerase. Enzymes digesting the RNA part of DNA-RNA hybrids (RNAse H) are known in higher organisms; their involvement in DNA replication is possible but so far unproven.

Progress in the isolation and characterization of further enzymes and proteins involved in DNA replication can be expected from two experimental systems. One is genetic analysis of the process, i.e., isolation and characterization of conditionally lethal, mostly temperature-sensitive, mutants in DNA replication. This approach has been applied very successfully to analysis of the replication apparatus of bacterial and phage systems (*Kornberg, A.*, 1974) and could be expected to be equally important for unraveling the replication process in eukaryotes. Unfortunately only few eukaryotic systems are easily amenable to genetic analyses. One such system is the yeast, *Saccharomyces cerevisiae,* and indeed several temperature-sensitive mutants of this organism are known already which affect DNA synthesis (*Hartwell,* 1971, 1973; *Hereford* and *Hartwell,* 1974); their biochemical analysis has yet to be carried out in detail. A second fruitful approach may be the analysis of the replication process of small DNA molecules such as DNA from the tumor virus polyoma. Recent evidence suggests that this DNA, if injected into *Xenopus laevis* eggs, is faithfully replicated (*Laskey* and *Gurdon,* 1973) and cell-free extracts from *Xenopus* were found to be active in the replication of viral DNA (*Hobish* and *Pigiet,* 1977). This system, as well as studies on the replication of polyoma and SV40 DNA in nuclei from infected cells in vitro will be useful for an analysis of the factors and enzymes involved in this reaction (*Waqar* et al., 1977; *Edenberg* et al., 1977).

5. Regulation of DNA Replication

DNA synthesis must be regulated at various steps. The first one is, of course, the initiation of replication which is tightly regulated in the cell cycle and takes place at the transition of cells from the G1-phase to the S-phase of the cell cycle. Further regulatory steps assure that clusters of replicons are initiated at particular times in the S-period and that all the DNA of the nucleus is synthesized once and only once during the S-phase. Moreover, replication must be coupled in some way to the production of histones and other chromosomal proteins that are necessary for the formation of chromatin and chromosomes.

5.1 Initiation of the S-Phase

Most substances that control cell proliferation (such as hormones, mitogens, serum factors, mitotic inhibitors) function by controlling the initiation of DNA replication, i.e., the progression of cells from the G1-period to the S-phase of the cell cycle (*Clarkson* and *Baserga,* 1974). These substances usually do not act directly on the process of replication initiation itself but rather seem to affect some step or steps in the G1-phase with the consequence that DNA synthesis is initiated (*Holley,* 1975; *Baserga,* 1976; *Prescott,* 1976). This is indicated by studies on cell cycle control in vivo (see *Shields,* 1976) as well as by genetic analyses of the cell division cycle in yeast (*Hartwell* et al., 1974). Many of the substances controlling the G1-S progression of cells first lead to an increase in the synthesis of RNA (in particular of ribosomal RNA) and to changes in chromosomal proteins (acetylation or phosphorylation of specific chromosomal proteins) (see *Baserga,* 1976). These events occur some time before DNA synthesis is initiated. It is likely that similar controls operate in the undisturbed cell cycle of most eukaryotic cells.

Current models of the regulation of the cell cycle are based on two properties of the cell cycle which were observed in many different cell types and seem to be generally valid (*Smith* and *Martin,* 1973; *Robinson* et al., 1976; *Shilo* et al., 1976; *Brooks,* 1976; *Armelin* and *Armelin,* 1977; *Jimenez de Asua,* 1977; *Shields,* 1977):

1. Of the four periods of phases of the cell cycle (G1, S, G2, M, see Fig. 5), S, G2 and M are traversed by a given cell type at a constant time every division cycle.

2. The length of the fourth phase, G1, varies greatly even between individual cells of a population of cells of the same type.

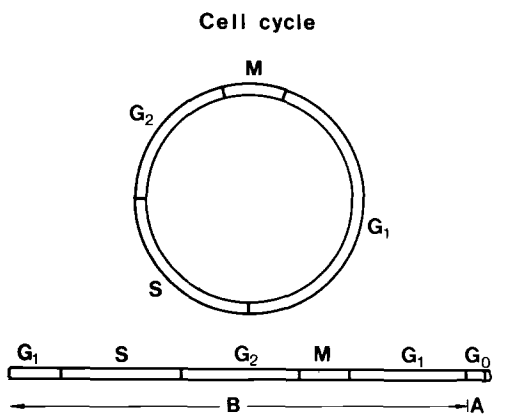

Fig. 5. The cell cycle. For details see text

More detailed examinations of the cell cycle have led *Smith* and *Martin* (1973) to divide the cycle into two states, A and B, where the latter is fixed in time and includes S, G2, M, and part of G1. The A state represents the variable part of G1 (often called G_0) during which cells are awaiting a random triggering event before proceeding into the next cycle. It is the variable length of the A state which is responsible for the great variability of G1 and for the asynchrony of cell division in a cell population. In steady-state conditions, the probability (the transition-probability) of a cell leaving the A state and entering the B phase in any unit of time is constant.

These proposals, which are supported by many experimental observations with a variety of cell types, must be taken into account when considering the regulation of initiation of DNA synthesis and S-phase. It is not known yet at which point (early or late) in G1 the end of the A state (or G_0) is situated and which events follow immediately after transition of cells from the A to the B state. It is likely, however, that this transition does not directly involve the initiation event of DNA replication. It is tempting to speculate that the transition probability is influenced by a variety of factors (mitogens, hormones, growth factors, nutrients) which all effect the cell in some way leading eventually to the switch-on of a cellular programm that ultimately results in the initiation of DNA replication.

There is strong evidence that the ability of a cell to start DNA synthesis is under positive control. This is supported, for instance, by cell fusion experiments which have shown that when cells that synthesize DNA are fused with dormant cells which do not replicate the DNA, both types of nuclei in the heterokaryon are capable of DNA synthesis (*Harris* et al., 1966). Analogous experiments suggest that the controls that trigger DNA synthesis are not species specific (*Graves,* 1972). Further support for the positive control model of the induction of DNA synthesis comes from cell fusion experiments involving HeLa cells at different stages of the cell cycle (*Rao* and *Johnson,* 1970). Thus if cells which are in the G1-phase of the cell cycle (and which do not synthesize DNA) are fused with cells which are in the S-phase, both nuclei are induced to synthesize DNA. Cells which are in the G2-phase, on the contrary, cannot be induced to synthesize DNA in a similar way by fusion with S-phase cells. Hence, there is a restriction in G2 nuclei which prevents the reinitiation of the S-phase. The nature of this restriction is unknown. In several in vitro studies involving isolated nuclei from cells at different stages of the cell cycle it was found that the differences in DNA synthetic activity between inactive and actively synthesizing cells is still retained in the isolated nuclei from these cells (*Hershey* et al., 1973; *Friedman,* 1974; *Benbow* and *Ford,* 1975; *Benz* and *Strominger,* 1975; *Jazwinski* et al., 1976; *Jazwinski* and *Edelman,* 1976): i.e., nuclei from DNA-synthesizing cells incorporate precursors

into DNA more rapidly than nuclei from inactive cells. Several studies have been performed with the aim to isolate, from the cytoplasm of S-phase cells, protein factors that would stimulate nuclei from G1-phase cells to synthesize DNA (*Seki* and *Mueller*, 1976; *Krokan* et al., 1977). While definitive prove for the existence of such a protein and a characterization of its activity in vitro has yet to come, recent studies on this system are encouraging.

A number of enzymatic activities and precursor-pools change when cells move from the G1-phase to the S-phase of the cell cycle. This is particularly true for the pool of the desoxyribonucleoside triphosphates which is low during G1 and increases steadily during S-phase (*Nordenskjöld* et al., 1970; *Walters* et al., 1973; *Skoog* et al., 1973; *Skoog* and *Bjursell*, 1974). The dCTP pool most closely follows the course of DNA synthesis which led to the suggestion that this pool might serve a regulatory function in DNA replication (*Skoog* et al., 973). Many enzymes involved in DNA synthesis and in the production of DNA precursors likewise show increased activity in cells stimulated to synthesize DNA (see Sect. 4). This is true for DNA polymerases, DNA ligase, thymidine kinase, and other enzymes involved in the synthesis of desoxypyrimidine triphosphates. However, the increase in specific activity of most of these enzymes during the G1-S progression is only by a factor of two to three. These enzymes are, therefore, probably not limiting in the G1-phase and it is unlikely that the level of the activity of these enzymes plays an important regulatory role in the initiation of DNA replication.

5.2 Temporal Order of the S-Phase

Once initiated, DNA replication is further controlled by a temporal order to the S-phase. DNA sequences in the cell are not replicated at random times. DNA sequences that replicate during a particular portion of one S-phase do replicate within the same portion of succeeding S-phases (*Mueller* and *Kajiwara*, 1965; *Plaut* et al., 1966; *Braun* and *Wili*, 1969; *Amaldi* et al., 1973). Early in S-phase, the average G + C content of the newly synthesized DNA is lower than late in the S-phase and heterochromatin is always replicated late in S (*Lima -de-Faria-* and *Jaworska*, 1968; *Tobia* et al., 1970; *Bostock* and *Prescott*, 1971). The temporal order of DNA synthesis within the S-phase has recently been proven on a somewhat finer scale for several genes in yeast which were found to replicate in a defined sequence (*Burke* and *Fangman*, 1975). Inhibition of DNA replication by substances such as 5-fluordeoxyuridine or hydroxyurea often interferes with the normal regulation of the S-phase (*Adegoke* and *Taylor*, 1977). Studies using such inhibitors to synchronize cells at the onset of the S-phase may therefore lead to erroneous and abnormal results.

6. Synthesis of Chromosomal Proteins

In mammalian cells and in lower eukaryotes, histones are synthesized only during the S-phase (*Robbins* and *Borun*, 1967: *Takai* et al., 1968; *Gallwitz* and *Mueller*, 1969; *Butler* and *Mueller*, 1973; *Moll* and *Wintersberger*, 1976). Inhibition of DNA synthesis rapidly blocks the synthesis of histones. The nature of the coupling between histone synthesis and DNA synthesis has been the subject of many investigations but is still largely unknown. It has repeatedly been suggested that histone mRNA is transcribed only when DNA is replicated (*Borun* et al., 1967; *Breindl* and *Gallwitz*, 1973. 1974) and much experimental support for a transcriptional control of histone synthesis, mediated by S-phase-specific chromosomal proteins, was reported (*Stein* et al., 1976; *Park* et al., 1976; *Jansing* et al., 1977). Recent evidence indicates, however, that the primary transcription product of histone genes in the nucleus is produced at any time in the cell cycle. Only the maturation of the RNA precursor and the formation of polysome-bound histone mRNA seem to be restricted to the S-phase (*Melli* et al., 1977; *Stein* et al., 1977). It thus appears that the control of histone synthesis and the coupling of histone and DNA synthesis takes place after the synthesis of the histone mRNA precursor. When DNA replication stops, the appearance of polysome-bound histone mRNA likewise strops but preexisting cytoplasmic histone mRNA is rapidly degraded (*Gallwitz* and *Mueller*, 1969; *Butler* and *Mueller*, 1973; *Breindl* and *Gallwitz*, 1974). Strangely enough, degradation of histone mRNA is much more rapid when DNA synthesis is blocked than when RNA synthesis is inhibited (*Borun* et al., 1967; *Gallwitz* and *Mueller*, 1969). Searches for cytoplasmic proteins which are involved in the block of histone synthesis and in degradation of histone mRNA in cells in which DNA replication is inhibited have so far been unsuccessful (*Jacobs-Lorena* et al., 1973; *Breindl* and *Gallwitz*, 1974). It was suggested that an excess of histones itself might cause an inhibition of histone synthesis (*Butler* and *Mueller*, 1973; *Weintraub*, 1973).

Unlike histones, most nonhistone chromosomal proteins are synthesized throughout the cell cycle (*Gerner* and *Humphrey*, 1973). It cannot be excluded, however, that there may be a few minor species of nonhistone proteins which are also linked in their synthesis to the synthesis of DNA. This possibility is of some interest because among these proteins there might be some which play a part in the control of the timing of DNA synthesis in the S-phase and in the assembly of chromatin and chromosomes from newly synthesized DNA and histones (scaffold proteins, *Adolph* et al., 1977).

The assembly process of chromatin has been investigated recently and several groups came to the conclusion that newly synthesized DNA does

not preferentially combine with newly synthesized histones (*Jackson* et al., 1976; *Seale,* 1976, 1977; *Hildebrand* and *Walters,* 1976; *Freedlender* et al., 1977). Newly synthesized histones combine randomly with newly synthesized and with old DNA. On the other hand, all histones within one octamer of the nucleosomes seem to be either new or old ones; mixing of new and old histones in the octamer seems not to occur (*Leffak* et al., 1977). More detailed information on the mechanism of chromatin assembly might come from studies using small DNAs such as polyoma or SV40 DNA and cell extracts from *Xenopus laevis* eggs. These extracts were shown to convert viral DNA into chromatin in vitro (*Laskey* et al., 1977). It was also shown that this process not only requires the proper amounts of histones but in addition some proteins present in the egg extract which appear to be necessary for the assembly process. It was observed that heating the extract destroys its capacity to convert viral DNA into chromatin. Readdition of histones to the heated extract, however, does not restore its activity. Thus, heat labile protein(s) present in the egg extract is (are) required, in addition to the histones, for the assembly. The system should prove useful for a more detailed in vitro characterization of chromatin assembly and its requirements and regulation.

It has been known for some time that inhibition of protein synthesis in higher cells causes a decline in the rate of DNA replication, but there is still considerable uncertainty and controversy about the extent of the effect and its mechanism (*Littlefield* and *Jacobs,* 1965; *Muldoon* et al., 1971; *Hyodo* et al., 1971; *Weintraub* and *Holtzer,* 1972; *Gautschi* et al., 1973; *Gautschi,* 1974; *Seki* and *Mueller,* 1975; *Seale* and *Simpson,* 1975; *Hand,* 1975b; *Garcia-Herdugo* et al., 1976; *Evans* et al., 1976; *Stimac* et al., 1977). Generally, the degree of inhibition of DNA synthesis is roughly equal to the degree of inhibition of protein synthesis both in cultured cells and in regenerating rat liver. There is disagreement concerning the question whether an inhibition of protein synthesis interferes primarily with the initiation of DNA replication or with DNA chain growth. By analogy with observations in bacterial systems, where protein synthesis inhibition blocks the initiation of a new round of chromosome replication but does not interfere with ongoing DNA replication (*Lark,* 1969), many investigators interpreted the effects of protein synthesis inhibitors on the replication of eukaryotic chromosomes in a similar manner. In the yeast, *Saccharomyces cerevisiae,* protein synthesis seems indeed to be required only for the initiation of the S-phase; once initiated, replication can proceed to completion in the absence of protein synthesis (*Hereford* and *Hartwell,* 1973; *Williamson,* 1973; *Golombek* et al., 1974; *Slater,* 1974). Similar results were obtained with small DNA tumor viruses (*Manor* and *Neer,* 1975). Since histones in yeast, like in all other eukaryotes, are synthesized trhoughout the S-phase of the cell cycle (*Moll* and *Wintersberger,*

1976), DNA synthesis in this organism can be uncoupled from concomitant histone synthesis. As mentioned earlier, however, yeast may represent a special case because all replicons in this organism seem to be initiated very early in the S-phase. In most other eukaryotes, initiation of clusters of replicons takes place throughout the whole S-period and if this process requires protein synthesis this could certainly explain the fact that inhibition of protein synthesis in these cells at any point during the S-phase leads to a block in DNA replication. This rather simple interpretation is, however, probably an oversimplification. Recent extensive studies of several groups have provided strong evidence that a variety of protein synthesis inhibitors (including cycloheximide, puromycin, emetin, pactamycin, 2,4-dinitrophenol, and several amino acid analogs) all cause a reduction in the rate of DNA synthesis in different cell systems (mouse L-cells, Chinese hamster ovary cells, HeLa cells). Using DNA fiber autoratiography to measure accurately the rate of replication fork movement, it was found that the rate of fork movement, and thereby the rate of chain elongation, is reduced shortly after the addition of the protein synthesis inhibitor and the decline of this rate accounts for the decline in the rate of DNA synthesis (for extensive discussion of this problem see *Edenberg* and *Huberman*, 1975, and *Stimac* et al., 1977). Thus, these studies indicate that protein synthesis inhibitors primarily affect the elongation rate of DNA replication and that only after longer periods of blocked protein synthesis might inhibition of initiation of DNA replication also contribute to the decline in the rate of overall DNA synthesis. The mechanism of the close coupling between the level of overall protein synthesis within the cell and the level of overall DNA synthesis is not completely clear. It is possible that there exists a specific class of proteins whose rate of synthesis is limiting for DNA replication. This specific class of proteins might turn over very rapidly or it might be used up stoichiometrically as replication proceeds, which would explain the need for continuous synthesis of these proteins during the S-phase. It is unlikely that the proteins in question are identical with histones, as suggested some time ago (*Weintraub*, 1972), because it was found that many of those histones which are bound to newly synthesized DNA are old ones and that these are always present in excess over newly synthesized DNA. One of the arguments in favour of the histone hypothesis was that DNA replication is drastically inhibited by the arginine analog, canavaine (*Weintraub*, 1972b). Arginine is known to be present in high concentrations in histones. Canavanine, therefore, could be expected to preferentially inhibit histone synthesis and by that mechanism affect DNA replication. However, it was found more recently that 5-methyl tryptophane, an analogue of the amino acid tryptophane (an amino acid which is absent from histones), likewise inhibits DNA synthesis to the same extent as it inhibits overall protein synthesis (*Stimac* et al., 1977). This finding

makes it unlikely that inhibition of protein synthesis interferes with DNA replication primarily by its effect on histone synthesis. In conclusion then, this interesting problem is still unsolved and further experiments have to be designed to unravel the mechanism of the coupling of protein and DNA synthesis.

7. Fidelity of DNA Replication

DNA replication must be extremely accurate. Any mistake made by the replication system will result in a mutation. In fact, the observed fidelity is such that only one mistake is made per $10^6 - 10^9$ base pairs replicated. This may be one of the reasons why the replication apparatus has to be so complex. Most likely various parts of the replication apparatus, and not only the DNA polymerases, are responsible for this high fidelity. It is, however, known that DNA polymerases themselves copy the template strand with exceedingly high accuracy. Mistakes made by these enzymes in vitro are in the order of one per $10^4 - 10^5$ nucleotides polymerized. As demonstrated by studies with DNA polymerases in bacterial and phage systems, the fidelity of these enzymes can be changed by mutation in the gene coding for the enzyme (*Muzyczka* et al., 1972; *Bazill* and *Gross*, 1973; *Gillin* and *Nossal*, 1976). This can lead to both reduced as well as increased fidelity. In these prokaryotic systems, the built-in $3' \rightarrow 5'$ exonuclease activity certainly helps to maintain the low error frequency of the enzymes because these polymerases will effectively remove their own polymerization errors.

