THE CULTURE, GENERAL PHYSIOLOGY, MORPHOLOGY, AND CLASSIFICATION OF THE NON-SULFUR PURPLE AND BROWN BACTERIA

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1. INTRODUCTION

"Eine Untersuchung des Stoffwechsels der heterotrophen Purpurbakterien erschien nicht nur deshalb, weil nähere Kenntnisse fehlten, als dankbare Aufgabe, sondern weil zu erwarten war, dass im Falle einer Kohlensäurereduktion durch diese Bakterien die Reaktion ganz anders verlaufen musste als bei den grünen Pflanzen und den Thiorhodaceen. Und je mehr Varianten des Assimilationsvorganges wir kennen, um so eher können wir hoffen, Aufklärung über seinen Mechanismus zu erhalten."

H. Gaffron, (1), p. 2.

The studies on the green and purple sulfur bacteria have led to the demonstration that the normal metabolism of these organisms represents a truly photosynthetic process which differs in two major respects from that of the green plants. Their biochemical activities have been interpreted as a photochemical carbon dioxide reduction with hydrogen which is ultimately derived, not from water as in green plants, but from hydrogen sulfide (2, 3).

In the first publication on this subject (2) I intimated that the sulfur-free purple bacteria, or *Athiorhodaceae* in the nomenclature of Molisch (4), which require organic substances for growth, might constitute a group of organisms in which the function of hydrogen sulfide as ultimate hydrogen donor for the photochemical carbon dioxide reduction might be here fulfilled by organic compounds.

The tentative statement made in 1929 (2, p. 168) " . . . . it is probable that the continued study of the metabolism of this group will give additional information with regard to the further possibilities of photosynthesis" was strongly supported by the experiments of H. Gaffron, published in 1933 and 1935 (1, 5). In these it was shown beyond a doubt that a carbon dioxide reduction is actually accomplished by representatives of the *Athiorhodaceae*, and that this is fully dependent upon both illumination and the presence of organic substrates or molecular hydrogen.

With the unambiguous demonstration by Foster (6) that the function of the organic substrate is indeed that of hydrogen donor for photosynthesis, the main outlines of the biochemical characteristics of this group were placed on a firm foundation. Meanwhile Muller, in 1933, had shown (7) that *Thiorhodaceae*, too, can utilize simple organic compounds instead of hydrogen sulfide for photosynthesis. Herewith the fundamental similarity in the metabolism of *Thio-* and *Athiorhodaceae* was established.

The various aspects of the problem of photosynthesis by bacteria have recently been reviewed in some detail (8). It is, therefore, superfluous to deal here with this phase. However, for studies on bacterial photosyntheses the use of representatives of the non-sulfur purple bacteria is, in many cases, preferable to that of *Thiorhodaceae*. This is partly due to the fact that many of the former are far less sensitive to such external influences as the presence of small amounts of oxygen, and hence are more easily handled. Also, the strains of bacteria belonging to this group have exhibited a capacity for attacking a greater variety of substances than have those sulfur purple bacteria which have been studied in this respect. But different cultures of *Athiorhodaceae* display quite considerable differences in their biochemical potentialities. Although this has occasionally been recognized, a general survey of this aspect has never been published.

During the studies on bacterial photosynthesis, carried out in the last fifteen years, a large number of strains of non-sulfur purple bacteria have been isolated in pure culture.

A more detailed investigation of the general morphology and physiology of this group has, therefore, been undertaken. It has led to the recognition of definite types which can be distinguished by both morphological and physiological criteria. As a consequence it has also become possible to attempt a more up-to-date system of classification of these organisms.

It is hoped that the publication of the results may make these bacteria more generally known to microbiologists, and thus lead to more intensive studies of the many fascinating problems which they still present.