As mentioned earlier most of the eukaryotic DNA polymerases lack such an associated $3' \rightarrow 5'$ exonuclease but the mistake frequency of these enzymes in vitro is still less than 10^{-4}. In vivo it is probably further reduced by the action of separate proof-reading exonuclease and DNA-binding proteins which may be part of a replication and repair complex. Also the error frequency of eukaryotic DNA polymerases can be changed by mutations in the enzyme (*Springgate* and *Loeb*, 1973). Moreover, the mistake frequency of DNA polymerases can be considerably increased in vitro by adding various intercalating dyes, mutagenic substances, or metal ions to the assay system (*Sirover* and *Loeb*, 1974, 1976, 1977). It is interesting that a correlation was established between the potency of various substances or metals as inductors of mistakes in DNA polymerase reactions and their cancerogenic activity in vivo. This supports the hypothesis (*Burnett*, 1974) that mutations induced by errors in the process of DNA replication may be at least one of the causes of cancer. *Linn* et al. (1976) have reported evidence for a decrease in the fidelity of DNA polymerases during aging of cells and suggest that the increased error frequency thus induced within the cell may be responsible at least in part for the process of aging (see also *Burnett*, 1974).

8. Mechanism of Gene Amplification

Several genes or groups of genes occur in the genome of eukaryotes in many identical copies; genes exhibiting this property are called redundant genes (*Tartof*, 1975). Among redundant genes are those coding for ribosomal RNAs (28 S RNA, 18 S RNA, 5.8 S RNA and 5 S RNA), tRNAs, histones and antibodies. Also, the so-called "satellite" DNA comprises highly repetitive DNA sequences which are organized as tandemly repeated units of sometimes rather simple sequences. These multigene families may have arisen in the evolution by gene duplications involving crossing over events (*Smith*, 1973).

Quite distinct from the organization and evolution of redundant genes is the process called "gene amplification". This process involves the production of supernumerary copies of genes which then occur as extrachromosomal, intranuclear DNA. In other words, in contrast to redundant DNA, amplified DNA occurs in the nucleus in a form not integrated into chromosomes. The best studied example of DNA amplification involves the DNA coding for ribosomal RNA in *Xenopus* (*Brown* and *Dawid*, 1968; *Gall*, 1968; *Kalt* and *Gall*, 1974). This DNA is produced in large amounts in the pachytene stage of oogenesis in the frog leading to a 1000-fold amplification of rDNA which is then contained in many nucleoli spread over the inside of the germinal vesicle envelope. Isolated, amplified rDNA of *Xenopus* oocytes consists of 2%–5% circular molecules, the remainder is linear. All of this DNA carries exclusively the genes coding for rRNA as shown by the formation of characteristic patterns upon partial denaturation. About 20% of the circles contain an attached tail of duplex DNA with a length that varies up to several times the contour length of the circle. These observations indicate that gene amplification occurs by a rolling circle mode of DNA replication (*Hourcade* et al., 1973; *Rochaix* et al., 1974), as shown schematically in Figure 6. Proof for the rolling circle replication of rDNA came from electron microscope autoradiographs which show that the rolling circle structures are in fact replicating structures.

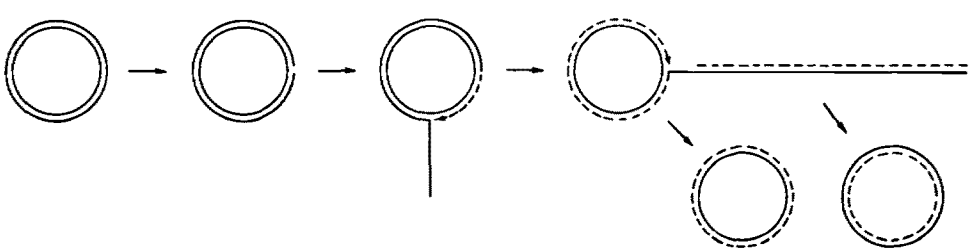

Fig. 6. Rolling circle mode of replication as examplified by the mechanism of amplification of DNA coding for ribosomal RNA in amphibia

In sum, all current evidence is consistent with the view that amplification is initiated from chromosomal rDNA and proceeds by replication of rolling circle intermediates. The amplified rDNA, unlike chromosomal rDNA, contains no methylcytosine (*Dawid* et al., 1970). This modification may be of importance, specifying which rDNA is to be destroyed or preserved at the end of oogenesis. The rolling circle mechanism of DNA replication requires a free circular molecule of rDNA as a template. What is the origin of the first circle? Several possibilities may be suggested: one is that a specific rDNA episome exists aside the chromosomal rDNA and provides the initial template (*Wallace* et al., 1971). This possibility, though unlikely, has not yet been strictly eliminated. The second possiblity is that an excision of chromosomal rDNA would generate a free circle. Such an excision, resembling the induction of phage lambda from the prophage stage, would over a period of time deplete the number of rDNA copies in the chromosomal DNA unless a subsequent reinsertion of excised rDNA occurred or unless crossing over events restored the original redundancy of rRNA genes. A third possibility is that a reverse transcriptase copies a precursor rRNA (*Crippa* and *Tocchini-Valentini,* 1971). Since the circles and rolling circle intermediates contain the spacer segment of rDNA and since the spacer itself is not transcribed (see *Perry,* 1976), this possibility appears highly unlikely. Lastly, simple disproportionate replication of chromosomal rDNA could also generate free circular DNA segments containing a complete rRNA precursor gene (*Tartof,* 1975). If a second replication would be initiated within a replicative bubble of an rRNA gene, this second replication would generate a free extrachromosomal rDNA segment which by a variety of means could be circularized to a free rDNA circle.

Ribosomal DNA amplification serves to provide a large enough number of templates for the synthesis of rRNA which has to take place at an exceedingly high rate during oogenesis in order to keep up with the production of the large amounts of ribosomes that have to be present in the mature oocyte. Since a typical growing somatic cell can produce about 4×10^4 ribosomes per day, it would require several hundred years to produce the 10^{12} ribosomes that occur in the mature oocyte. An about 1000-fold rDNA amplification makes oogenesis possible in months rather than centuries and may explain the widespread occurrence of rDNA amplification among many organisms.

While there is thus no doubt that gene amplification plays an important role in providing the large number of templates for rRNA synthesis during oogenesis, it is rather clear now that this process does not represent a general mechanism for the specialization of differentiated cells. In several cases where this possibility was tested (e.g., in the case of globin synthesis in erythrocytes) it was found that differentiation did not involve an ampli-

fication of genes but rather regulatory mechanisms at the level of transcription and translation (*Bishop* et al., 1972; *Harrison* et al., 1972; *Leder* et al., 1973).

Contrary to the observations in this well studied example of a differentiated function, there is now very strong evidence for the amplification of the structural gene for the enzyme dihydrofolate reductase in variant murine cell lines selected for resistance to the drug methotrexate (*Alt* et al., 1978). These variants contain up to 250 times the normal amounts of dihydrofolate reductase (the enzyme inhibited by methotrexate) due to a similar increase in the amounts of mRNA for the enzyme and of genes coding for this mRNA. In some of the variant cell lines the amplified genes are unstable, i.e., gene copies are reduced to normal levels if the cells are grown in the absence of the drug, in other cases, however, they appear to be stable. It is unknown so far, whether the supernumerary genes are located tandemly arranged within a chromosome or whether they occur extrachromosomally. Nor is it known whether they arise through a replication process or through recombination events.

Although there is at present no evidence that processes leading to selective multiplications of structural genes play a role in cell differentiation, the experiments with methotrexate resistant cell lines clearly show that mechanisms do exist which make such reactions possible.

9. Replication of Mitochondrial DNA

9.1 General Mechanism of Mitochondrial DNA Replication

The mode of replication of mitochondrial DNA in animal cells has recently been established by ordering the various replicative intermediates seen in the electron microscope. The overall mechanism appears to be that of a modified Cairns type of replication, involving replication bubbles, rather than the rolling circle mechanism described above (*Kirschner* et al., 1968; *Kasamatsu* et al., 1971; *Robberson* et al., 1972; *Wolstenholme* et al., 1973). The DNA present in the mitochondria of animal cells is a circular molecule with a circumference of about 5 μm, corresponding to a MW of about 10^7 daltons. Replication of this molecule starts at a fixed point and occurs unidirectionally with a high degree of asymmetry (*Kasamatsu* and *Vinograd,* 1973; *Berk* and *Clayton,* 1974) (Fig. 7). After initiation, one strand of the double-stranded molecule is replicated first while the opposite strand remains single stranded. This gives rise to a so-called "displacement loop" (D-loop) which can be seen in electron micrographs of molecules of mitochondrial DNA. D-loops are expanded by further elongation of the newly synthesized DNA strand which can proceed to a considerable

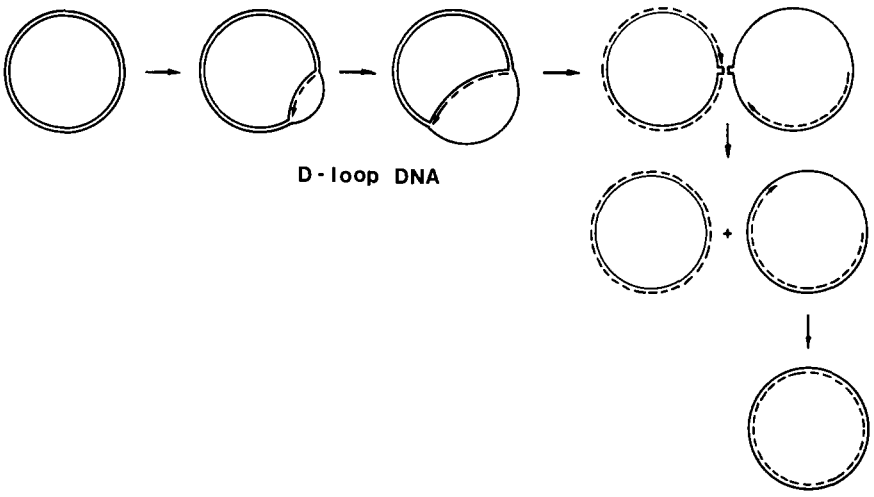

Fig. 7. Asymmetric replication of circular mitochondrial DNA involving displacement loop (D-loop) structures

extent before replication of the opposite strand of the parental DNA is initiated. The displacement synthesis has been shown to be strand specific. In mouse cells, for instance, it is always the H (heavy) strand that is synthesized first (the strand designation, heavy and light, relates to the buoyant densities in alkaline CsCl gradients).

As also demonstrated in the replication of papova virus DNA (*Jaenisch* et al., 1971), replicating circular mitochondrial DNA molecules are covalently closed and superhelical in the nonreplicating part (*Robberson* and *Clayton*, 1972). Despite the asymmetry, the replication of mitochondrial DNA is semiconservative (*Flory* and *Vinograd*, 1973). A unique feature of mitochondrial DNA replication is that displacement synthesis temporarily terminates after a small percentage of the genome has been replicated, forming small D-loops which apparently are awaiting some signal for extension (*Kasamatsu* et al., 1971).

When isolated from cells in culture, or from tissues containing dividing cells, a large fraction of mitochondrial DNA consists of replicating molecules with D-loops. These replicating intermediates contain a stretch of the H strand synthesized during the early part of the replication process. This small DNA may be released by partial denaturation yielding a homogeneous single-stranded DNA (some 450 nucleotides long and sedimenting with about 7 S) and a closed circular molecule of parental DNA. The size of the released piece of DNA corresponds to the size of the D-loop (*Kasamatsu* et al., 1971; *Arnberg* et al., 1971).

In view of the importance of short oligoribonucleotides to serve as primers of DNA synthesis, isolated 7 S DNA was examined for the presence of terminal ribonucleotides after radioactive labeling with polynucleotide kinase (*Kasamatsu* et al., 1973). The terminal radioactivity remained attached after treatment of the DNA with alkali suggesting the absence of ribonucleotides at the 5′ terminus. This indicates that either mitochondrial DNA replication is initiated by deoxyribonucleotides or, more likely, that the initiating ribonucleotides are rapidly removed from the 5′ terminus of 7 S DNA within the cell when the D-loop is waiting for extension.

Partially purified mitochondria are capable of incorporating DNA precursors into mitochondrial DNA, indicating that mitochondrial DNA replicates autonomously (*Wintersberger,* 1966; *Parson* and *Simpson,* 1967, 1973; *Ter Schegget* and *Borst,* 1971a,b). Incorporation of thymidine triphosphate into mitochondrial DNA depends on the addition of all four deoxyribonucleoside triphosphates and particularly on the maintenance of the ATP level in the mitochondria. Inhibition of oxydative phosphorylation or of electron transport inhibits the incorporation. Several investigators observed that labeled deoxyribonucleoside triphosphates were incorporated primarily into a species of DNA which upon denaturation sediments as a small 7 S polynucleotide while the parental DNA, which remained a closed circular molecule, was found to be unlabeled. It was thus established that under in vitro conditions, using isolated mitochondria, D-loop DNA is synthesized; apparently, however, extension of the D-loop does not usually take place under these in vitro conditions.

Experiments using synchronized cells have shown that mitochondrial DNA synthesis is not restricted to the S-phase but also takes place at other times in the cell cycle (*Picamottoccia* and *Attardi,* 1972). Since the amounts of mitochondrial DNA per cell or the ratio of nuclear to mitochondrial DNA are held constant, there must exist mechanisms to control the quantity of mitochondrial DNA synthesized.

Studies using yeast mutants which are temperature sensitive in nuclear DNA replication provided strong evidence that nuclear and mitochondrial DNA synthesis share common proteins. Mutants defective in the elongation reaction in nuclear DNA replication turned out to be at the same time temperature sensitive in mitochondrial DNA synthesis (*Wintersberger* et al., 1974; *Newlon* and *Fangman,* 1975). Conversely, mutants which are temperature sensitive in the initiation of nuclear replication were, at least for some time, normal in mitochrondrial DNA synthesis (*Newlon* and *Fangman,* 1975). As expected, therefore, the initiation of DNA replication is the event which shows the greater specificity.

9.2 Enzymology of Mitochondrial DNA Replication

More than 10 years ago, mitochondria were found to contain a DNA polymerase (*Wintersberger,* 1966; *Meyer* and *Simpson,* 1968; *Kalf* and *Chih,* 1968). Extensive studies with yeast have provided evidence that the mitochondrial enzyme is different from the nuclear polymerases and that this enzyme is coded by nuclear DNA and synthesized by cytoplasmic and not by mitochondrial ribosomes (*Wintersberger* and *Wintersberger,* 1970b). This polymerase is, therefore, one of the many proteins which are incorporated into the mitochondria from the cytoplasm during mitochondrial biogenesis. In recent years, mitochondrial DNA polymerases have been highly purified from many eukaryotes, including mouse cells (*Radsak* and *Seidel,* 1976), HeLa cells (*Radsak* et al., 1976), rat liver (*Tanaka* and *Koike,* 1977), and yeast (*Wintersberger* and *Blutsch,* 1976). The MW of these enzymes was found to be around 120,000 and, as first shown for yeast mitochondrial polymerase, the enzymes seem to consist of two subunits of a MW of about 60,000. It is not known so far whether these subunits are identical. Despite the similarity in size of mitochondrial DNA polymerases from mammalian cells and from yeast, there exists one distinct difference: HeLa cell and other mammalian mitochondrial DNA polymerases were recently shown to be identical with the DNA polymerase γ found earlier in nuclei and cytoplasm of animal cells (*Bolden* et al., 1977; *Bertazzoni* et al., 1977; *Hübscher* et al., 1977); these enzymes are capable of copying ribopolynucleotide templates. The yeast enzyme, on the other hand, was found to be almost completely inactive with such templates under a variety of conditions (*Wintersberger* and *Blutsch,* 1976). It is worth mentioning that yeast mitochondrial DNA polymerase exhibits a significantly lower fidelity in vitro than nuclear enzymes from the same cell (*Wintersberger,* unpublished). This property could provide a basis for the high rate of spontaneous mutations of mitochondrial DNA observed in this unicellular organism.

Almost nothing is known about other enzymes and proteins involved in mitochondrial DNA replication. Certainly a DNA ligase and a relaxing enzyme must be needed: the first one at least for closing the ring after replication (even if the special mechanism of mitochondrial DNA replication should otherwise dispende with such an enzyme), the latter one to remove the superhelical turns present in the DNA molecule.

10. Concluding Remarks and Outlook

Several basic questions concerning the mechanism of DNA replication in eukaryotes have been answered during the last 10 years. Most important is the finding that eukaryotic chromosomes are subdivided into a large

number of replication units. Batteries of such units are initiated at particular times in the S-phase of the cell cycle, probably at specific initiation sites. Replication proceeds bidirectionally until two neighbouring replication bubbles merge. The size of replication units varies among different organisms and also depends on the developmental stage of a particular cell. The rate of fork movement (the elongation rate), on the other hand, is fixed for a given cell: The duration of S-phase, therefore, is largely determined by the number of replicons active at a certain time. The events at the replication fork itself are less clear. There is no doubt that replication occurs by the formation of small intermediates, Okazaki fragments, at least at the lagging (net direction of synthesis $3' \rightarrow 5'$) strand. Primary replication intermediates sediment with about 4 S, they are smaller than Okazaki fragments in bacterial systems and their size may be determined by the structural organization of the DNA in chromatin or by a built-in property of eukaryotic DNA polymerases. Strong evidence suggests that at least some Okazaki fragments are initiated by the synthesis of short (about ten bases long) oligoribonucleotides, but it is not yet known whether this is true for all Okazaki fragments.

The enzymology of eukaryotic DNA replication is only at its beginning. Considerable knowledge on the properties of the various DNA polymerases has accumulated in the past few years but our ideas on the detailed function of these enzymes are so far only suggestive. Much less is known about the other enzymes and proteins required. It seems to me that this is a long neglected, but very important, area in cell biology and biochemistry. Knowledge of the enzymatic properties of various parts of the replication apparatus will be extremely important for an understanding of the regulation of DNA replication in the cell cycle. Several possibilities for cell cycle regulation can now be considered. Apart from the timing of the synthesis of particular regulatory proteins in the cell cycle, chemical modifications of preexisting enzymes and proteins must always be taken into consideration as alternative modes of regulation. Such modifications are frequently found to be one of the very first reactions following a mitotic stimulus (e.g., in phytohemagglutinine-stimulated lymphocytes). These are enzymatic reactions and knowledge of the specificity and regulation of the enzymes involved would greatly help to understand cell cycle controls.

Much information on the regulation of DNA replication and cell division may be gained from the detailed analysis of a system involving cells lytically infected or transformed by small DNA tumor viruses. The great advantage of this model system lies in the fact that the mitogen, the T-antigen, is a viral gene product the structure and properties of wich will soon be known in some detail. T-antigens are localized primarily in the nucleus of the infected or transformed cell and thus one may hypothesize

that they interfere directly with regulatory circuits on the chromatin of the host cell. To learn how T-antigens of SV40, polyoma, or adenoviruses exert their functions will not only help to understand the mechanism of the replication of, and the transformation by, DNA tumor viruses but at the same time will bring us an important step closer to the unraveling of the complex mechanisms controlling DNA replication and cell division.

Equally important may be the application of genetic methods to the solution of the detailed mechanism and control of DNA replication. Favorite organisms for such an approach are lower, unicellular eukaryotes, particularly yeasts which are easily amenable to genetic analyses. Conditionally lethal cell cycle and DNA replication mutants have already been isolated in the yeasts *Saccharomyces cerevisiae* and *Schizzosaccharomyces pombe*. The biochemical analysis of such mutants is only at its beginning but can be expected to yield important informations.