2. DELIMITATION OF THE GROUP OF NON-SULFUR PURPLE BACTERIA

> "Vergleicht man Molischs Ergebnisse mit denen der übrigen Beobachter, namentlich Winogradskys, so kommt man bei vorurteilsloser Würdingung aller Umstände zu dem Schlusse, dass sich eine Scharfe Trennung zwischen schwefelhaltigen und schwefelfreien Purpurbakterien empfiehlt. Die typischen Vertreter beider Gruppen gehören offenbar ganz verschiedenen ökologischen Kategorien an und weichen in wesentlichen Punkten ihres normalen Stoffwechsels weit voneinander ab. . . . Die hier betonte scharfe Gegenüberstellung kommt in Molischs Schrift nicht überall klar zum Ausdruck."
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> Joh. Buder (9), p. 531.

It is advisable to outline what, in this treatise, will be understood by non-sulfur purple bacteria. In the past much confusion has resulted from the use of ambiguous names and inadequate definitions. This was largely the result of an incomplete understanding of the physiological activities of the various purple bacteria, and it should now be possible to avoid such difficulties by a careful appraisal of present-day information.

In 1907 Molisch (4) created the order *Rhodobacteria* for those organisms which he considered "purple bacteria." They were characterized as bacteria containing a complex pigment system, made up of a green pigment, "bacteriochlorin," and one or more red pigments, "bacteriopurpurin." The order was subdivided into two families, the *Thiorhodaceae* and *Athiorhodaceae*. Diagnostically, these were distinguished by the occurrence of sulfur droplets in the cells of organisms belonging to the former family, whereas representatives of the latter always appear without sulfur droplets inside the cells.

Buder, in the report of his extensive studies on the purple bacteria (9), was the first to call special attention to the fact that these two groups of purple bacteria are primarily distinguishable by a fundamental difference in their metabolism. According to his views the *Thiorhodaceae* would represent organisms with an autotrophic, the *Athiorhodaceae* such with an heterotrophic mode of life; the former oxidizing hydrogen sulfide, the latter organic substances. This idea has been amply confirmed by the later investigations.

It should here be observed that the sulfur purple bacteria—as Winogradsky (10, 11) had already shown in 1887—contain sulfur globules only when growing in media containing sulfide. They use up this storage product during periods when they are exposed to a sulfide-free environment. Since, then, the morphological characterization is not always strictly valid, subject as it is to environmental influences, it would seem better to use the intrinsic physiology as a basis for differentiation rather than its transitory morphological expression. On the other hand, it is well to remember that in nature one rarely ever encounters typical *Thiorhodaceae* except under conditions where sulfide is present, so that sulfur-free specimens of this group may be considered as artifacts of the laboratory.

Although this last consideration would appear to make the morphological and physiological characterizations equivalent for practical purposes, another observation clearly favors Buder's approach. There have, namely, been found in nature small, rod-shaped purple bacteria which are physiologically speaking true *Thiorhodaceae* because they are capable of development in strictly mineral media with sulfide (3). Yet these organisms do not conform to the criteria set up by Molisch for this family because, even with an abundant sulfide supply, they never store sulfur droplets in their cells. The intermediate oxidation

product, sulfur, does not here become trapped inside the bacteria, but it accumulates in the external environment. Morphologically these microörganisms can therefore never be recognized as sulfur bacteria, while on the basis of Buder's physiological differentiation they should properly be grouped with the *Thiorhodaceae*. I should like to point out here that this consideration in an earlier publication (3) has given rise to a misunderstanding on the part of Pringsheim (12) who interpreted it as meaning that I opposed a subdivision of the purple bacteria into *Thio-* and *Athiorhodaceae*, whereas I merely intended to bring out the difficulties inherent in a purely morphological diagnosis and their ready elimination as a result of a physiological evaluation.

A more serious point is raised by the existence of types which appear to be intermediate between the two large physiological groups in the purple bacteria. Though Buder (9) failed to find experimental evidence in favor of such a concept, first introduced by Nadson (13), he felt compelled to consider it as a theoretical possibility, and states: "Sollten aber künftige Ergebnisse jene Angaben bestätigen, so wurden derartige Formen einen Übergang zwischen den beiden Extremen bilden. Wir hätten es dann mit mixotrophen oder fakultativ autotrophen Formen zu tun. So etwas wäre jedenfalls denkbar and hätte auch schon Analoga unter den Schizomyceten" (p. 536).

Now, as mentioned in the Introduction, it has been conclusively demonstrated that all the representatives of the typical *Thiorhodaceae* so far investigated in this respect are, in pure culture, not dependent upon the presence of sulfide, but can utilize organic substrates instead (7; see also 14, 15, 16). At first sight this might seem to erase the clear-cut physiological differentiation which Buder stressed. But it must be remembered that in natural environments the true sulfur purple bacteria do not come to the fore unless the medium contains sulfide, as has been clearly brought out by Winogradsky, who winds up his review with the claim: "Il n'y aurait, en conséquence, encore aucune raison valable de nier la nature essentiellement autotrophe des *Thiorhodaceae*" (17, p. 974).

Consequently it appears that a rigorous separation of the purple bacteria in Molisch's two groups of *Thio-* and *Athiorhodaceae* would be feasible provided: *a*. it is based upon physiological rather than morphological criteria, and *b*. due allowance is made for potentialities residing in the representatives of the first-mentioned group, but demonstrable only with pure cultures.

It is in this sense that the non-sulfur purple bacteria will here be treated. Even so, as will become apparent later, there exist cases in which it is rather difficult to assign to the organism in question a definitive position.

Whereas many bacteria of this group appear in cultures as a pink, violet, to deep-red growth, there also occur types which, though exhibiting essentially the same physiological characters, produce yellowish to dark-brown masses of organisms. It is probable that it is these bacteria which Utermöhl (18) has noted during his plankton investigations, and to which he refers as " . . . . eine neue, den Purpur- und Chlorobakterien vergleichbare Reihe farbstofffführender Bakterien . . . . , die ihrer braunen Färbung wegen wohl als Phaeobakterien bezeichnet werden können." But Utermöhl was not the first to find such "Phaeobacteria." Ewart, as early as 1897 (19), described such an organism under the name "*Streptococcus varians*"; and Molisch (4), in his descriptions of various (non-sulfur) purple bacteria, mentions a few examples of brown and reddish-brown pigmentation. Much later Gaffron (5), referring to his cultures of "brown bacteria," states: "Diese Bakterien sind sehr wahrscheinlich schon von anderen Forschern beobachtet worden. Eine Litteraturstelle ist mir nicht bekannt" (p. 307).

Organisms of this type are quite common in crude cultures of non-sulphur purple bacteria, as observed also by Czurda and Maresch (20). During the past fifteen years, numerous strains have been isolated and studied, morphologically as well as physiologically. In view of the fact that these bacteria contain a pigment system consisting of a functional bacteriochlorophyll accompanied by various yellow and red carotenoid pigments, there seems to be no valid reason for excluding them from the group of purple bacteria, even

though the cultures present colors which can certainly not be called "purple." They will, therefore, be included in the present treatise.

A discussion of the taxonomy and nomenclature of the group will be postponed till morphological and physiological characteristics of the several representatives have been described. Meanwhile, the organisms will be variously designated as "non-sulfur purple bacteria," "brown bacteria," or "*Athiorhodaceae*." Too much significance should not be attached to these names, however. They will merely serve to characterize the group of bacteria which forms the subject of this study.

3. THE CULTURE OF THE NON-SULFUR PURPLE AND BROWN BACTERIA

> "Es war klar, dass trotzdem noch nicht alle Schwierigkeiten der Kulturmethodik beseitigt sein müssten. Ein Hinblick auf die Grünalgen genügt, um zu zeigen, wie trotz der gleichbleibenden Art und Weise des Kohlenstoffgewinnes doch noch eine so grosse Mannigfaltigkeit der ökologischen Ansprüche der Einzelformen vorkommen kann, dass nur ein Teil von ihnen heute einer sicheren Kultur zugänglich ist."
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> Czurda and Maresch, (20), p. 99.