Finally there is one aspect which has received relatively little attention so far but which will probably turn out to be extremely important; this concerns the relationship between chromatin replication and cell differentiation. The differentiated state of a cell is stable over many generations. Since much of the information for the control of gene expression is encoded in the structure of chromatin, there should be strict rules by which this information is transferred from mother to daughter cells. The propagation of specific chromosomal information, therefore, could be a direct result of the semiconservative replication of DNA and of the distribution of chromosomal proteins during replication. Moreover, the stability of the differentiated state of a cell, layed down in the stability of chromatin structure, demands that any change in this structure can occur only during chromatin replication. This could form the basis of the requirement for the defined number of cell cycles a stem cell has to go through in order to develop into a particular differentiated cell. Each division cycle could result in a specific change in the chromatin structure (triggered from outside the cell, e.g., by hormones), giving the daughter cell a potential not present in the mother cell. Whether this cell makes use of all its transcriptional potential may well depend on several other factors in addition to the structure of chromatin. However, in the absence of a specific structural organization of a particular gene, this gene might well be never expressed. In view of these considerations much effort will be needed to analyse the distribution of chromosomal proteins during DNA replication in differentiating cells.

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The Kinin System: Its Relation to Blood Coagulation, Fibrinolysis and the Formed Elements of the Blood *

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Contents

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List of Abbreviations

BAEe	Benzoyl arginine ethyl ester
DFP	Diisopropylfluorophosphate
EACA	Epsilon amino caproic acid
EDTA	Ethylene diamine tretraacetate
Factor XIIa	Activated (intact) factor XII
Factor XIIf	Fragmented factor XII
HMW	High molecular weight
LBTI	Lima bean trypsin inhibitor
LMW	Low molecular weight
MW	Molecular weight
PF/dil	Permeability factor, dilute
pI	Isoelectric point
PKA	Prekallikrein activator
PTT	Partial thromboplastin time
SBTI	Soy bean trypsin inhibitor
SDS	Sodium dodecyl sulfate
TAMe	Tosyl arginine methyl ester
TLCK	Tosyl lysine chloromethyl ketone

I. Introduction

About 50 years ago, *E.K. Frey* (*Frey* and *Kraut,* 1928), a surgeon in Munich, found that intravenous injection of normal urine causes hypotension and an increase in heart rate. The findings confirmed earlier observations by *Abelous* and *Bradier* (1909). *Kraut* and *Werle* (1930), coworkers of *Frey,* isolated a hypotensive substance from pancreas and named it kallikrein (Gr. kallikreas = pancreas). It was first believed that kallikrein is secreted from the pancreas into the blood where it circulates in an inactive form and is later reactivated and excreted in the urine. Later studies demonstrated that a mixture of plasma and kallikrein causes contraction of an isolated segment of guinea pig ileum and the spasmogen was referred to as "darmkontrahierende Substanz" or gut-contracting substance (*Werle* et al., 1937). *Werle* and *Berek* (1948) substituted the name kallidin for darmkontrahierende Substanz and referred to the precursor as kallidinogen. Kallidin is known today as the decapeptide lysyl-bradykinin.The nonapeptide, bradykinin, was discovered independently by *Rocha e Silva* et al. (1949), by demonstrating that incubation of the snake venom *Bothrops jararaca* or trypsin with a pseudoglobulin fraction of plasma leads to the formation of a potent vasodilator and smooth mucle-stimulating substance. These investigators referred to the precursor as bradykininogen. Today the term kininogen is used for the precursor of all kinins. Later studies demonstrated that kallikrein of glandular tissues is a different enzyme than plasma kallikrein (*Webster* and *Pierce,* 1963). The glandular kallikreins generate kallidin or lysyl-bradykinin, whereas plasma kallikrein generates bradykinin from kininogen.

Activation of the plasma kinin system is a complex process in which the ultimate step is cleavage of bradykinin from kininogen by plasma kallikrein. It is the activation of prekallikrein to kallikrein by activated factor XII (factor XIIa) or Hageman factor, but particularly the mechanism of activation of factor XII that has received considerable attention over the past 25 years. The impetus for these studies was the observation that the end product, bradykinin, is a potent vasoactive substance; very small doses being capable of eliciting the vascular phenomena of acute inflammation.

Early observations on the activation of the kinin system were made by *Miles* et al. between 1953 and 1958 (see *Miles,* 1964). These studies dealt with a permeability globulin referred to as *PF/dil,* or permeability factor dilute; a putative substance, capable of inducing enhanced vascular permeability on dilution of serum or plasma. The substance was not demonstrable when the plasma was diluted in plastic or siliconized vessels. Plasma kallikrein had been shown to be capable of releasing kinin from both fresh and heated plasma. In the latter only kininogen is present, the enzymes having been inactivated. *Mason* and *Miles* (1962) added PF/dil to both fresh and to heated plasma. Since kinin could be released only from fresh plasma they postulated that PF/dil converted prekallikrein to kallikrein in the fresh plasma and that the kallikrein then released kinin from kininogen. In heated plasma the prekallikrein had been inactivated. Based on earlier observations of *Armstrong* et al. (1955, 1957) and *Margolis* (1957, 1958), *Miles* (1964) proposed that activated factor XII acted on pro-PF/dil, converting it to PF/dil, which in turn activated prekallikrein to kallikrein; the latter releasing kinin from kininogen. *Armstrong* and coworkers and *Margolis* demonstrated a relationship between a pain-producing substance, kinin-formation, and the contact phase of blood coagulation, in which activation of factor XII played a central role. *Miles* based his hypothesis also on observations he had made with *Ratnoff* (*Ratnoff* and *Miles,* 1964). They injected highly purified factor XIIa (*Ratnoff* and *Davie,* 1962) intradermally into guinea pigs and observed an increase in vascular permeability, detectable as extravasation of intravenously injected Evans blue. A subthreshold dose of factor XIIa, which caused no bluing, did cause dye leakage when first mixed with diluted noncontacted plasma. *Ratnoff* and *Miles* believed that factor XIIa induced formation of a permeability factor in plasma.

Of the early studies, most informative were those of *Margolis* (1958, 1960, 1966). His observations carried out with crude preparations are well in keeping with new data on purified components of the kinin system. *Margolis* used a special nomenclature. In referring to these various substances, the current terminology will occasionally be given in parenthesis. *Margolis* found that kinin-formation in plasma by surface contact required

factor XII (*Margolis,* 1958). Factor XII became adsorbed to glass or another surface and, following activation, activated another substance referred to as "component A" (plasma kallikrein). Component A was shown to remain unadsorbed, i.e., in the plasma where it could generate kinin from kininogen. Contact exposure could not sonsume all the kininogen in plasma. *Margolis* first exposed plasma for 5–10 min to glass. After separation of the glass and standing for 3–4 h both component A, i.e., kinin-forming activity (plasma kallikrein) and kinin decayed. Today we know that this is due to the effect of certain inhibitors. New exposures of this plasma for 2–4 min to glass induced more component A or kinin-forming activity, but no kinin could be generated. The kinin-forming activity could be demonstrated in plasma after separation from the glass beads by incubating the exposed plasma with fresh nonexposed plasma which contained kininogen. The inability to release kinin upon a second exposure of plasma to glass was not due to a lack of kininogen, since glandular kallikreins could generate kinin (kallidin) from such plasma (*Keele,* 1960). It was therefore proposed by *Margolis* (1960) that the first exposure of plasma to glass depleted it of a substance which he called "component B" (HMW kininogen). A later section of this review describes the different susceptibilities of the two kininogens; HMW kininogen, in contrast to LMW kininogen, is rapidly cleaved by plasma kallikrein (see Sect. IV). Several investigators, including *Margolis* (1966), believed that component A was plasma kallikrein and this was substantiated in more recent studies. However, the nature of component B remained obscure for some time. *Vogt* (1965, 1966) found that it is a special kininogen different from the one which remained after contact activation and which was susceptible only to glandular kallikreins. Recent data indicate that component B is HMW kininogen.

Vogt's (1965, 1966) hypothesis that plasma contains two distinct kinin-forming systems could not be substantiated. However, *Vogt* et al. (*Wendel* et *al.*, 1972; *Vogt* and *Dugal,* 1976) presented data to explain the hypothesis, as will be discussed in Section III which deals with prekallikrein and kallikrein.

The studies carried out between 1953 and about 1967 were done with plasma after various treatments. For almost a decade an increasing number of studies were published concerning partially purified and highly purified components of the kinin system. These studies led to good, but as yet incomplete, understanding of the plasma kinin system and its interrelation to blood coagulation and fibrinolysis (Fig. 1). When factor XII becomes activated it activates factor XI, which in turn converts factor IX to IXa; the latter acts on factor VIII, converting it to VIIIa, which activates factor X. This is the intrinsic pathway. In the extrinsic pathway of blood coagulation, factor VII acts directly on factor X after tissue damage and release of tissue thromboplastin.

With respect to the inflammatory reaction, the implication of the kinin system and vasoactive peptides as mediators is still conjectural since direct evidence is lacking.

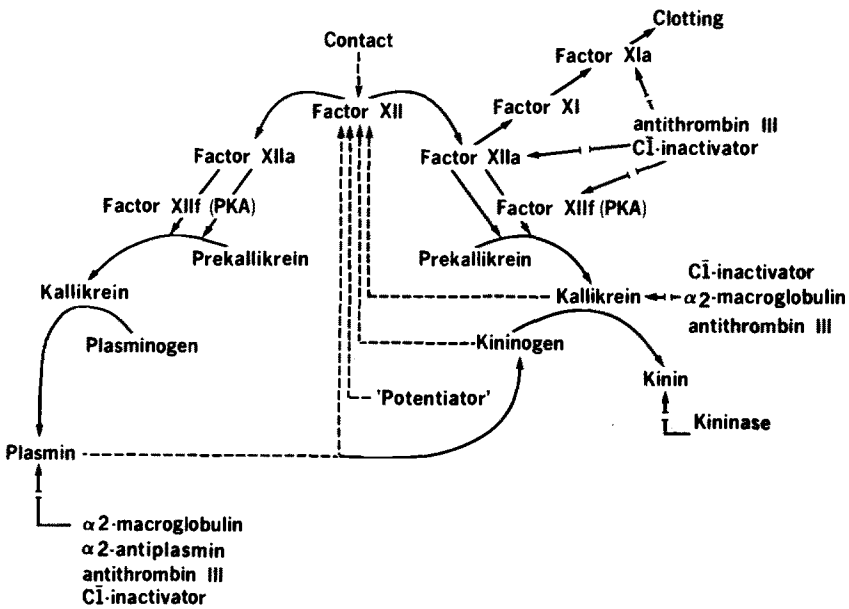

Fig. 1. Schematic representation of the plasma kinin system, its relation to fibrinolysis and the initial stage of the intrinsic clotting system. Inhibitors are represented by interrupted arrows

II. Factor XII

A. Isolation and Characterization

Factor XII or Hageman factor deficiency was discovered in 1954 by *Ratnoff* (see *Ratnoff*, 1966). As indicated above, implication of this blood clotting factor in kinin-generation is attributable to *Margolis* (1958).

Isolation and purification of factor XII was attempted from about 1960 onward. The first highly purified factor XII was isolated from *human plasma* by *Ratnoff* and *Davie* (1962), who achieved over 5000-fold purification by a series of chromatographic steps. This preparation served for a large number of studies. During the past 5–6 years, a number of investigators obtained factor XII preparations of varying degrees of purity (*Cochrane* et al., 1976; *Movat*, 1978b). The most recent procedures for obtaining homogeneous factor XII were reported by *Griffin* and *Cochrane* (1976a) and by *Chan* and *Movat* (1976). *Griffin* and *Cochrane* used anion and

cation exchange chromatography and *Chan* and *Movat* added an immunoabsorbent column (Figs. 2 and 3). Factor XII was isolated also from *animal plasmas,* namely from bovine (*Schoenmakers* et al., 1965; *Temme* et al., 1969; *Komiya* et al., 1972; *Fujikawa* et al., 1977) and from rabbit plasma (*Cochrane* and *Wuepper,* 1971b). In the recent publication of *Fujikawa* et al. (1977) bovine plasma was passed through a series of chromatographic steps, including affinity chromatography on heparin-Sepharrose, arginine-Sepharose, and benzamindine-Sepharose.

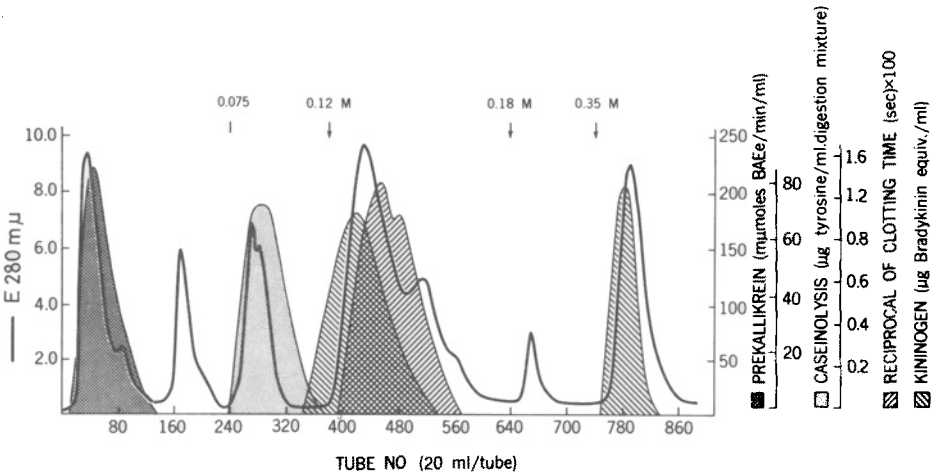

Fig. 2. Elution pattern of components of the kinin system on QAE-Sephadex at pH 8. Prekallikrein elutes in the excluded peak, plasminogen (caseinolysis) when the NaCl is raised to 0.075 M, followed by factor XII (clotting) and LMW kininogen (with 0.12 M NaCl) and finally HMW kininogen (with 0.35 M NaCl). (From *Habal* and *Movat,* 1976a)

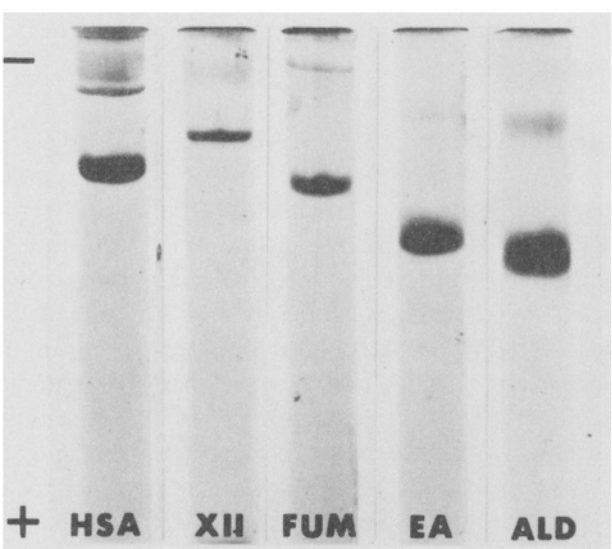

Fig. 3. SDS-disc gel electrophoresis of factor XII, together with the marker proteins of human serum albumin (HSA; MW 69,000), fumarase (FUM; MW 49,000), egg albumin (EA: MW 43,000) and aldolase (ALD; MW 40,000). The second (higher MW) band of HSA represents the dimer (MW 138,000). (From *Chan* and *Movat,* 1976)

The physicochemical properties of human factor XII are well characterized. Factor XII is a β-globulin with an isoelectric point of 6.0–6.5. By gel filtration, a MW of approximately 110,000 was estimated and the sedimentation coefficient varies between 4.5 and 5.5 S in the various reports (*Donaldson* and *Ratnoff,* 1965; *Cochrane* and *Wuepper,* 1971b; *Kaplan* et al., 1971; *Soltay* et al., 1971). Subsequent findings based on SDS disc gel electrophoresis (*Cochrane* et al., 1972; *Bagdasarian* et al., 1973a) and on the sedimentation coefficient, partial specific volume, and diffusion coefficient (*Movat* et al., 1973a) indicated a MW of about 90,000. More recent studies, using SDS-disc gel electrophoresis, indicate a still lower MW, i.e., 80,000 (*Revak* et al., 1974) and 76,000–78,000 (*Chan* and *Movat,* 1976), respectively. Factor XII consists of a single polypeptide chain.

The amino acid composition of factor XII showed a reasonable degree of similarity in experiments carried out in two different laboratories (*Revak* et al., 1974; *McMillin* et al., 1974).

The concentration of factor XII in human plasma has been estimated to be 29 μg/ml (range, 15–47 μg/ml in 17 subjects), as determined by quantitative radial immunodiffusion (*Revak* et al., 1974).

Bovine factor XII was earlier found to have a sedimentation coefficient of 7.0 S, and a MW of 82,000 (*Schoenmakers* et al., 1965) or 89,000 (*Komiya* et al., 1972). The recent studies of *Fujikawa* et al. (1977) indicate that bovine factor XII is a glycoprotein with a MW of 74,000, containing 13.5% carbohydrate, including 3.4% hexose, 4.7% N-acetylhexosamine, and 5.4% N-acetylneurminic acid. It consists of a single polypeptide chain with an NH_2-terminal homologous to the reactive site of a number of proteinase inhibitors. The amino acid sequence of the C-terminal was found to be homologous with the active site of several plasma serine proteases.

B. Activation of Factor XII

Factor XII can be activated in fluid phase by incubating it with a proteolytic enzyme such as trypsin or plasmin or by contact with a negatively charged surface such as glass, kaolin, or collagen to which it becomes absorbed. The two mechanisms have been referred to as fluid phase and solid (contact) phase activation (*Cochrane* et al., 1973).

Essential in both fluid and solid phase activation are proteolytic enzymes. In this respect it is important to define "activation". Factor XIIa is referred to as the activated clotting factor XII.

Cochrane and *Wuepper* (1971b) used trypsin for the *fluid phase* activation of purified factor XII and assayed the capacity of factor XIIa to convert prekallikrein to kallikrein and to correct the coagulation defect of

factor XII-deficient plasma. Trypsin cleaves factor XII to XIIf (prekallikrein activator, PKA). It induces two measurable changes in the factor XII molecule. As the amount of trypsin incubated with factor XII increases there is simultaneous decrease in partial thromboplastin time (PTT) with factor XII-deficient plasma and an increase in prekallikrein activation (*Soltay* et al., 1971). Plasmin also fragments factor XII, inducing PKA-activity, associated with preablumin bands (*Kaplan* and *Austen,* 1971). Similar to trypsin, plasmin induces simultaneously a decrease in clot-promoting activity and a conversion of prekallikrein to kallikrein (*Burrowes* et al., 1971). It would appear that limited proteolysis induces maximum activation of factor XII for clotting of factor XII-deficient plasma, whereas proteolysis associated with complete cleavage of factor XII induces maximum activity for activation of prekallikrein. However, the enzymatic activity resides in fragment XIIf in both systems, since the fragment is capable of activating prekallikrein as well as factor XI (*Heck* and *Kaplan,* 1974; *Movat* and *Özge-Anwar,* 1974). Kallikrein is another enzyme which plays a role in the activation of factor XII (*Cochrane* et al., 1973). Its function is discussed below in conjunction with contact activation.

A number of substances can activate factor XII. As already indicated, factor XII can be activated in fluid and in solid phase (*Cochrane* et al., 1973). The most common activators used in the laboratory are glass, kaolin, and celite (*Margolis,* 1966; *Ratnoff,* 1966). Other agents include elagic acid (*Ratnoff* and *Crum,* 1964), cellulose sulfate, carageenan (*Kellermeyer* and *Kellermeyer,* 1969; *Schwartz* and *Kellermeyer,* 1969), chondroitin sulfate, and articular cartilage (*Moskowitz* et al., 1970). Various connective tissue components have been shown to be capable of absorbing and activating factor XII in plasma including collagen, elastin, and basement membrane (*Niewarowski* et al., 1965; *Wilner* et al., 1968; *Cochrane* and *Wuepper,* 1971a; *Harpel,* 1972). Other substances with possible in vivo significance are uric acid (*Kellermeyer* and *Breckenridge,* 1965), L-homocysteine (*Ratnoff,* 1968), and bacterial lipopolysaccharides (*Pettinger* and *Young,* 1970; *Kimball* et al., 1972; *Morrison* and *Cochrane,* 1974).

Contact activation also seems to require proteolysis. When purified factor XII is exposed to celite, little activation takes place and the eluates contain mostly unactivated factor XII (*Movat* and *Özge-Anwar* et al., 1974), whereas exposure of whole plasma to celite is associated with activation and fragmentation of factor XII (*Özge-Anwar* et al., 1972). Therefore, probably additional plasma factors are required for the activation of factor XII. The discovery of certain deficiencies of components of the kinin system in recent years led to a better understanding of these factors and their role in contact activation. Fletcher trait, a deficiency in prekallikrein is described in Section IV (*Wuepper,* 1973; *Saito* et al., 1974a; *Weiss* et al., 1974). A deficiency in HMW kininogen has been described under

several names (*Saito* et al., 1975; *Wuepper* et al., 1975; *Colman* et al., 1975; *Donaldson* et al., 1976; *Webster* et al., 1976), the name of the factor referring to patients in which the deficiency was encountered (Fitzgerald, Flaujeac, Williams). Figure 4 illustrates the effect of various deficiencies on clotting and fibrinolysis. Independent studies of *Schiffman* and *Lee* (1974, 1975) showed that a substance referred to as "contact activation cofactor"

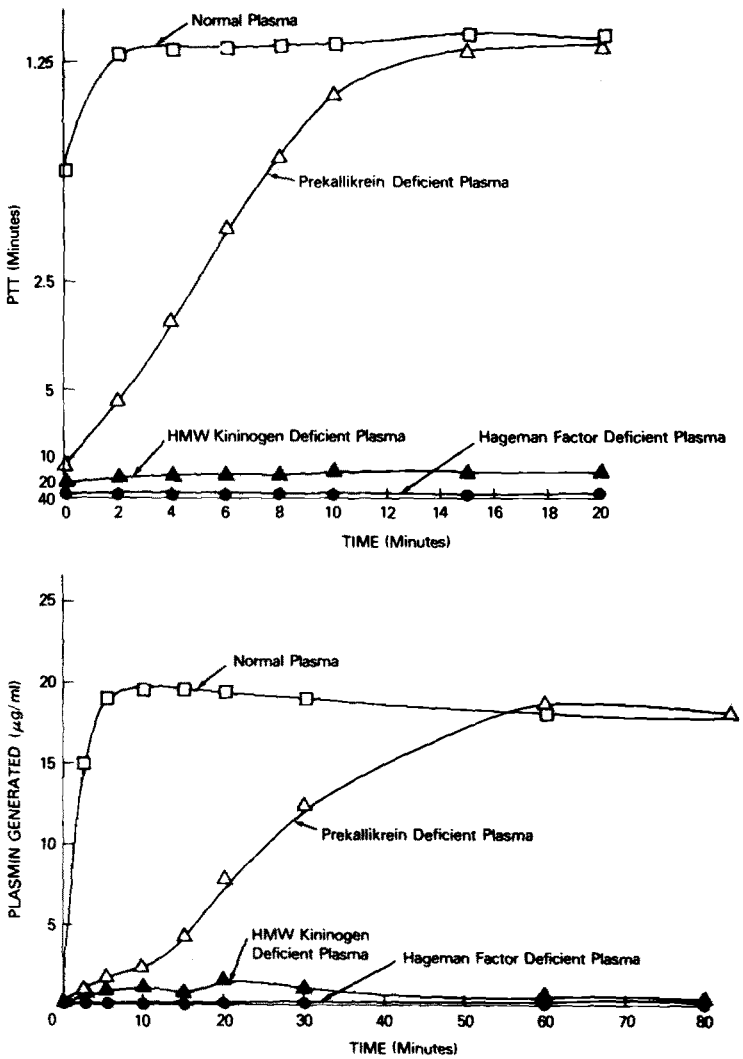

Fig. 4. *Top:* Partial thromboplastin time (PTT) showing the clotting (in minutes) upon exposure to kaolin of normal plasma and of various deficient plasmas, as indicated. Note that the clotting of HMW kininogen-deficient and of factor XII-deficient plasmas is not corrected upon prolonged exposure, but plasma deficient in prekallikrein gradually becomes corrected during contact with a surface. *Bottom:* Similar observations measuring plasmin generated using a fibrinolytic (fibrin plate) assay. (From *Meier* et al., 1977)

is required for the rapid and full activation of factor XI by factor XIIa. This substance was subsequently identified with Fitzgerald factor or HMW kininogen (*Schiffman* et al., 1975).

The studies with deficient plasmas were followed by investigations with isolated components (*Kaplan* et al., 1976a; *Chan* et al., 1976, 1978; *Meier* et al., 1977). Surface-bound factor XII requires the addition of HMW kininogen and an unidentified potentiator for full activation of prekallikrein to kallikrein (Fig. 5) (*Chan* et al., 1977b). The rate of activation of factor XII and of prekallikrein conversion is dose dependent. Increasing (stoichiometric) amounts of HMW kininogen yield a proportionate increase in the amount of prekallikrein that is activated to kallikrein (*Chan* et al., 1976, 1978). Similar observations were made with activation of factor XI

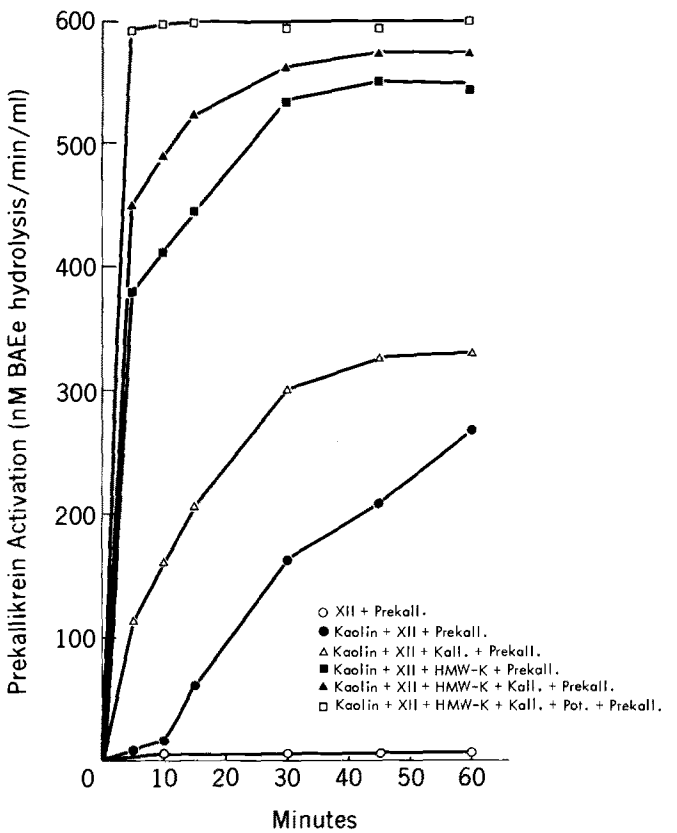

Fig. 5. Rate of activation of prekallikrein by surface-bound factor XII. Factor XII was incubated for 2 min with kaolin followed by another incubation for 10 min with the other reactants shown in the illustration, except prekallikrein. Prekallikrein was then added and the entire mixture incubated for the times indicated. The kallikrein generated from prekallikrein was assayed for BAEe hydrolysing activity. Abbreviations: Prekall. = prekallikrein; Kall. = kallikrein; HMW-K = high molecular weight kininogen; Pot = potentiator (for further details see *Chan* et al., 1976 and 1977b)

(*Schiffman* and *Lee*, 1975; *Griffin* and *Cochrane*, 1976b; *Griffin* et al., 1976; *Saito*, 1977). *Griffin* et al. (1976) proposed a model in which factor XII and HMW kininogen form a complex on a negatively charged surface, placing factor XII into a conformation which is highly susceptible to proteolytic cleavage by kallikrein. As soon as kallikrein is generated by activated factor XII, more factor XII becomes activated. What remains to be answered is how the process is initiated when all components are unactivated. The fact that mere exposure to a negatively charged surface brings about configurational changes in the factor XII molecule (*McMillin* et al., 1974) may be the answer, but not a definite one since *McMillin* et al. did not study the function of factor XII. In fact, recent findings of *Meier* et al. (1977) indicate that mere exposure to celite (Supercel) does not induce incorporation of ^{3}H-labeled DFP. Similar observations were made by *Griffin* (1977). Factor XII exposed to kaolin took up only trace amounts of [^{3}H]-DFP, whereas trypsin-treated factor XII, or factor XII exposed to a surface in the presence of kallikrein and HMW kininogen, took up the radiolabel in the 28,000 MW fragment. Thus, initiation of the contact activation of factor XII remains an open question. For the present, is it simplest to assume that contact activation begins as a nonenzymic process in which minor conformational changes in the factor XII molecule are sufficient to activate traces of prekallikrein. The generated kallikrein can then activate more factor XII in the presence of HMW kininogen and thus initiate a vicious cycle.

It was first believed that so-called kinin-free HMW kininogen (the major fragment remaining after kinin-releasing cleavage of HMW kininogen) can augment the activation of factor XII (*Colman* et al., 1975; *Kaplan* et al., 1976b; *Webster* et al., 1976). However, it was subsequently demonstrated that human HMW kininogen cleaved by trypsin, plasmin, or plasma kallikrein cannot enhance the contact activation of factor XII (*Chan* et al., 1976, 1978). When increasing amounts of kallikrein were incubated with HMW kininogen, there was an inverse relationship between the kinin generated from the kininogen and the ability of the treated kininogen to activate factor XII. Thus, the intact HMW kininogen molecule is required for the enhanced contact activation of factor XII. Independent studies with bovine HMW kininogen prepared by Japanese investigators demonstrated first that kinin-free kininogen had only 30% of the activity of the intact molecule (*Waldmann* et al., 1976) and later that it was almost free of any such activity (*Matheson* et al., 1976). The fragments of bovine HMW kininogen had no enhancing effect. While this manuscript was being prepared, *Schiffman* et al. (1977) again presented evidence that kininogen, presumed to be kinin-free, could enhance the activation of factor XII. However, the amounts of kinin released from the HMW kininogen were very small, indicating perhaps incomplete cleavage of the kininogen.

During both fluid phase and solid phase activation, fragmentation of the factor XII molecule takes place. The smallest fragments of MW 30,000–40,000 were recognized first as prekallikrein activators (PKA) without knowledge that they derive from factor XII (*Movat* et al., 1968, 1969b). *Kaplan* and *Austen* (1970) were the first to propose that PKA was derived from factor XII. When they fractionated human serum (in which factor XII activation has already taken place) on DEAE-cellulose five peaks were recovered which generated kinin when added to fresh plasma. Of these, peak 1, the most cationic, was plasma kallikrein. Peaks 2 and 3 probably represented activated intact or partially fragmented factor XII, which shortened the PTT of factor XII-deficient plasma. Peaks 4 and 5 were weak in correcting the clotting deficiency in factor XII-deficient plasma, but readily activated prekallikrein to kallikrein. Peaks 2–5 were designated PKA and believed to derive from factor XII (peak 2). The most anionic peaks (4 and 5) migrated as prealbumin bands during alkaline disc gel electrophoresis and had an estimated MW of 30,000–40,000 by gel filtration. Following another study, *Kaplan* and *Austen* (1971) noted "a progressive decrease in size, increase in net negative charge, increased prekallikrein-activating activity, and decreased ability to correct Hageman factor deficiency". Similar observations were made by *Poon* (1970), who by gel filtration found a MW of 125,000 for activated intact factor XII, 75,000 and 60,000 for intermediate fragments which were more anionic, and 37,000 for the most anionic PKA. Because of the fragmentation, PKA was designated fragment XIIf (*Özge-Anwar* et al., 1972). *Bagdasarian* et al. (1973a,b) isolated from plasma an active intermediate fragment of MW 70,000 with the mobility of an α-globulin. Intact factor XII has β-electrophoretic mobility and the fully fragmented XIIf migrates further anodally as a prealbumin. Intermediate fragments were demonstrated also by *Chan* (1975), using both gel filtration and sucrose density gradient ultracentrifugation. Three peaks demonstrable by ultracentrifugation sedimented at 5.1, 3.7 and 2.7 S; the fastest sedimenting being factor XII and the slowest PKA or XIIf (Fig. 6).

Contrary to these findings, *Cochrane* and coworkers detected only two forms of biologically active factor XII, either intact or fully fragmented. Rabbit factor XII, which by gel filtration had a MW of 110,000, converted after treatment with trypsin into a 32,000 MW fragment (*Cochrane* and *Wuepper*, 1971b). Using SDS disc gel electrophoresis and trypsinization of radiolabeled rabbit factor XII, three subunits of 30,000 MW each were subsequently demonstrated (*Cochrane* et al., 1972). When human ^{125}I-labeled factor XII was treated with trypsin, plasmin or plasma kallikrein, the molecule was cleaved into fragments with estimated MW of 52,000 and 28,000. Sucrose density gradient ultracentrifugation and functional assays for prekallikrein activation indicated that the 2.6 S peak,

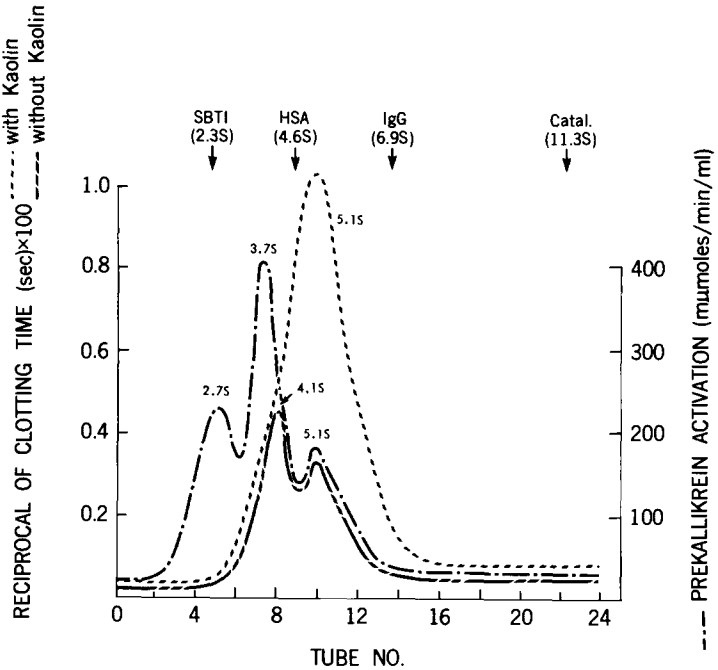

Fig. 6. Sucrose density gradient ultracentrifugation of untreated factor XII (5.1 S) and of plasmin-treated factor XII (2.7–5.1 S). The fragments were assessed in the same way as untreated factor XII by clotting of factor XII-deficient plasma (the unactivated XII with kaolin; the plasmin-treated without kaolin) and by their effect on prekallikrein (esterolysis). The slowest sedimenting (2.7 S) fragment had an effect on prekallikrein, but did not enhance the clotting. (From *Chan*, 1975)

probably corresponding to the 28,000 MW fragment, was the only one containing activity (*Revak* et al., 1974). Three fragments (28,000; 40,000; 12,000) were further characterized immunologically, taking advantage of the fact that the 28,000 MW fragment does not bind to kaolin (*Revak* and *Cochrane*, 1976). *Cochrane* and coworkers found prekallikrein-activating activity in either the 28,000 MW fragment of the unfragmented, i.e., seemingly intact but activated 80,000 MW factor XII. To try to explain this they postulated cleavage or fragmentation at two closely situated sites (*Revak* et al., 1977). The cleavage by enzymes and the two newly proposed cleavage sites are illustrated in Figure 7. One cleavage was proposed outside a disulfide loop and the other within the loop. The latter, it was postulated, would result in an activated intact molecule. Only reduction of the disulfide group could bring about a separation of the 28,000 MW fragment.

Despite these differences there is good agreement about the physicochemical and biologic properties of the smallest fragment PKA or XIIf (*Movat* et al., 1969b, 1970; *Kaplan* and *Austen*, 1970; *Cochrane* and

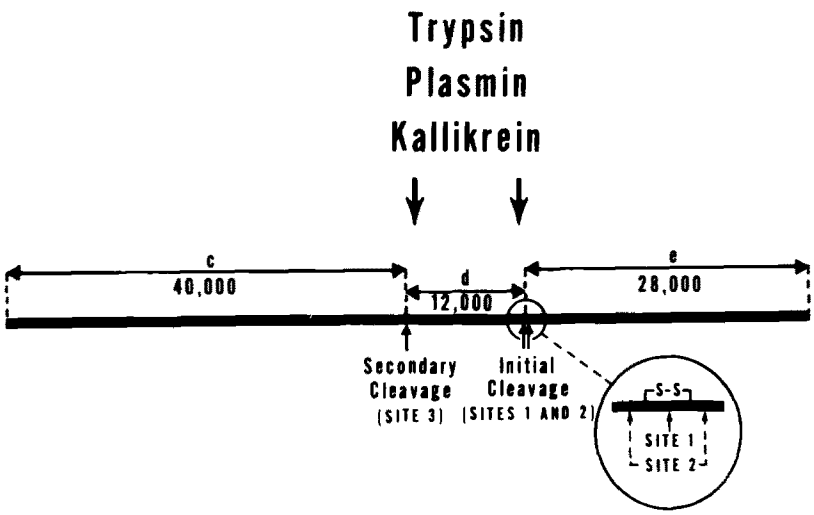

Fig. 7. Diagrammatic representation of cleavage of factor XII by proteases (trypsin, plasmin, plasma kallikrein). The proteases are believed to cleave at two sites, between fragment c and d and between d and e. The illustration also shows the possible cleavages giving rise to a free 28,000 MW fragment or one that remains attached to the molecule by an S-S bond described in the text. (From *Revak* et al., 1977)

Wuepper, 1971a,b; *Kaplan* et al., 1971; *Wuepper* and *Cochrane,* 1971; *Movat* et al., 1971; *Özge-Anwar* et al., 1972; *Treloar* et al., 1972; *Cochrane* et al., 1973; *Venneröd* and *Laake,* 1974). Factor XIIf is a highly anionic protein with an isoelectric point of 4.3–4.6, migrating like albumin in starch block or agarose and as prealbumin in alkaline polyacrylamide gel electrophoresis (Fig. 8). It is the smallest of the components of the kinin system with a sedimentation coefficient of 2.6–2.8 S and an estimated MW of 28,000–30,000. Fragment XIIf generates kinin from fresh plasma but not from heated plasma or purified kininogen. When injected intradermally it enhances vascular permeability. The kinin-formation in fresh plasma occurs indirectly, through conversion of prekallikrein to kallikrein. This is probably what leads to enhanced vascular permeability; the XIIf activates, in the interstitial tissue, prekallikrein to kallikrein, which cleaves bradykinin from kininogen and the bradykinin induces exudation of plasma. From the exuded plasma more kallikrein and bradykinin can be generated.