I. Crude and enrichment cultures

a. Early methods; complex media. In the publications of Molisch (4), Buder (9), Ljubimenko (21), Schneider (22), Czurda and Maresch (20), etc., a number of general methods has been described for obtaining crude cultures of purple bacteria. The principle of all these methods is the exposure of a mixture of mud, surface water, and such ill-defined substrates as hard-boiled eggs, bones, preferably with meat, dead animals, such as worms, mussels, and other invertebrates, packets of leaves, etc., in a tall glass cylinder to light. After some time, usually of the order of magnitude of 1 to 2 weeks, purple bacteria begin to develop in such containers, and the crude cultures thus obtained are used for subsequent experiments.

An important improvement in methodology was introduced by Seliber in 1928 (23) and consists of a continuous illumination with artificial light. The time involved in getting the purple bacteria to develop until they form visible accumulations is thereby cut down to 3 or 4 days. In nearly all subsequent studies (2, 3, 5, 6, 7, 20) the continuous illumination procedure has been used to great advantage.

As a rule, the results obtainable with these methods are entirely satisfactory if one aims at securing crude cultures of any representative or mixture of representatives of this group of bacteria. It will, however, be obvious that the most diversified processes of microbial decomposition take place in such containers, in an utterly uncontrollable manner, and give rise to a more or less large variety of metabolic products. And these, in turn, may influence the types and numbers of purple bacteria which gradually make their appearance. By using these methods, it is thus left entirely to chance which species or combination of species will become sufficiently predominant so that their isolation can be achieved by the ordinary means of cultures on or in solid media.

The investigations of Molisch have made it abundantly clear that the group of *Athiorhodaceae* comprises a number of rather different types, including more or less spherical, rod-shaped, curved, and spirally-wound organisms, and Molisch

was the first to attempt a more detailed classification by creating a number of genera and species for the organisms studied by him (4). Since that time studies on the non-sulfur purple bacteria have been carried out with impure cultures or with pure cultures which had usually been acquired incidentally. The latter have been more or less adequately described and identified with one or another of Molisch's species. Only very recently has an attempt been made, by Czurda and Maresch (20), to isolate various representatives of this group with a view to studying their morphological and physiological characteristics, and thus to supplement the old descriptions and system of classification. But changes in the methods for obtaining crude cultures of *Athiorhodaceae* were not introduced.

b. Improved methods: theoretical considerations. During the past several years much information has been collected concerning the fundamental metabolic activities of the non-sulfur purple bacteria. This made it seem likely that methods could be worked out which would make it possible to secure cultures of different representatives at will. Once it is realized that these organisms are capable of photosynthetic activity in the light, and in the absence of oxygen, when provided with simple organic compounds, it also seems logical to expect that different organic substances will form the substrate "*par excellence*" for different types of *Athiorhodaceae*. With a more exact knowledge of their nutrient requirements it would become feasible to devise enrichment media for obtaining cultures of the desired type or group. Such methods would also tend to eliminate from the crude cultures many of the organisms which, in the complex and undefined media mentioned above, often comprise a large proportion of the microbial population, but which do not belong to the purple bacteria. The presence of such contaminants frequently makes it very difficult to proceed satisfactorily with the isolation of pure cultures of *Athiorhodaceae*.