Whereas the nature and physicochemical properties of factor XII and of the enzymatically cleaved fragment XIIf or PKA are relatively well established, the nature of activated, seemingly intact, factor XIIa and the possible intermediate fragments remains unknown. Factor XIIa seems to be the activated form of the factor XII molecule which is adsorbed to negatively charged surfaces. Whether it has been fragmented and is held together by an S-S band as postulated by *Revak* et al. (1977) remains to be ascertained.

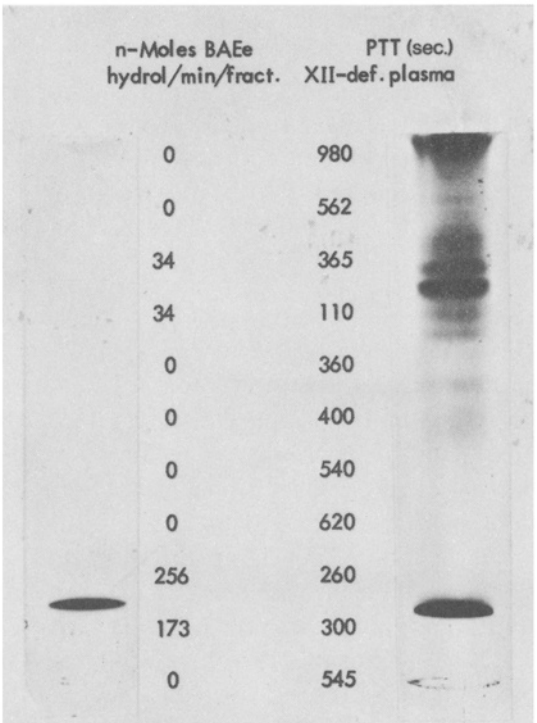

Fig. 8. Preparative disc gel electrophoresis. The stained gel on the right represents partially purified celite eluates and the one on the left the isolated purified fragment XIIf. The two columns show prekallikrein activation (BAEe hydrolysis) and clotting of factor XII-deficient plasma of replicate gel slices from gels similar to the one shown on the right

C. Factor XI

Of the substrates of factor XII, prekallikrein is the more important one in conjunction with the kinin system and is discussed in Section III.

Another substrate of factor XII is factor XI or plasma thromboplastin antecedent, the deficiency in this factor having been first recognized by *Rosenthal* et al. (1953). Separation from factor XII was achieved in 1960 by *Schliffman* et al. More recently, partially purified (*Kaplan* and *Austen,* 1972; *Wuepper,* 1972; *Movat* et al., 1972) preparations were obtained, followed by more highly purified ones (*Saito* et al., 1973; *Heck* and *Kaplan,* 1974; *Movat* and *Özge-Anwar,* 1974; *Schiffman* and *Lee,* 1974) and finally by homogeneous ones (*Bouma* and *Griffin,* 1977).

Factor XI was first estimated to have a MW of 160,000–180,000 by gel filtration. By SDS disc gel electrophoresis factor XI has a MW of 160,000 and when reduced 83,000. Activated factor XI (XIa) also has a MW of 160,000, but when reduced the two chains of 83,000 are cleaved into subunits of 50,000 and 33,000, respectively (*Bouma* and *Griffin,* 1977). Similar observations were made earlier by *Wuepper* (1972) and *Kaplan* et al. (1976a). The isoelectric point is about 9.0.

The first to demonstrate activation of factor XI by factor XIIa was *Ratnoff* et al. (1961). Activation is also achieved by trypsin (*Wuepper,* 1972; *Saito* et al., 1973) and fragment XIIf (*Heck* and *Kaplan,* 1974; *Movat* and *Özge-Anwar,* 1974). It was by studying the activation of factor XI by contact activated intact factor XII that *Schiffman* and *Lee* (1974) discovered a substance required for this activation (contact activation cofactor), which proved to be HMW kininogen (see above).

D. Inhibition of Factor XII

Inhibition of activated factor XII has been demonstrated by a number of investigators (see *Cochrane* et al., 1976). Factor XII has been reported to be susceptible to inhibition by lima bean trypsin inhibitor (LBTI) (*Schoenmakers* et al., 1965). Since LBTI does not affect kallikrein, the inhibition of activated factor XII by this inhibitor could be used in studies dealing with prekallikrein activation (*Wuepper,* 1972). Other inhibitors, such as soy bean trypsin inhibitor (SBTI), can not be tested on factor XIIa since they inhibit the substrate which is activated by XIIa.

Natural plasma proteinase inhibitors are more important from a pathophysiologic point of view. $C\bar{1}$-inactivator inhibits factor XIIa (*Forbes* et al., 1970) and the fragment XIIf (*Özge-Anwar* et al., 1972; *Schreiber* et al., 1973a). Another of the proreinase inhibitors capable of inactivating factor XII is antithrombin III (*Stead* et al., 1976; *Chan* et al., 1977a). *Stead* et al. estimated by SDS disc gel electrophoresis the MW of factor XIIa as 75,000, that of antithrombin III as 61,500 and the stoichiometric complex of 1:1 as 117,000. Of the various proteinase inhibitors tested by *Chan* et al., only $C\bar{1}$-inactivator and antithrombin III inhibited surface-bound activated factor XII; α_1-antitrypsin and α_2-macroglobulin had no effect.

Of the fragments formed when HMW kininogen is cleaved by plasma kallikrein, fragment 1–2 and fragment 2 (histidine-rich) strongly inhibited contact activation of factor XII similarly to hexadimethrine bromide (*Iwanaga* et al., 1976; *Oh-ishi* et al., 1977a). These findings were interpreted to represent a negative feedback for the contact activation of factor XII, in contrast to kallikrein and plasmin which provide a positive feedback. The fragments of HMW kininogen are discussed in Section IV.

III. Prekallikrein and Kallikrein

The early observations and the differentiation between glandular and plasma kallikrein was discussed in the introduction. This section deals with plasma kallikrein.

A. Isolation and Characterization

Becker and coworkers (*Kagen* et al., 1963; *Becker* and *Kagen,* 1964) obtained the first crude *human plasma kallikrein* free of other components of the kinin system. Further improvements in the purification followed (*Colman* et al., 1969; *Movat* et al., 1970, 1971; *Fritz* et al., 1972a) and kallikrein was eventually obtained in homogeneous form (*Sampaio* et al., 1974).

Similar to the active enzyme, *plasma precallikrein* (the zymogen) was recovered from human plasma first in functionally pure form (*Kaplan* et al., 1971; *Movat* et al., 1972; *Laake* and *Venneröd,* 1973, 1974; *Revak* and *Cochrane,* 1976). The use of anion exchange, cation exchange, and molecular sieve chromatography followed by immunoabsorption with anti-IgG yielded homogeneous preparations (Fig. 9) (*Weiss* et al., 1974; *Habal* et al., 1974; *Kaplan* et al., 1976a,b; *Mandle* and *Kaplan,* 1977a).

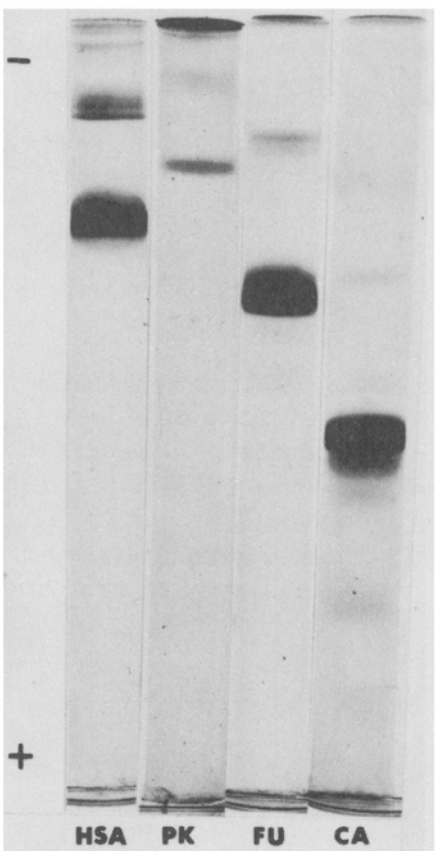

Fig. 9. SDS disc gel electrophoresis of prekallikrein (PK), together with the marker proteins human serum albumin, monomer and dimer (HSA), fumarase (FU) and carbonic anhydrase (CA)

Kallikrein and prekallikrein were isolated also from *animal plasmas.* Bovine kallikrein was isolated in partially purified form after activation with glass (*Nagasawa* et al., 1967) or casein (*Yano* et al., 1970). A homogeneous preparation with a 1300-fold purification was reported by *Takahashi* et al. (1972a). Kallikrein was isolated in highly purified form also from hog serum (*Fritz* et al., 1972a). A well characterized and homogeneous prekallikrein was purified by *Wuepper* and *Chochrane* (1972) from rabbit plasma.

Although there are some variations between reports, the *physicochemical properties* of human prekallikrein and kallikrein may be summarized as follows. Both the zymogen and the enzyme have a sedimentation coefficient of 5.2 S and a MW of 100,000–108,000 (Fig. 10). The migration is that of a fast γ to slow β globulin by starch block or agarose electrophoresis at alkaline pH. Corresponding to this, the isielectric point is between 7.7 and 9.4. A microheterogeneity has been demonstrated by *Wendel* et al. (1972) and by *Laake* and *Venneröd* (1974), the arginine esterase and kinin-forming activities overlapping.

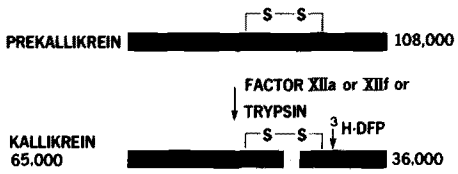

Fig. 10. Gross structure of prekallikrein and kallikrein. The zymogen can be activated by factor XIIa, fragment XIIf or trypsin, giving rise to two chains. Of those the 36,000 MW chain seems to contain the active center, since it binds labeled DFP

Bovine kallikrein and prekallikrein have similar MWs to the human equivalents and an isoelectric point of about 7.0. However, rabbit prekallikrein, according to *Wuepper* and *Chochrane* (1972), has a MW of about 100,000 and a sedimentation coefficient of 4.5 S which upon activation to kallikrein become about 89,000 and 4.0 S respectively. Rabbit prekallikrein has a relatively low isoelectric point of 5.9, which becomes 5.4 upon conversion to kallikrein.

B. Activation of Prekallikrein

Human prekallikrein can be converted to kallikrein by factor XIIa or by trypsin. The proposal that plasmin can activate prekallikrein (*Vogt,* 1964) had to be modified as this effect of plasmin was shown to occur indirectly through activation of factor XII (*Kaplan* and *Austen,* 1971; *Burrowes* et al.,

1971; *Movat* et al., 1973a). Activation of human prekallikrein is associated with limited proteolysis without demonstrable change in the MW, sedimentation coefficient, or isoelectric point. Both prekallikrein and kallikrein have a MW of 88,000 by SDS disc gel electrophoresis. However, while reduction and alkylation does not change the MW of prekallikrein, reduction of kallikrein gives rise to two fragments. A heavy chain of MW 52,000 and a light chain of MW 36,000 or 33,000 (Fig. 10). Thus, the limited proteolysis by factor XIIa or fragment XIIf generates two fragments linked by disulfide bonds (*Kaplan* et al., 1976a,b; *Mandle* and *Kaplan,* 1977b). This is in keepring with similar observations made with bovine (*Takahashi* et al., 1972b) and rabbit prekallikrein (*Wuepper,* 1972). The data of *Mandle* and *Kaplan* (1977b), based on incorporation of [^{3}H]-DFP indicate that the active center is in the light chain.

Rabbit prekallikrein, as described above, undergoes more marked proteolytic cleavage. The first observation of *Wuepper* and *Cochrane* (1972) were extended by *Wuepper* (1972). He showed, by SDS disc gel electrophoresis, a MW of 90,000 for rabbit prekallikrein and fragments of 80,000 and 10,000 for kallikrein. However, further cleavage was noted since fragments of 56,000, 26,000 and 10,000 were recovered after reduction of kallikrein and it was concluded that there must be two sites of cleavage, one of which is probably within a disulfide bridge.

C. Complex Formation Between Kallikrein or Prekallikrein and Other Components of Plasma

As described in the introduction, early investigators in the field believed that kallikrein is secreted in the pancreas, circulates complexed in the blood and is excreted in the urine. However, subsequent findings clearly separated glandular from plasma kallikreins, two entirely different enzymes. More recent data indicate that plasma kallikrein and prekallikrein may circulate in the blood complexed to other proteins. The complexing of kallikrein may explain the thesis of two different kininogenases or kallikreins proposed by *Vogt* et al. (*Vogt* et al., 1967; for a review see *Eisen* and *Vogt,* 1970).

Plasma kallikrein is a basic protein with a MW of about 100,000. Kininogenase activity in acidic, HMW fractions was detected first in guinea pig (*Movat* et al., 1968, 1969a) and then in human serum (*Movat* et al., 1970, 1971). One of the complexes eluted immediately after the void volume (MW ~ 800,000) and another with a MW of about 240,000 (arginine esterase and kinin generation) when subjected to gel filtration on Sephadex G-200. When subjected to starch block electrophoresis, the activity eluting as a macroglobulin migrated as a γ-globulin (similar to

plasma kallikrein) and the ~240,000 MW complex as an α-globulin. These findings are more or less in keeping with the observation of *Vogt* and coworkers (*Wendel* et al., 1972; *Vogt* and *Dugal*, 1976), who examined these phenomena in detail.

Wendel et al. (1972) exposed plasma to quartz and after washing, eluted the absorbed protein with 1.0 M NaCl. By electrofocusing, the kininogenase activity was detected in two peaks; at pH 4.0–5.7 and at pH 7.5–10,0. The acidic peak had a MW of about 180,000 and the basic one < 100,000. The acidic peak was subjected to ammonium sulfate precipitation and anion exchange chromatography. Following this it focused between pH 7.8 and 9.1 and the MW decreased to about 97,000, i.e., it had acquired the properties of plasma kallikrein, some of wich was already detectable in the initial focusing. Thus, *Wendel* et al. demonstrated that plasma kallikrein may be complexed with a noninhibitory α-globulin, from which it can be separated during DEAE-cellulose chromatography when the starting buffer has a very low ionic strength. It is conceivable that the 180,000–240,000 MW kallikrein demonstrated by *Movat* et al. (1970, 1971) and *Wendel* et al. (1972) represents complexing with HMW kininogen, as described below for prekallikrein. In the complexed form with prekallikrein, the HMW kininogen would be intact, whereas in a kallikrein-HMW kininogen complex the latter could be partly fragmented (see Sect. IV, dealing with kininogens).

Vogt and *Dugal* (1976) examined the kininogenase activity which *Movat* et al. (1971) had found to elute between the void volume and the marker apoferritin. *Harpel* (1970, 1973, 1976) had demonstrated a complex formation between plasma kallikrein and α_2-macroglobulin. *Vogt* and *Dugal* found that in acetone-treated plasma most of the kininogenase activity eluted in the α_2-position during anion exchange chromatography or gel filtration. Similar to free kallikrein, the enzyme complexed with α_2-macroglobulin released kinin more efficiently from HMW than from LMW kininogen, but the kininogenase activity of the complexed form was lower than that of the free enzyme. However, in plasma, free kallikrein was rapidly inactivated by inhibitors whereas the complex, protected from inactivation, was capable of cleaving kinin slowly from LMW kininogen after the HMW kininogen had been consumed (Fig. 11). The observations of *Wendel* et al. explain in part the existence of a second kininogenase in plasma. The action of the α_2-macroglobulin-kallikrein complex explains these observations further and the effect of the complexes in plasma stresses their possible significance in vivo.

Plasma prekallikrein also can circulate in complexed form. The first to demonstrate this, without knowing what the complexing protein was, were *Nagasawa* and *Nakayasu* (1973). They demonstrated a complex with an approximate MW of 300,000 and an isoelectric point of 4.3 (the iso-

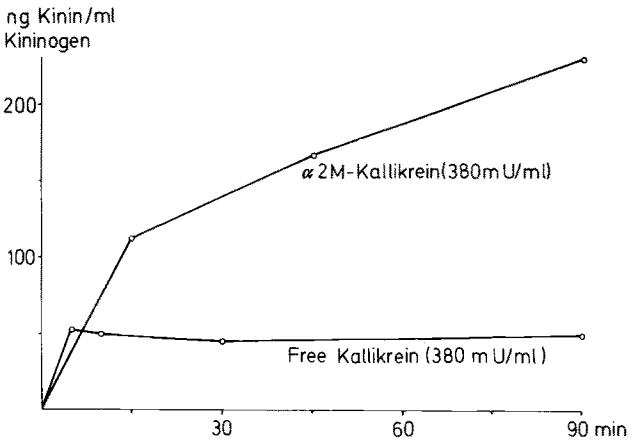

Fig. 11. Release of kinin from plasma by isolated free plasma kallikrein and by kallikrein complexed to α_2-macroglobulin (α_2-M-kallikrein) and thus protected from inhibitors. (From *Vogt* and *Dugal*, 1976)

electric point of HMW kininogen is 4.5; *Habal* and *Movat*, 1976a). These investigators isolated from plasma an acidic protein. When this protein was mixed with prekallikrein a 180,000 MW complex was formed. *Mandle* et al. (1976) subjected prekallikrein to gel filtration, demonstrating a MW of 115,000. Under similar conditions HMW kininogen had a MW of 200,000 and a mixture of the two a MW of 285,000. They confirmed these observations by using purified ^{125}I-labeled prekallikrein and HMW kininogen. No complex formation could be demonstrated when HMW kininogen-deficient plasma was mixed with the prekallikrein. Because during anion exchange chromatography the basic prekallikrein and the acidic HMW kininogen dissociated, it was assumed that they were noncovalently linked, representing a charge interaction. This complex formation is of interest because the two components have to interact (after activation of prekallikrein) to liberate bradykinin. Furthermore, on surfaces, kallikrein and HMW kinonogen can bring about and enhance the activation of factor XII.

IV. Kininogens

A. Isolation and Characterization

Until a few years ago, but even in some recent publications on studies on the kinin system, plasma heated to at least 60°C for 1–2 h was used as a source of kininogen. In such heated plasma, kinin-forming enzymes and their zymogens, as well as kinin-degrading enzymes are activated. Heating was even used as a step in the purification of kininogens as reported in some publications; one of the difficulties during kininogen purification being activation of zymogens. As described above, HMW-kininogen can form complexes with prekallikrein.

The interest in kininogens arose mainly from the observations of *Jacobsen,* who described two kininogens in the plasma of several species including man: a LMW and a HMW form (*Jacobsen,* 1966a,b; *Jacobsen* and *Kriz,* 1967). This was in keeping with the description of two kininogens reported by *Vogt* and coworkers (*Vogt,* 1966; *Vogt* et al., 1967).

The LMW form of kininogen has been isolated from human plasma by a number of investigators (*Webster* and *Pierce,* 1963; *Pierce* and *Webster,* 1966; *Brocklehurst* and *Mawr,* 1966; *Spragg* et al., 1970; *Spragg* and *Austen,* 1971, 1974; and *Hamberg* et al., 1975). Both a LMW and a HMW kininogen were isolated by *Jacobsen* and *Kriz* (1967), *Seidel* (1973), *Habal* and *Movat* (1972), *Habal* et al. (1974) and by *Pierce* and *Guimarães,* (1976). The latter authors described a series of kininogen peaks when subjected to anion exchange chromatography, the early eluting ones corresponding to LMW kininogen and the late eluting, i.e., anionic ones, to HMW kininogen.