Consequently several attempts have been made to apply these theoretical deductions to the development of more satisfactory and specific enrichment culture methods for the non-sulfur purple bacteria. Considering the photosynthetic activity as the outstanding physiological characteristic of all the purple bacteria, and the successful accomplishment of photosynthesis in the presence of a simple organic substance as the specific feature of the *Athiorhodaceae*, it was believed that a strictly mineral medium with an adequate supply of carbon dioxide, to which a single organic compound had been added, should suffice to ensure the development of the latter group of organisms from an appropriate inoculum, when exposed under anaerobic conditions to light. The results of these experiments, although not entirely satisfactory from the point of view of immediate success in the desired respect, are sufficiently instructive to justify a brief discussion. This is the more true because, particularly in the light of our present knowledge of the nutrient requirements of the *Athiorhodaceae*, they may well lead to the rapid development of more adequate methods.

c. Experimental results with different pure organic substances. For these experiments a standard mineral medium was used consisting of distilled or tap water with 1 g $(NH_4)_2SO_4$, 0.5 g K_2HPO_4, 0.2 g $MgSO_4$, 2 g NaCl and 5 g $NaHCO_3$

per liter. This basal medium was then supplemented by the addition of a single organic substance in a final concentration of 0.15–0.2%, after which the reaction was adjusted to pH 7.0 with H_3PO_4. The organic compounds used were a number of individual members of the groups of simple alcohols, polyalcohols, fatty acids, hydroxy acids, dibasic acids, amines, and amino acids. A few cultures with complex nutrients, peptone and yeast extract, were always included. The various media were dispensed into glass stoppered bottles, inoculated with a small amount of surface water or mud-suspension, and the completely filled and stoppered bottles then incubated in a light cabinet (cf. (3)) at a temperature of 25° to 30°C under continuous illumination with a series of 25-40 watt electric bulbs.

Cultures of photosynthetic, red and brown colored bacteria develop fastest in the media with peptone and yeast extract. In 2 to 3 days one may usually count on an abundant growth of purple bacteria in the bottles. Next, the red organisms appear in media with ethanol, glycerol, mannitol, formate, acetate, succinate, malate, alanine, and asparagine; good cultures are regularly obtained in 4 to 5 days. The development of purple bacteria in media with higher fatty acids and other hydroxy acids is considerably slower; such compounds as isocaproic and heptylic acids often do not yield satisfactory cultures at all. Subsequent experiments have shown that this is due to too high an initial concentration of the fatty acids; the use of these substrates in concentrations of 0.05% or less yields excellent cultures in less than a week.

Microscopic examination of the various enrichment cultures revealed the presence of large numbers of easily distinguishable *Thiorhodaceae*. *Thiocystis* and *Chromatium* species occurred regularly, *Thiosarcina* not infrequently. In not too old cultures these bacteria present themselves stuffed with sulfur globules; a definite indication of the presence, and hence of the formation, of hydrogen sulfide in the media. The explanation of this fact is obvious; the enrichment media used allow of the development of sulfate-reducing bacteria (*Vibrio desulfuricans* and related species, cf. Baars (25)) which, by the dehydrogenation of the organic substrates with sulfate as the ultimate hydrogen acceptor, produce the sulfide. Transfers of such cultures to the same media under anaerobic conditions, but incubated in the dark, where the development of the purple bacteria is completely suppressed, have served to verify this statement; in all cases flourishing cultures of sulfate-reducing bacteria were obtained.

Thus it appeared that the enrichment cultures contained sulfate-reducing bacteria and, as a consequence of their development, *Thiorhodaceae*, in addition to one or more types of non-sulfur purple and brown bacteria. That members of the last-mentioned group were present was demonstrated by making use of their strongly phototactic behavior (4, 9, 24), a property not exhibited by the sulfate-reducing bacteria. Although, as all previous investigators have noticed, the small individual cells of *Athiorhodaceae* do not appear colored when examined with the microscope, the pigmentation is unmistakable in aggregations. It may at first seem arbitrary to conclude that such colored accumulations are composed of non-sulfur purple bacteria, based as it is on the observation of red

or brown clumps of organisms exhibiting phototaxis, and not containing sulfur droplets. A confusion with certain members of the red sulfur bacteria would be quite possible since the small *Thiorhodaceae*, described in 1931 as the "pseudomonas-type" (3), would behave in the same manner. However, the actual isolation of typical non-sulfur purple bacteria from all such cultures, and of a size and shape which agrees remarkably well with that of the cells in the phototactically induced aggregates, tends to justify the above conclusion.