Kininogens were also purified from *animals plasmas.* The best studied kininogens are those of bovine plasma. LMW kininogen was isolated in the early 1960s by *Habermann* (*Habermann* and *Rosenbusch,* 1962, 1963; *Habermann,* 1963, 1970). Japanese investigators in turn isolated both forms of kininogens (*Suzuki* et al., 1967; *Yano* et al., 1967a,b, 1971; *Komiya* et al., 1974a). From rabbit plasma, first only LMW kininogen was isolated (*Pashkina* and *Ergova,* 1966; *Cochrane* and *Wuepper,* 1971a), but recently *Ergova* et al. (1976) described the isolation of two kininogens. Two kininogens have also been obtained from horse plasma (*Henriques* et al., 1967).

For a number of years a controversy existed, some believing that there is only one kininogen (LMW) in plasma, the HMW form having been looked upon as an artefact (*Habermann,* 1970; *Spragg* et al., 1970; *Spragg* and *Austen,* 1971; *Cochrane* and *Wuepper,* 1971a; *Hamberg* et al., 1975). Others insisted on the existence of two forms (*Jacobsen,* 1966a,b; *Jacobsen* and *Kriz,* 1967; *Yano* et al., 1971; *Habal* and *Movat,* 1972; *Habal* et al., 1974; *Pierce* and *Guimarães,* 1976; *Habal* and *Movat,* 1976a). The controversy became entirely solved when a kininogen deficiency was described, stressing the importance of HMW kininogen (*Saito* et al., 1975; *Colman* et al., 1975; *Wuepper* et al., 1975; *Donaldson* et al., 1976; *Webster* et al., 1976).

The *physicochemical properties* of the kininogens have been studied most thoroughly in bovine plasma but some data are available on the properties and gross structure of human kininogens. Human LMW kininogen is a single polypeptide chain, the MW of which has been estimated to be 50,000–52,000 (*Hamberg* et al., 1975; *Habal* et al., 1975) or 78,000 (*Nagasawa* and *Nakayasu,* 1976). Its isoelectric point is 4.7, migrating as an α-globulin in alkaline disc gels. The sedimentation coefficient is 3.85–

4.0 S. It is a glycoprotein. Human HMW kininogen has an apparent MW of about 200,000 by gel filtration in an aqueous medium. In the presence of a dissociating agent such as guanidine HCl, a MW of 108,000 was determined by chromatography through Sepharose 4B (*Habal* et al., 1975). In the presence of SDS in disc gels, MWs of 128,000 (*Nagasawa* and *Nakayasa*, 1976) was estimated (Fig. 12). The sedimentation coefficient is 4.2 S. The isoelectric point is 4.5 and the migration that of a β-globulin in alkaline disc gels.

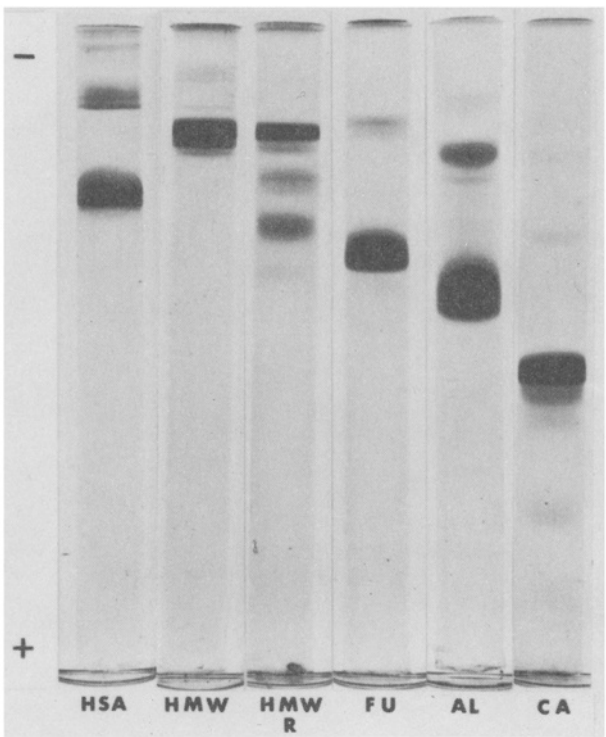

Fig. 12. SDS disc gel electrophoresis of HMW kininogen prepared by the two-step procedure of *Habal* and *Movat* (1976b). The gel marked HMW-R was reduced and alkylated. Marker proteins: human serum albumin (HSA) (monomer and dimer), fumarase (FU), aldolase (AL) and carbonic anhydrase (CA)

Bovine kininogens are much better characterized (*Suzuki* and *Kato*, 1976). LMW kininogen with a sedimentation coefficient of 3.66 S was estimated to have a MW of 48,000. The diffusion coefficient is 6.16, the partial specific volume 0.688, the intrinsic viscosity 0.063, and the isoelectric point 3.3. The last finding, based on earlier observations, is not in good agreement with its behaviour during ion exchange chromatography when compared to HMW kininogen. The N-terminal is serine and the C-terminal, alanine. Bovine LMW kininogen is a single chain polypeptide which has a total of 373 amino acid residues and has 19.8% carbohydrate.

Bovine HMW kininogen is much smaller than the human equivalent having an estimated MW of 76,000 by sedimentation equilibrium and 80,000 by gel filtration through Sepharose 4B equilibrated with guanidine HCl. The sedimentation coefficient is 3.8 S, the partial specific volume 0.718, and the isoelectric point 4.5. Its N-terminal is masked and the C-terminal is leucine. Bovine HMW kininogen is a single polypeptide chain which has 581 amino acid residues and a carbohydrate content of 12.6%.

B. Cleavage of Kininogens by Kininogenases

Two species of bovine HMW kininogen have been described, kininogen a and b (*Komiya* et al., 1974a,b). These have the same number of peptides on tryptic digestion, differing only by one proteolytic cleavage at the C-terminal end of bradykinin without loss of bradykinin or any other fragments. Only a few peptide bonds seem to have been lost in kininogen b, which derives from kininogen a. Thus, limited proteolysis has occurred in kinonogen b. Bradykinin and a peptide, designated fragment of peptide 1–2 are located within the intrachain disulfide loop (*Komiya* et al., 1974b; *Han* et al., 1976a,b,c; *Kato* et al., 1976a,b). The N-terminal of fragment 1–2 is connected with the C-terminal of bradykinin within the disulfide loop of bovine HMW kininogen (Fig. 13). The changes in the kininogen molecule

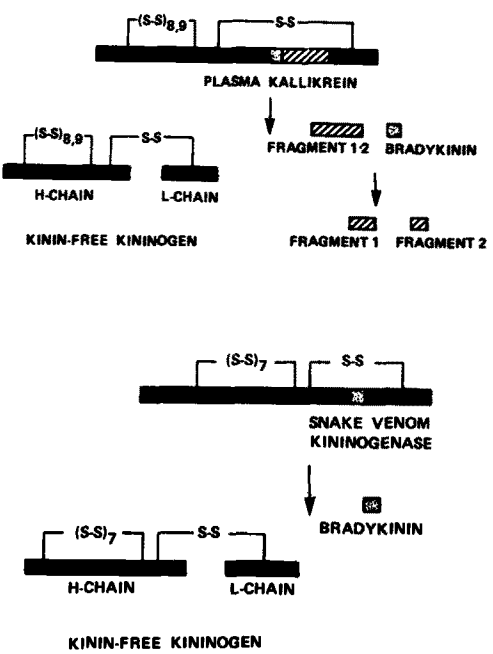

Fig. 13. Fragmentation of bovine HMW (top) and LMW (bottom) kininogens by plasma kallikrein and snake venom kininogenase, respectively. (Based on *Kato* et al., 1976a)

after digestion with kallikrein have been monitored by SDS disc gel electrophoresis (*Kato* et al., 1976a). Fragment 1–2 is cleaved as well as bradykinin and upon further incubation it is cleavage into fragment 1 and fragment 2. Fragment 1 is a 8000 MW glycopeptide (*Han* et al., 1976b). Fragment 2 is a 4584 MW histidine-rich peptide (*Han* et al., 1976a). When the digested HMW kininogen was chromatographed on a long column of Sephadex G-75, kinin-free kininogen appeared in the void volume and fragment 1–2 was retarded. Upon reduction and alkylation the kinin-free kininogen (MW 66,000) gave rise to two bands, suggesting that it consists of two polypeptide chains held together by a disulfide bond. The two chains were separated, consisting of a heavy (MW 48,000) and a light chain (MW 16,000) (*Kato* et al., 1976a,b). Changes in LMW kininogen were studied after digestion with snake venom kininogenase of *Agkistrodon haly blomhoffi* (Fig. 13). LMW kininogen consists of a heavy chain of 344 residues (MW 48,000) and a light chain of 47 residues (MW 4800). As with human kininogens (*Habal* and *Movat,* 1972; *Habal* et al., 1974; *Pierce* and *Guimarães,* 1976) there is immunologic cross reactivity between bovine LMW and HMW kininogens. The main structural differences between the two kininogens lie in the C-terminal portion next to the bradykinin moiety.

C. Phlogistic Activity of Fragment 1–2

Fragment 1–2 has a very transient mild vascular permeability enhancing effect. However, there is some controversy with respect to this activity. *Oh-ishi* et al. (1977a) first reported that the fragment inhibited contact activation of factor XII and had negligible kinin-like activity, i.e., smooth mucle contraciton, hypotension, and effect on vascular permeability. When similar material was tested by *Matheson* et al. (1976), a very rapidly developing and transient increase in vascular permeability was reported. The potency of the peptide was intermediate between that of bradykinin and histamine when tested in rabbits. Furthermore, it had a synergistic effect when injected together with bradykinin. In a further investigation, *Matheson* et al. (1977) cleaved fragment 1–2 into fragments 1 and 2 with kallikrein. The permeability-enhancing effect was recovered in the histidine-rich fragment 2. A C-terminal arginine was shown to be essential for biologic activity. *Oh-ishi* et al. (1977b) reinvestigated the vascular permeability enhancing effect of the fragments. Fragment 1–2 and fragment 2 had about 1% of the activity of bradykinin. On a molar basis it was almost equipotent to histamine in the rabbit. Unlike *Matheson* et al., *Oh-ishi* et al. detected an additive rather than potentiating effect when the fragments were mixed with bradykinin. These investigators extracted the extravasated

blue dye and determined it colorimetrically, whereas *Matheson* et al. had measured the diameter of the blue spots. When mixed with prostaglandin E_2 there was significant enhancement of the permeability-inducing effects of both the fragments and of bradykinin. The findings with prostaglandin are in keeping with the observations of *Johnston* et al. (1976) and *Kopaniak* et al. (1978) that prostaglandins of the E series augment the permeability induced by mediators such as bradykinin of histamine, by inducing hyperemia. Due to the enhanced blood flow, more exudation can occur through the leaky vessels.

D. Kininogen Deficiency

A number of cases of kininogen deficiency were described almost simultaneously, starting with one designated Fitzgerald trait in which there were abnormalities in the contact phase of blood coagulation (PTT), of kinin generation, and of fibrinolysis.

Colman and coworkers reported abnormally low levels of prekallikrein, plasminogen proactivator, and kininogen (William's factor) in a patient (*Colman* et al., 1975, 1976). An entity referred to as Flaujeac trait was first referred to as HMW kininogen deficiency by *Wuepper* et al. (1975). The coagulation deficiency designated Fitzgerald trait has been recognized earlier (*Waldmann* and *Abraham,* 1974; *Saito* et al., 1974b) and later described to be associated with abnormalities of fibrinolysis, kinin generation, and PF/dil activity (*Saito* et al., 1975). A fourth patient (Washington) likewise had kininogen deficiency and laboratory abnormalities similar to Fitzgerald trait (*Donaldson* et al., 1976). A fifth case, referred to as Reid trait, was described recently (*Lutcher,* 1976). Independently *Schiffman* and *Lee* (1974) recognized a factor which they designated "contact activation cofactor". This factor was recovered from plasma in purified form and found to be identical to Fitzgerald factor (*Schiffman* et al., 1975). With the exception of Fitzgerald trait, which lacks only HMW kininogen, all others have a deficiency in both kininogens. As described under contact activation of factor XII, HMW kininogen is essential for this activation and thus for the activation of the kinin system. In all these deficiencies there is a true lack of kininogen protein, which is absent also by immunochemical assays, rather than the presence of an abnormal (inactive) protein.

The role of HMW kininogen in the contact activation of factor XII was described in detail in Section II.B.

V. The Fibrinolytic System

A. Plasminogen and Plasmin

The source of plasminogen in the past was either Cohn fraction III or euglobulin precipitates of plasma. Highly purified plasminogen could be prepared by a series of chromatographic steps (*Robbins* and *Summaria*, 1970). Following the publication of *Deutsch* and *Mertz* (1970), most investigators used affinity chromatography on lysine-Sepharose (*Liu* and *Mertz*, 1971; *Sodetz* et al., 1972; *Summaria* et al., 1972; *Wallén* and *Wiman*, 1972; *Burrowes* et al., 1972). Although some investigators use only the affinity chromatography, others precede this step by anion exchange and follow it by molecular sieve chromatography (Fig. 14).

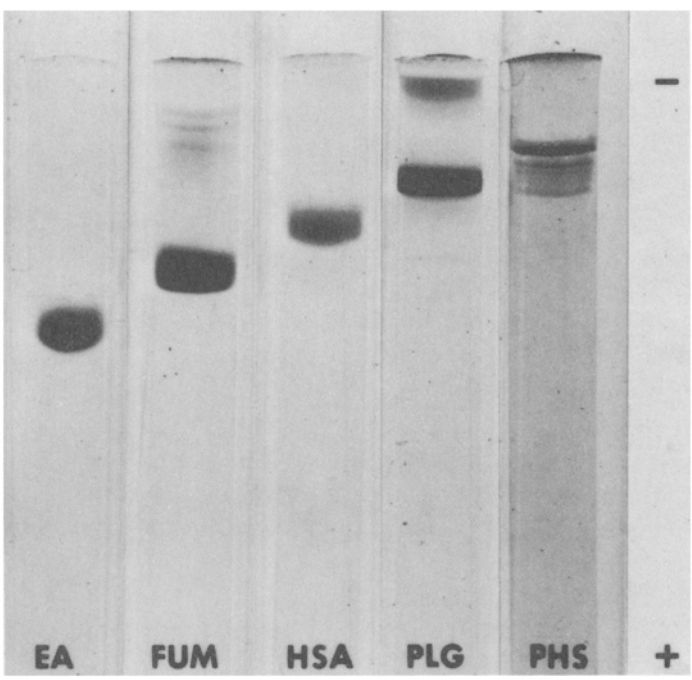

Fig. 14. SDS disc gel electrophoresis of plasminogen (PLG) and of the markers egg albumin (EA), fumarase (FUM), human serum albumin (HSA) and phosphorylase (PHS; MW 94,000). (From *Habal* et al., 1976)

Human plasminogen is a single chain monomeric plasma protein consisting of multiple forms, as demonstrated by isoelectric focusing and by alkaline disc gel electrophoresis in the presence of epsilon amino caproic acid. It migrates as a β-globulin in agarose at alkaline pH. The

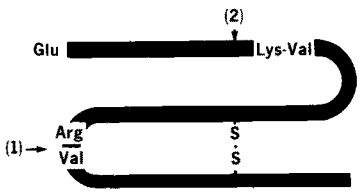

Fig. 15. The gross structure of Glu-plasminogen and its activation. When activated to plasmin by urokinase, two peptide bond cleavages occur. One occurs in the interior of the molecule (arrow 1) at an arginyl-valine bond, which converts the single chain plasminogen to a two-chain plasmin molecule, held together by at least one disulfide bond. Loss of a small peptide of MW 8000 also occurs at the N-terminal during activation (arrow 2), changing the N-terminal from glutamic acid to lysine. (Based on *Kosow*, 1976)

molecular forms which focus between pH 6.2 and 6.6 have glutamic acid as their N-terminal (*Rickli* and *Cuendet*, 1971; *Wallén* and *Wiman*, 1972; *Summaria* et al., 1973). Those focusing between pH 7.2 and 8.3 have an N-terminal lysine residue (*Summaria* et al., 1972, 1973). The two forms have been referred to as Glu-plasminogen and Lys-plasminogen. The sedimentation coefficient of Glu-plasminogen is 5.0 S and of Lys-plasminogen 4.4 S, the MWs are 83,800 and 82,400, the partial specific volumes 0.706 and 0.709, and the frictional coefficients 1.54 and 1.63, respectively (*Robbins* et al., 1975). Swedish investigators reported slightly different values, i.e., sedimentation coefficients of 5.1 S and 4.8 S, MWs of 92,000 and 90,000, and frictional coefficients of 1.50 and 1.56 for Glu-plasminogen and Lys-plasminogen, respectively (*Sjöholm* et al., 1973). The carbohydrate content of plasminogen is less than 2%. *Robbins* et al. (1975) and *Sjöholm* et al. (1973) detected a sedimentation coefficient of 4.3 S for plasmin, but the former estimated the MW to be 76,500 and the latter 81,000. The partial specific volume was determined to be 0.714–0.713 and the frictional coefficient 1.64 and 1.55, respectively. Plasmin consists of a heavy of H chain (MW 48,000) and a light or B chain (MW 25,700). By radioimmunoassay, the concentration of plasminogen in plasma was estimated to be 206 ± 36 μg/ml (*Rabiner* et al., 1969) and by radial immunodiffusion 464 ± 75 μg/ml (*Magoon* et al., 1974).

Robbins et al. (1967) have demonstrated that activation of plasminogen to plasmin is associated with cleavage of an arginyl-valine bond (Fig. 15). There is some controversy whether release of a peptide is essential for the activation of plasminogen (*Kosow*, 1976). A peptide of about 8000 MW is lost from the N-terminal end during activation (*Wiman* and *Wallén*, 1973; *Walther* et al., 1975) (Fig. 15). When the activation with urokinase was done in the presence of bovine trypsin inhibitor, a plasmin was isolated with a MW and N-terminal identical to that of plasminogen

It is believed that the inhibitor prevented the autocatalytic action of plasmin, which could have brought about the cleavage of the N-terminal peptide.

Activation of plasminogen can also be induced nonenzymatically with streptokinase instead of urokinase. Of the various mechanisms postulated, the most likely is that streptokinase forms a complex with plasminogen (plasmin) and this complex acts as a plasminogen activator which is capable of hydrolysing the arginyl-valine bond (*Kosow,* 1976).

B. Activation

Plasmin, the enzyme which induces fibrinolysis is derived from plasminogen through activation by a number of substances. Plasminogen activators may be extrinsic to plasma, i.e., derive from cells (urokinase) or bacteria (streptokinase). Recently, a plasminogen activator was isolated from macrophages (*Unkeless* et al., 1974b) and other cells, including cells that have undergone malignant transformation (*Unkeless* et al., 1974a). For the plasma kinin and fibrinolytic systems, an activator derived from plasma is more significant.