d. Evaluation and applications. These results paved the way for a more appropriate method of enriching *Athiorhodaceae*, in which the simultaneous development of sulfate-reducing organisms and, in consequence, of sulfur purple bacteria could be eliminated. Since the growth of *Vibrio desulfuricans* and its relatives depends upon the presence of sulfate, the substitution of NH_4Cl and $MgCl_2$ for the sulfates in the standard mineral medium served the purpose; sulfate reduction did not proceed in such media, and *Thiorhodaceae* did not appear. It must be remembered that the media prepared with this modified mineral solution are essentially sulfur-free; and it soon appeared that the *Athiorhodaceae* develop only scantily upon repeated transfers. But if, after two or three subcultures, transfers are made into sulfate-containing solutions, the new cultures generally fail to show development of sulfate reducing bacteria and of purple sulfur bacteria. The first few subcultures have thus served the purpose of so modifying the initial ratio of *Athiorhodaceae* to sulfate reducers and sulfur purple bacteria that only the first group of microörganisms comes to a full development.

Inspection of such enrichment cultures containing different organic substances clearly indicated the fundamental correctness of the premises which had led to their use. The macroscopical appearance often revealed characteristic differences of color and mode of development; and this was fully supported by careful microscopical examination. Media with fumarate, malate, citrate, and ethanol invariably contained an abundance of red-colored *Spirillum* species; the more complex media with peptone and yeast extract usually showed the largest variety of types, with a preponderance of brown representatives of the *Athiorhodaceae*. The rod-shaped organisms, reminiscent of Molisch's genera *Rhodobacillus* and *Rhodovibrio* were encountered in the cultures with ethanol and with the higher fatty acids. It thus became evident that more or less specific enrichment cultures for different types of the non-sulfur purple and brown bacteria would be practicable. The systematic investigation of the physiological characteristics of the large number of pure cultures of this group, to be discussed later, has supported the evidence here obtained, and has furnished additional information with the aid of which it has now become possible to develop strikingly specific culture media for the enrichment of its various members.

A peculiar behavior of the enrichment cultures discussed above should be noted at this place. Successive transfers of such cultures with a single organic compound to homologous media often showed a progressively poorer development of *Athiorhodaceae*, both in the total number of organisms and of types. It seemed that in successive transfers the growth of these organisms came to depend more and more on the simultaneous propagation of non-photosynthetic

bacteria. Also, the differences in flora between the cultures in various media, originally so pronounced, gradually tended to disappear. Only in the media with peptone and yeast extract did the *Athiorhodaceae* continue to flourish without diminution in numbers or types, and from such cultures they could be isolated without difficulty.

The reason for this strange behavior has become understandable as a result of the later studies with pure cultures. Suffice it to say here that all the representatives of the photosynthetic non-sulfur bacteria appear to require, in addition to an organic hydrogen donor, one or more accessory growth factors. Thus, the simple mineral media with a single organic substance are inadequate for the development of *Athiorhodaceae*. It is probable that the necessary growth factors are furnished in the crude cultures by the simultaneous growth of other types of bacteria, including the *Thiorhodaceae*. My own experiments have provided adequate experimental support for this contention. And the curious observations of Czurda and Maresch (20) on the influence of colonies of *Chromatium* or *Thiocystis* species on the development of neighboring cells of *Athiorhodaceae* can be most satisfactorily accounted for on this basis. Also, it is well to bear in mind that several of the media used by no means exclude the simultaneous growth of non-photosynthetic organisms under anaerobic conditions. The existing information on the fermentation of polyalcohols, such as glycerol and mannitol, of lactate, fumarate, malate, citrate, etc., by various obligatory and facultative anaerobes (see, for example, 26, 27, 28, 29) makes this obvious. In addition, it is now common knowledge that microöorganisms, capable of developing in a medium devoid of the typical growth factors, do synthesize these substances themselves, and often in amounts far in excess of their own "needs."