Similar to activation of the kinin system, activation of the intrinsic fibrinolytic system is factor XII dependent, i.e., no activation occurs in factor XII-deficient plasma (*Niewiarowski* and *Prou-Wartelle,* 1959; *Iatridis* and *Ferguson,* 1961). Factor XII does not activate plasminogen directly and it has been proposed that it may induce the formation of plasminogen activator from a precursor (*Iatridis* and *Ferguson,* 1962). Such a substance was isolated in crude form by *Ogston* et al. (1969) and designated "Hageman factor cofactor". It was recovered from celite eluates of plasma and appeared in the excluded peak during anion exchange chromatography. Of the two active substances present in such fractions, kallikrein was eliminated as a possible source since Hageman factor cofactor, unlike kallikrein, was not susceptible to $C\bar{1}$-inactivator. Activated factor XI was eliminated by demonstrating recovery from factor XI-deficient plasma. *Kaplan* and *Austen* (1972) recovered a plasminogen proactivator from the excluded peak by anion exchange chromatography. It was separable from factor XI by gel filtration and from prekallikrein by cation exchange chromatography on SP-Sephadex. The proactivator could be activated with factor XIIa or XIIf. Like *Ogston* et al., *Kaplan* and *Austen* found their plasminogen activator to be noninhibitable by $C\bar{1}$-inactivator. Later *Kaplan* et al. (1976b) detected two peaks of plasminogen activatory activity, one overlapping with prekallikrein and the second one with factor XI. The latter was interpreted to be a dimer. In Fletcher trait (prekallikrein-deficient) plasma only the 180,000 MW plasminogen activator was recovered. *Laake* and

Venneröd (1974) could detect only one plasminogen proactivator which overlapped in all chromatographic steps with prekallikrein. The microheterogeneity described for prekallikrein paralleled the plasminogen activator activity. When kallikrein was passed through a column of Sepharose 4B to which $C\bar{1}$-inactivator had been linked, both prekallikrein and plasminogen activator became adsorbed to the column. A highly purified and homogeneous preparation of kallikrein also possessed plasminogen activator activity, so *Laake* and *Venneröd* concluded that plasminogen activator is identical to kallikrein. In another publication, *Venneröd* and *Laake* (1976) presented evidence that prekallikrein-deficient plasma is devoid of plasminogen-proactivator activity. Recently, *Kaplan* and coworkers concluded that the plasminogen activator of 95,000 MW is indistinguishable from prekallikrein and that the 180,000 MW component could be identical to factor XI (*Mandle* and *Kaplan,* 1977b; *Kaplan,* 1977). Already in 1969 *Colman* presented some evidence that plasma kallikrein can activate plasminogen (*Colman,* 1969).

The mechanism of the factor XII-dependent activation has not been investigated, but since kallikrein, which is now believed to be the activator, is an enzyme it may act like urokinase, i.e., by cleaving two peptide bonds (Fig. 15).

C. Effect of Plasmin on the Kinin System

As described in the section dealing with factor XII, plasmin induces a positive feedback by cleaving and activating factor XII. In whole plasma this pathway seems, however, to be of minor significance, possibly because of the rapid inactivation of plasmin in the fluid phase.

At one time it was believed that plasmin is the principal kinin-forming enzyme and several investigators presented evidence that plasmin can act directly on kininogen (*Lewis,* 1958; *Eisen,* 1963; *Back* and *Steger,* 1965; *Hamberg,* 1968; *Gapanhuk* and *Henriques,* 1970). Other workers were not able to demonstrate kinin cleavage from partially purified kininogen by plasmin (*Bhoola,* 1960; *Vogt,* 1964; *Buluk* and *Malofiejew,* 1965; *Haustein* and *Marquardt,* 1966). Older literature with partially purified preparations indicates that plasmin generates kinin much more slowly than does plasma kallikrein (*Eisen* and *Vogt,* 1970). More recently, the effect of highly purified plasmin on highly purified kininogens was investigated (*Habal* et al., 1976). When the preparations of plasmin and kallikrein were rendered equipotent with respect to arginine esterase activity, kallikrein was found to cleave HMW kininogen more rapidly than did plasmin. Plasmin acted equally well on both kininogens. Bradykinin was the peptide recovered when kininogen was incubated with plasmin; a finding which contradicted an earlier report of *Gapanhuk* and *Henriques* (1970).

D. Inhibition of Plasmin

Plasmin is inhibited by DFP, SBTI, LBTI, TLCK, Trasylol and by the plasma proteinase inhibitors, $C\bar{1}$-inactivator (*Harpel,* 1970), α_2-macroglobulin and antithrombin III (*Highsmith* and *Rosenberg,* 1974; *Habal* et al., 1976). In the older literature one encounters reference to "slow" and "fast" inhibitors of plasmin in plasma (*Vogel* et al., 1966). It appears well established now that α_2-macroglobulin and $C\bar{1}$-inactivator inhibit plasmin rapidly. Antithrombin III is a slow inhibitor in the absence and a fast inhibitor in the presence of heparin. Alpha$_1$-antitrypsin too is a slow inhibitor (*Heimburger* et al., 1971). Its effect on plasmin is negligible compared to that of the other inhibitors (*Habal* et al., 1976). Recently, a new α_2-plasmin inhibitor was described (*Moroi* and *Aoki,* 1976, 1977; *Müllertz* and *Clemmensen,* 1976).

The activator of plasminogen, present as proactivator in plasma, was first believed to be inhibited by α_2-macroglobulin only (*Schreiber* et al., 1973b). However, since never data indicate that the activator is plasma kallikrein, it is inhibited by all inhibitors which have an effect on kallikrein.

E. Plasminogen-Independent Fibrinolysis

When plasma was depleted of plasminogen by passage through an affinity chromatography column, *Moroz* and *Gilmore* (1976a,b) were able to detect fibrinolysis by a sensitive ^{125}I-labeled fibrin solid phase assay. Various inhibitors, such as DFP, SBTI, LBTI and EACA, did not inhibit the fibrinolytic activity of the depleted plasma at concentrations which inhibit plasmin. This fibrinolytic activity was factor XII-independent and not affected by contact. The increase in the fibrinolytic activity was associated with a reciprocal decrease in the activities of the classic and alternative pathways of complement. *Moroz* and *Gilmore* concluded that in normal plasma fibrinolytic activity was relatively independent of plasmin as the ultimate fibrinolytic enzyme, that factor XII-dependent pathways are of minor importance and that the plasmin-independent fibrinolysis may include proteinases involved in complement activation and other nonplasmin proteinases. The findings of *Moroz* and *Gilmore* remain to be repeated in other laboratories.

VI. Kinin Formation by Proteases of Neutrophil Leukocytes

Most of the studies concerned with kinin-generation have dealt in the past with enzymes (kallikreins) derived from tissues (e.g., pancreas) or from plasma (kallikrein, plasmin). A third source of kininogenases are the lysosomal enzymes of neutrophil leukocytes. The lysosomal enzymes are released whenever these cells phagocytoze.

A. Proteases of Neutrophil Leukocyte Lysosomes

A large number of proteolytic enzymes are present in neutrophil lysosomes (*Metchnikoff,* 1968; *Opie,* 1922; *Grob,* 1949). The neutral proteases of human neutrophils have been reviewed in 1972 by *Janoff* (1972b), but several have been added since then. In experimental animals, the granules contain mainly acid cathepsins, some of which can generate kinin at low pH (*Greenbaum* and *Kim,* 1967; *Chang* et al., 1972; *Greenbaum* et al., 1973; *Greenbaum,* 1976). A cartilage-degrading enzyme and a histonase acting at neutral pH have also been demonstrated (*Weissmann* and *Spielberg,* 1968; *Davies* et al., 1971), although some degree of nonspecific proteolytic activity at neutral pH is present in the lysosomes of rabbit neutrophils (*Wasi* et al., 1966).

The best studied in human neutrophils is an *elastase-like enzyme* (*Janoff* and *Scherer,* 1968; *Janoff,* 1970, 1972a, 1973; *Ohlsson* and *Olsson,* 1974a; *Schmidt* and *Havemann,* 1974; *Feinstein* and *Janoff,* 1975b; *Taylor* and *Crawford,* 1975; *Baugh* and *Travis,* 1976; *Kruze* et al., 1976; *Movat* et al., 1976a). Elastases have been demonstrated also in neutrophils of horse (*Dubin* et al., 1976; *Koj* et al., 1976), pig (*Kopitar* and *Lebez,* 1975) and dog blood (*Delshammar* and *Ohlson,* 1976; *Ardelt* et al., 1976). The elastase isolated from human spleen is identical to the elastase of neutrophils (*Starkey* and *Barrett,* 1976). The elastase-like protease of neutrophils is a highly basic protein consisting of three to five charge isomers. Its isoelectric point ranges between pH 10.0 and 11.8 (*Movat* et al., 1976a) and its MW has been found by most investigators, using SDS disc gel electrophoresis, to be just under 30,000 (26,000–28,000), although *Ohlsson* and *Olsson* (1974a) and *Feinstein* and *Janoff* (1975b) reported 33,000–35,000. By gel filtration in aqueous buffer, the MW was estimated to be only 20,000–23,000. The S-value is 2.7. Although referred to as elastase, this neutral protease has a weak elastinolytic activity but it readily degrades cartilage chondromucoprotein (*Janoff* and *Blondin,* 1970; *Ignarro* et al., 1973; *Malemud* and *Janoff,* 1975; *Janoff* et al., 1976; *Keiser* et al., 1976). On chrondromucoprotein it probably acts in conjunction with the chymotrypsin-like enzyme described below. The elastase-like

protease is readily inhibited by DFP and to a lesser degree by SBTI and Trasylol. A series of chloromethyl ketone inhibitors inhibit elastase (*Tuhy* and *Powers,* 1975). The most specific is MeO-Suc-Ala-Ala-Pro-Val CH_2Cl (*Powers* et al., 1977; *Powers,* 1977). However, while it inhibited the alanine esterase activity, it only partially inhibited the kinin-generating capacity (*Wasi* et al., 1978). Of the natural proteinase inhibitors, the elastase-like enzyme is inhibited by α_1-antitrypsin, α_2-macroglobulin and antithrombin III (*Ohlsson,* 1971; *Janoff,* 1972a; *Ohlsson* and *Olsson,* 1974b; *Movat* et al., 1976a).

The second well-characterized neutral protease of human neutrophil lysosomes is a *chymotrypsin-like enzyme* (*Rindler* et al., 1973, 1974; *Rindler-Ludwig* et al., 1974; *Schmidt* and *Havemann,* 1974; *Gerber* et al., 1974; *Rindler-Ludwig* and *Braunsteiner,* 1975; *Feinstein* and *Janoff,* 1975a). The same enzyme isolated from spleen has been referred to as *cathepsin G* (*Barrett,* 1975). The MW has been estimated to be 20,000–23,000. By cationic disc gel electrophoresis some investigators (*Schmidt* and *Havemann,* 1974; *Rindler-Ludwig* et al., 1974) demonstrated three isoenzymes, of which two migrated faster than lysozyme; others observed a single band (*Feinstein* and *Janoff,* 1975a). An interesting observation was made by *Rindler-Ludwig* and coworkers, that cathepsin G is only slightly soluble at physiologic NaCl concentration, but soluble at 1.0 M NaCl. This is useful for the separation from elastase. The enzyme is inhibited by some of the above mentioned chloromethyl ketone inhibitors, of which Z-Gly-Leu-Phe-CH_2Cl is the most specific (*Powers,* 1977). The plasma proteinase inhibitors α_1-antitrypsin and α_2-macroglobulin, as well as α_1-antichymotrypsin inhibit cathepsin G (*Ohlsson* and *Åkesson,* 1976).

Collagenase, a metalloproteinase, was detected by *Lazarus* and colleagues in granulocyte lysosomes (*Lazarus* et al., 1968, 1972). The monomers were cleaved into 1/3 and 1/4 length fragments, the cleavage point being probably 1/4 of the length from the C-terminal end of the collagen molecule. The specific cleavage products induced by the collagenase could be further degraded by a nonspecific protease present in lysosomal lysates. This latter enzyme was inhibited by serum. More recently, two collagenases, antigenically related, have been obtained in highly purified form (*Ohlsson* and *Olsson,* 1973). Both enzymes had a sedimentation coefficient of 4.5 S and a MW of 76,000, being composed of two subunits with MWs of 42,000 and 33,000, respectively. The collagenases were susceptible to inhibition by α_1-antitrypsin and α_2-macroglobulin. The two collagenases differed in their migration in disc gels due to differences in a few residues. The collagenases also degraded proteoglycans and fibrinogen. Since collagenase is metal dependent (zinc, calcium), it is readily inhibited by chelating agents such as EDTA, 1,10-phenanthroline, and by thiol compounds.

The localization of enzymes in the granules or lysosomes of the neutrophil has been studied by zonal sedimentation and isopycnic equilibration in sucrose gradients. By this procedure, the lysosomes can be separated into azurophil granules which have an average density of 1.23 and specific granules with an average density of 1.19 (*Bainton* et al., 1971; *Bretz* and *Baggiolini,* 1974). Application of this method to the above three enzymes points to the localization of the elastase-like enzyme and of cathepsin G (chymotrypsin-like) in the azurophil granules (*Dewald* et al., 1975; *Viescher* et al., 1976) and of the collagenase in the specific granules (*Murphy* et al., 1977).

Cathepsins A–E are, according to *Barrett* (1975), a heterogeneous group of peptide hydrolases which are all cell-derived and have an acid pH optimum. Of these, cathepsins B_1, D and E are endopeptidases and A, B_2, and C are exopeptidases. The latter enzymes comprise the aminopeptidases cleaving single amino acids at the N-terminal and carboxypeptidases cleaving at the C-terminal of a protein.

An *aminopeptidase* of neutrophil leukocytes capable of cleaving the amino acids lysine and methionine from the kinin-peptides, lysyl-bradykinin, or methionyl-lysyl-bradykinin was described in the writer's laboratory. The enzyme has a neutral pH optimum, an isoelectric point of 4.5, and by gel filtration an estimated MW of 200,000 (*Pass* et al., 1978). The aminopeptidase has been localized in granules distinct from those which contain the elastin- and chymotrypsin-like activity (azurophils), i.e., derived from the specific granules (*Folds* et al., 1972).

B. Interaction Between Neutrophil Proteases and Plasma Substrates

Acid proteases or cathepsins B, D, or E can cleave kinins from crude kininogen at acid pH (*Greenbaum* and *Kim,* 1967; *Chang* et al., 1972; *Greenbaum,* 1972), but the substrate has been subsequently shown to be distinct from the kininogen (*Greenbaum* et al., 1973; *Greenbaum,* 1976), which is the source of the three known vasoactive peptides (K-9, K-10, K-11). The peptides recovered by *Greenbaum* are referred to as "leukokinins" and are distinct from the plasma kinins.

The formation of leukokinins is a relatively slow process, occurring at acid pH and thus peptide generation in vivo is difficult to visualize. A rapid kinin-forming activity was described by *Melmon* and *Cline* (1967) in experiments in which intact neutrophils were incubated with whole plasma or a crude kininogen preparation. The model with the human neutrophils is a complex system and attempts to repeat the original experiment were unsuccessful (*Webster,* 1973). The availability of highly purified kininogens (*Habal* and *Movat,* 1972; *Habal* et al., 1974) and the fact

that human neutrophils contain neutral proteases, led the writer and coworkers to study the possibility that proteases of neutrophils release kinin from kininogen (*Movat* et al., 1973b). The release occurred rapidly at neutral pH and amounted to about 20% of the peptide releasable (expressed as bradykinin equivalents) by trypsin from the same kininogen preparation. With LMW kininogen it was essential that the substrate be free of α_1-antitrypsin, α_2-macroglobulin and kininase. When the fragmented neutrophils were subjected to differential centrifugation, the kinin-forming activity was detected in the granule or lysosomal fraction, whereas the cell sap or cytosol fraction contained a kinin-inactivating enzyme of kininase which was inhibited by heavy metals. The kinin-forming enzyme was subsequently purified, characterized and found to be similar to the above described elastase-like enzyme (*Movat* et al., 1976a). Yet more recent data indicate that the enzyme is distinct and merely cochromatographed with elastase (*Wasi* et al., 1978). With the whole lysosomal lysate (which contains also the kinin-converting aminopeptidase; *Pass* et al., 1978), the generated peptide behaved like bradykinin when chromatographed on CM-cellulose (*Movat* et al., 1976a) by the method of *Habermann* and *Blennemann* (1964). When the purified protease was used, the peptide eluted in an intermediate position between bradykinin and Met-Lys-bradykinin, though closer to the hendecapeptide (*Movat* et al., 1976b). The peptide generated was not bradykinin but could be converted to it, as demonstrated pharmacologically. When the generated peptide was treated with trypsin or the above described neutrophil-derived aminopeptidase (*Pass* et al., 1978), its potency increased when tested on the rat uterus.

Olsson and coworkers described and isolated highly cationic proteins of leukemic myeloid cells (*Olsson* and *Venge*, 1972, 1974). Of the seven proteins demonstrated by electrophoresis in agarose at acid pH, the four most cationic ones hydrolysed tyrosine esters, indicating a chymotrypsin-like activity (*Odeberg* et al., 1975). The chymotrypsin-like proteins, upon incubation with the complement components C1s, C4, C3 and C5, induced conversion of these components (*Venge* and *Olsson*, 1975). Of these, C3 and C5 gave rise to chemotactic activity, which however, disappeared upon prolonged incubation. The chemotactic activity was not very marked, about half that of positive controls (casein 5 mg/ml) and only about twice that of C3 or C5 itself. Furthermore, unlike C5a, C3a (a fragment of C3) is believed not to be chemotactic (*Müller-Eberhard*, 1976). In vivo, however, a chemotactic response to purified C3a has been observed (*Damerau* et al., 1978). Further, C3 was well as C5 may release other fragments with chemotactic activities in addition to C3a and C5a (*Vogt*, 1974; *Romualdez* et al., 1976). C3 cleavage products generated by the kinin-generating neutrophil protease enhance vascular permeability and upon short incubation a spasmogen is generated (*Movat* et al., 1976c).

The cleavage products were demonstrated by acid disc gel electrophoresis and sucrose density gradient ultracentrifugation of radiolabeled C3. The conversion of C3 was obvious also from immunoelectrophoretic analysis. *Johnson* et al. (1976) also demonstrated by immunoelectrophoresis conversion of C3 and C5 upon treatment with the elastase-like enzyme.

VIII. Plasma Proteinase Inhibitors

The inhibition of the various enzymes of the kinin system was discussed briefly when these enzymes were described. This section will be an overview of the properties of the plasma proteinase inhibitors which act on the plasma-kinin system. Unlike synthetic and plant inhibitors that are often used in the laboratory, the natural proteinase inhibitors of plasma have in vivo significance, since they keep the kinin and related systems in homeostatic balance. Pathologic states usually arise from activation of the kinin system, but lack of an inhibitor may also lead to abnormal function and disease states, e.g., angioedema due to a lack of $C\bar{1}$-inactivator. This indicates a continuous spontaneous low-rate activation of kallikrein which may be significant also for the initiation of, e.g., contact activation (see Sect. II.B).

On a weight basis, α_1-antitrypsin and α_2-macroglobulin are in the highest concentration in plasma, but on a molar basis α_1-antitrypsin is by far the most abundant inhibitor. The combining ratios have also to be taken into consideration (*Heimburger* et al., 1971; *Heimburger,* 1975).

If plasma is contact-activated, most of the kallikrein becomes inactivated by $C\bar{1}$-inactivator, antithrombin III and α_2-macroglobulin, but some remains active. In fact, α_2-macroglobulin forms complexes with various enzymes in which the active center is not blocked (*Shulman,* 1955; *Meyers* and *Burdon,* 1956; *Haverback* et al., 1962; *James* et al., 1966; *Dyce* et al., 1967). The catalytic sites of enzymes complexed with α_2-macroglobulin are still capable of acting on low MW substrates and to a lesser degree on higher substrates. The latter are excluded probably by steric hindrance. This type of inhibition is explained by the "trap" mechanism of *Barrett* and *Starkey* (1973), which requires the attack of α_2-macroglobulin by a proteolytic enzyme, resulting in a conformation change entrapping the protease. Although the residual activity is lower than that of the original enzyme, the complex has catalytic effect when the α_2-macroglobulin has interacted with either plasmin (*Harpel* and *Mosesson,* 1973) of kallikrein (*Vogt,* 1976a,b; *Vogt* and *Dugal,* 1976). The effect of the kallikrein-α_2-macroglobulin complex on kininogen is shown in Figure 11. Other enzymes, inactivated when plasma is contact-

activated, are plasmin and factor XIIa, both of which are inhibited by the same inhibitors as kallikrein (see Fig. 1).

The mode of action of plasma proteinase inhibitors is not fully understood. A constant feature of the inhibition of trypsin by non-plasma-derived inhibitors (e.g., SBTI of LBTI) is the hydrolysis of a peptide bond (cleavage of a single lysine or arginine residue) located within the disulfide loop of the inhibitor molecule (*Laskovski,* 1972). From this finding it was postulated that other proteinase inhibitors may have a similar susceptible peptide bond and hydrolysis of the peptide bond may be essential for the enzyme complex formation and the inibition (*Laskovski* and *Sealock,* 1971). When proteases interact with the plasma inhibitors the latter undergo cleavage. This has been observed when plasmin or plasma kallikrein interact with α_2-macroglobulin or $C\bar{1}$-inactivator (*Harpel,* 1973; *Harpel* and *Cooper,* 1975; *Harpel* et al., 1975) and when neutrophil leukocyte protease (elastase) interacts with α_2-macroglobulin or antithrombin III (*Movat* et al., 1976a).

Alpha$_1$-antitrypsin has little effect on the plasma kinin system, since it does not inhibit activated factor XII and plasma kallikrein and has little effect on plasmin (*Habal* et al., 1976), earlier reports of slow inactivation of kallikrein (*Fritz* et al., 1972b) were probably attributable to traces of antithrombin III (*Burrowes* and *Movat,* 1977). However, α_1-antitrypsin rapidly inactivates neutrophil elastase (*Ohlsson,* 1971; *Janoff,* 1972; *Ohlsson* and *Olsson,* 1974b; *Movat* et al., 1976a). The antitryptic activity of plasma was first reported in 1894 by *Ferni* and *Pernossi.* About 90% of the antitryptic activity is confined to the α_1-globulin zone and the bulk of this is due to α_1-antitrypsin. The inhibitor migrates in the α_1-zone by paper, acetate or agarose electrophoresis, but in the albumin region by alkaline disc gel electrophoresis. Its sedimentation coefficient is 3.8 S, the diffusion coefficient 5.2, the partial specific volume 0.646, the extinction coefficient 5.3, and the isoelectric point 4.9. It is a glycoprotein, containing 12.2% carbohydrate. Early data indicated a MW of 45,000 but all recent results obtained by various methods suggest 53,000–54,000 (*Hercz,* 1973; *Liener* et al., 1973; *Crowford,* 1973; *Pannell* et al., 1974; *Heimburger,* 1975; *Borrowes* and *Movat,* 1977). Earlier, based on the fact that α_1-antitrypsin forms 1:1 complexes with SBTI and that SBTI binds three times its weight, an approximate MW of 60,000 was calculated for the inhibitor (*Vogel* et al., 1966). The concentration in plasma is 2000–4000 μg/ml (*Heimburger,* 1975).

Alpha$_1$-antichymotrypsin has no effect on any of the components of the plasma kinin system, but it inhibits cathepsin G, the chymotrypsin-like protease of neutrophils (*Ohlsson* and *Åkesson,* 1976). Antichymotrypsin has a MW of 69,000, a sedimentation coefficient of 3.9 S, and a carbohydrate content of 24.6%.

Alpha$_2$-macroglobulin, first isolated by *Schultze* et al. (1955), has a sedimentation coefficient of 19.6 S (*Baumstark,* 1970) or 18.0 S (*Jones* et al., 1972; *Hamberg* et al., 1973), a diffusion coefficient of 2.41, a partial specific volume of 0.735 (*Heimburger* et al., 1971), and an isoelectric point of 5.3–5.4 (*Hamberg* et al., 1973). In the older literature, the MW is cited as 820,000 (*Schultze* and *Heremans,* 1966), but more recent findings indicate a MW of 725,000 (*Jones* et al., 1972) or even as low as 650,000 (*Saunders* et al., 1971). These later findings are in good agreement with the subunit structure of the inhibitor (*Jones* et al., 1972; *Harpel,* 1973). The carbohydrate content is 7.7% (*Heimburger,* 1975). Its mode of action is described above. The concentration in plasma is 1500–3500 μg/ml in men and 1750–4200 μg/ml women (*Heimburger,* 1975).

$C\bar{1}$-inactivator was discovered by *Ratnoff* and *Lepow* (1957) as an inhibitor of the activated first component of complement, as the original designation "C'1 esterase inhibitor" implies. It was purified by *Pensky* et al. (1961) and shown later (*Pensky* and *Schwick,* 1969) to be identical to the earlier-described α_2-neuraminoglycoprotein (*Schultze* et al., 1962). The sedimentation coefficient was estimated to be 3.67 S (*Schultze* et al., 1962; *Haupt* et al., 1970) of 4.2 S (*Pensky,* 1970). The MW of 139,000 by gel filtration (*Pensky,* 1970) is too high, presumably due to the high (34.7%) carbohydrate content. The value of 104,000 given by *Heimburger* (*Heimburger* et al., 1971; *Heimburger,* 1975) is in good agreement with data obtained by SDS disc gel electrophoresis (*Harpel* and *Cooper,* 1975; *Harpel* et al., 1975). By this method a major band (MW 105,000) and a minor band (MW 96,000) are detected. They show a reaction of identity by immunodiffusion. Reduction and alkylation do not change the MW, indicating a single polypeptide chain. Complexes with plasmin have a MW of 180,000 and 170,000, respectively, but those with plasma kallikrein, although demonstrated with radiolabeled material, have not been determined. As shown in Figure 1, $C\bar{1}$-inactivator acts on several enzymes of the kinin system.

Antithrombin III is one of the two inhibitors of thrombin. The second one is α_2-macroglobulin (*Lanchantin* et al., 1966; *Steinbuch* et al., 1968). Antithrombin activity of human plasma was first observed in 1905 by *Marawitz. Brinkhous* et al. in 1939 noted that heparin was effective as an anticoagulant only in the presence of a plasma factor. Later an intimate relationship between the antithrombin activity of plasma and heparin was observed, heparin enhancing up to 100-fold the trhombin-neutralizing activity of antithrombin. Recently, purified preparations of antithrombin III have been prepared (*Abilgaard,* 1968; *Rosenberg* and *Damus,* 1973; *Damus* and *Wallace,* 1974; *Miller-Anderson* et al., 1974; *Burrowes* and *Movat,* 1977). *Heimburger* used $Ca_3(PO_4)_2$ and *Rosenberg* and *Damus* $Al(OH)_3$ to adsorb the antithrombin from plasma. The desorbed anti-

thrombin III was passed by all investigators through a series of chromatographic steps, including affinity chromatography on heparin-Sepharose 4B. *Rosenberg* and *Damus* (1973) used isoelectric focusing as the final step to obtain a homogeneous preparation. *Burrowes* and *Movat* found that α_1-antitrypsin, having a similar charge and molecular weight, cochromatographed with antithrombin but could be separated on QAE-Sephadex at pH 7.4. The inhibitor has an α_2-electrophoretic mobility, a sedimentation coefficient of 3.8 S, an isoelectric point of 5.1 and a MW of 63,000–65,000. It forms complexes of MW 110,000, 123,000 and 139,000 with plasma kallikrein (*Venneröd* et al., 1976). With plasmin the complex formed has a MW of 142,000 (*Highsmith* and *Rosenberg,* 1974). Complexes with factor XIIa have also been described. Their MW was estimated to be 117,000 (*Stead* et al., 1976).

Alpha$_2$-plasmin inhibitor was described recently by 3 groups of investigators (*Collen,* 1976; *Moroi* and *Aoki,* 1976; *Müllertz* and *Clemensen,* 1976). These studies have subsequently been further extended (*Wiman* and *Collen,* 1977; *Highsmith* et al., 1977; *Edy* and *Collen,* 1977; *Moroi* and *Aoki,* 1977; *Harpel,* 1977; *Aoki* and *Moroi,* 1978; *Teger-Nilson* et al., 1978).

The molecular weight of the inhibitor (often referred to as α_2-antiplasmin or fast inhibitor of plasmin) has been estimated to be about 70,000. It is a single chain polypeptide with an estimated carbohydrate content of 14%. Its sedimentation constant is 3.45 S. Its charge and molecular weight indicated that it elutes with other inhibitors (α_1-antitrypsin, antithrombin III). However, affinity chromatography on plasminogen-Sepharose in addition to other steps yielded a homogeneous preparation. *Wiman* and *Collen* (1977) suggested a procedure in which Cohn fraction I was depleted of plasminogen (lysine-Sepharose), followed by plasminogen-Sepharose and this in turn by DEAE-Sephadex and finally concanavalin A-Sepharose.

The main characteristic of the inhibitor is that it inactivates plasmin instantaneously. The inhibitor has been compared to other known inhibitors of plasmin. *Harpel* (1977) reported that when the α_2-antiplasmin was allowed to compete with α_2-macroglobulin almost as much plasmin was bound to α_2-antiplasmin in mixtures containing a large excess of α_2-macroglobulin relative to plasmin or α_2-antiplasmin, as was bound in mixtures not containing α_2-macroglobulin. *Highsmith* et al. (1977) used trace amounts of ^{125}I-plasminogen and studied its distribution amongst the the various proteinase inhibitors in whole plasma, after activation to plasmin by urokinase or streptokinase. At 37° about 90% of the labeled plasmin was bound to α_2-antiplasmin and about 7% to α_2-macroglobulin. More plasmin was bound to α_2-macroglobulin at 22°.

VIII. Bradykinin and Related Kinins

It has been known since the studies of *Werle* et al. (1937) that glandular kallikrein could release from plasma a smooth muscle stimulating substance (darmkontrahierende Substanz). Studies in this field received a new impetus in the late 1940s, through the work of *Werle* and *Berek* (1948, 1950) and of *Rocha de Silva* et al. (1949). The former demonstrated that the earlier-described smooth muscle stimulating substance was a peptide which they designated kallidin. *Rocha de Silva* et al. independently showed that when trypsin or snake venom (*Bothrops jararaca*) was added to plasma a smooth mucle and vasodilator substance is generated, which they called bradykinin. Antihistamine had no inhibitory effect on it and the guinea pig ileum contraction took about seven times longer than with histamine (bradys, slow; kinein, to move).

The three principal kinins which can be generated from plasma by cleavage of kininogens include (a) bradykinin, consisting of nine amino acids, hence referred to as nonapeptide, (b) lysyl-bradykinin (kallidin), a decapeptide and (c) methonyl-lysyl-bradykinin, an hendecapeptide (*Schröder* and *Hempel,* 1964; *Lewis,* 1964).

The isolation of bradykinin from plasma is beautifully described by *Elliott* (1970). It was isolated by *Elliott* et al. (1960a, 1961), who first suggested that it consists of eight amino acids. However, on synthesis, this octapeptide was found to be inactive whereas the synthetic nonapeptide had all the activity of bradykinin isolated from plasma (*Boissonnas* et al., 1960). More or less simultaneously, *Elliott* et al. (1960b) found that they had missed a third molecule of proline in their original work and assigned to bradykinin the structure of the nonapeptide synthesized by *Boissonnas* et al. When the biologic properties of synthetic and natural bradykinin were compared, they were found to be identical (*Lewis,* 1960; *Konzett* and *Stürmer,* 1960). The structure of kallidin or lysyl-bradykinin was ascertained one year later by isolating it from bovine plasma treated with hog salivary kallikrein (*Werle* et al., 1961) and from human plasma treated with urinary kallikrein (*Pierce* and *Webster,* 1961). Some of the recovered peptide under these consitions is bradykinin. Glandular kallikreins cleave lysyl-bradykinin from kininogens, but plasma contains a kinin-converting enzyme or aminopeptidase, which converts the decapeptide into the nonapeptide (see below). Plasma kallikrein, on the other hand, releases the nonapeptide directly (*Webster* and *Pierce,* 1963; *Habermann* and *Blennemann,* 1964). Soon after the isolation of lysyl-bradykinin, the peptide was also prepared synthetically (*Nicolaides* et al., 1961). The third kinin, methionyl-lysyl-bradykinin was isolated from bovine plasma which had been acidified and reneutralized (*Elliott* and *Lewis,* 1965) and *Schröder* (1964) carried out its synthesis. The following is the structure of the three plasma kinins (the bradykinin is labeled 1–9):

H-Met-Lys-Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9-OH

The *biologic activities* of the three kinins are similar, the differences being quantitative (*Reiss* et al., 1971). The activities include in vitro contraction of the ileum (guinea pig, rabbit, rat, cat, and dog), contraction of the estrous rat uterus and relaxation of the rat and rabbit duodenum and of the rat colon. In vivo, kinins induce bronchoconstriction in the guinea pig, hypotension, increase in vascular permeability, and pain when applied to a blister base in man (*Lewis,* 1960; *Rocha e Silva,* 1963; *Schröder* and *Hempel,* 1964; *Reiss* et al., 1971).

Kinins are *inactivated* by enzymes known as kininases, the majority of which are carboxypeptidases (*Erdös* et al., 1976). The best characterized plasma kininase is carboxypeptidase N, which inactivates kinins by cleaving the C-terminal arginine. Kininase II or peptidyldipeptide hydrolase cleaves the C-terminal dipeptide. Plasma contains also an aminopeptidase which converts larger kinins into bradykinin by cleaving amino acids at the N-terminal (*Guimarães* et al., 1973). Methionine and lysine are cleaved also from the hendecapeptide and the decapeptide by an aminopeptidase derived from human neutrophil leukocytes (*Pass* et al., 1978).

IX. Concluding Remarks

The kinin system is complex. The central role is played by factor XII. Through its activation three cascading systems are set into motion: the intrinsic clotting, the fibrinolytic, and the kinin forming. The three are interrelated not only because factor XII initiates the activation of all three systems, but also because components of one system can interact with components of another. Deficiency of a component of one system affects also the other two. For example, plasmin, the principal enzyme of the fibrinolytic system, whose main function is fibrinolysis, can act also on kininogen to generate kinin. Furthermore, plasmin has a positive feedback; by acting on factor XII it can cause its activation and fragmentation. Prekallikrein, the zymogen of kallikrein which is the main kinin-forming enzyme, exerts a positive feedback on factor XII which is even more significant than that of plasmin. In prekallikrein deficiency, because there is inadequate feedback, there is a clotting abnormality and inadequate intrinsic activation of the fibrinolytic system. In fact, newer data indicate that prekallikrein is probably identical to the plasminogen activator (see Fig. 1).

As already indicated, activation of factor XII plays a central role in the activation of the three systems. Figure 16 illustrates the possible mechanisms which underlie contact activation of factor XII. Initially, mere contact of factor XII with a negatively charged surface (such as collagen

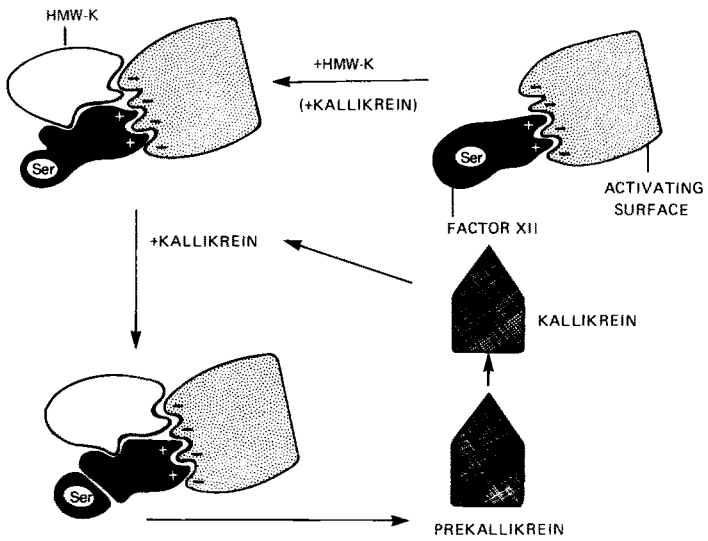

Fig. 16. Diagram of hypothetical events during contact activation of factor XII. Upon contact with a negatively charged surface some activation of factor XII occurs (upper right corner). Traces of prekallikrein are activated to kallikrein, which can act on a complex of factor XII and HMW kininogen (upper left corner). Further activation of factor XII generates more kallikrein, which can cleave fragment XIIf from the factor XII molecule (lower left corner). The fragment contains the active center (ser = serine). Fragment XIIf acts on prekallikrein, converting it to kallikrein (lower right corner) which causes a positive feedback by acting on factor XII

or cartilage) will cause a minimal activation of the factor XII molecule, converting traces into factor XIIa. This is sufficient to convert traces of prekallikrein to kallikrein. From here on the process is similar to the fluid phase activation, i.e., enzymatic. There is, however, one exception: While in fluid phase, kallikrein (unlike trypsin or plasmin) is not very efficient but in solid phase (i.e., contact activation) a cofactor, namely HMW kininogen, plays an enhancing role in the activation of factor XII and probably also in the formation of the XIIf-fragments. In this process a complex probably forms between the activating surface, factor XII, and HMW kininogen. The process is self-limiting because fragments 1–2 cleaved by kallikrein from HMW kininogen (a highly basic peptide) compete for the negatively charged surface which activates factor XII. As shown in Figure 1, plasmin through its activation can cause a positive feedback. Figure 1 also shows the balancing or homeostatic effects excercised by the various plasma proteinase inhibitors.

To date no physiologic role has been attributed with certainty to kinins. However, they are believed to play a role in pathologic mechanisms. They have been implicated as pharmacologic mediators of the vascular phenomena of acute inflammation and of systemic shock and disseminated

intravascular coagulation. A direct role of kinins in the inflammatory process has not been demonstrated. However, this is true also of most other mediators. Kinins do fulfill the criteria of a potential pharmacologic mediator (*Movat,* 1978a), i.e., kinins or the precursors leading to their formation are (a) widely distributed in various tissues of higher species, (b) are readily available and activatable, (c) can induce, when injected, the vascular phenomena of inflammation, (d) have been isolated from inflammatory lesions and (e) their effect can be suppressed by inhibitors. The final criterion, depletion and thereby prevention of its action, has not been demonstrated adequately.

A number of activators have been listed which could play a role in vivo. However, as indicated, potent inhibitors acting at all levels (see Fig. 1) have been described. Therefore, pathologic states could arise from excess activation which would be only partially counterbalanced by the inhibitors. Alternatively, the homeostatic balance could be tipped by inadequate inhibition. Inhibition can be visualized as inadequate in circumscribed areas. It is therefore possible that in inflammation, enzymes with kininogenase activity, derived from neutrophils, play an essential role. Neutrophils can be attracted chemotactically not only by complement components or bacterial products, but also by kallikrein. Through the release of lysosomal kininogenases and local formation of kinins, more plasma with components of the kinin system can escape into the tissues and the process can be perpetuated.

All these questions remain unanswered and it remains to apply all the knowledge acquired from in vitro studies to in vivo experiments to ascertain the possible role of the kinin system in physiologic or pathophysiologic conditions.

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