With this in mind it is a simple matter to prevent the regression in vigor of the enrichment cultures for the non-sulfur purple bacteria. All that is necessary is the addition to the simple culture solutions of small amounts of material rich in growth factors. Yeast extract has proved to be entirely satisfactory; liquid autolysate, prepared according to Orla-Jensen (30), is used in amounts of 1 to 5 ml per liter of medium, while 0.1 g of dehydrated yeast extract usually suffices. Such enriched media have yielded dependable and reproducible results.

e. Elimination of green algae. Occasionally the first inoculum contains large numbers of green flagellates, such as *Euglena* and *Chlamydomonas* species, which may cause difficulties in obtaining good cultures of the purple bacteria. This is particularly the case if, due to the composition of the medium, the development of the latter is slow; it has often been observed with media in which tartrate and malonate constitute the sole organic compounds. Due to the rapid production of oxygen by the green forms the conditions in such cultures soon cease to be anaerobic, and in consequence a varied flora of more or less common aerobic, non-photosynthetic bacteria makes its appearance. Transfers at different stages of the development of such cultures fail to eliminate the flagellates. This can, however, be readily achieved by making use of the elegant method, first employed by Gaffron (5) for the same purpose, of using short infra-red illumination (800–1000 mμ) as a source of radiant energy. Since Engelmann's original

observations (31) it has become firmly established that the purple bacteria, in contrast to the green plants, are capable of utilizing infra-red radiation for photosynthesis. (e.g., French (32, 33)). But in most cases the use of special light filters in order to prevent the growth of algae is superfluous because the purple bacteria develop so rapidly that they soon overgrow the green organisms. In one or two transfers, particularly if made from young cultures, the algae will in general have been diluted out.

f. Effect of pH. Apart from using different organic substrates for the enrichment of various representatives of the *Athiorhodaceae*, experiments have also been conducted in which the same solution, but adjusted to different pH values, was employed. Such cultures with an acetate medium at pH 5.6, 6.2, 6.6, 7.2, 7.4, 7.7, 8.0, and 8.6 have not only yielded a variety of pure cultures of non-sulfur purple bacteria, but also shown that differences in the reaction of the medium certainly affect various representatives of this group in different ways. An initial pH below 6, together with an acetate concentration of 0.2%, is not conducive to the growth of any one type, nor is a pH much above 8. But in an

TABLE 1

Growth of Rhodospirillum rubrum and of a brown member of the Athiorhodaceae in media containing 0.2% Na-acetate, 3 days after inoculation

ORGANISM \ pH	6.1	6.4	6.6	6.8	7.0	7.6
R. rubrum	3*	6	4	3	2	1
Brown bacterium	0	1	1	2	5	2

* The numbers indicate relative densities of organisms.

acetate medium, originally at pH 6.2, spirilla become abundant in a few days, while at pH 7 and higher brown bacteria tend to be the more numerous. This is quite in line with the outcome of experiments with pure cultures of a strain of *Rhodospirillum rubrum* and of a "brown bacterium" in acetate media at different initial pH, as shown in Table 1. However, the reaction of a medium in which *Athiorhodaceae* develop does not, as a rule, remain constant. And since the use of different substrates provides a more convenient as well as a more certain way for obtaining enrichment cultures of various types, extensive further experiments on the effect of pH on the microflora of enrichment cultures have not been carried out.

g. Enrichment cultures with pasteurized inocula. During the progress of the work on enrichment cultures, observations were occasionally made which suggested the presence of sporeforming bacteria in the media. Several subcultures from such media have been made after subjecting the original culture to pasteurization for 5 to 10 minutes at 60° to 80°C. In none of the transfers did purple bacteria ever develop.

Also inoculations of those sterile media which had proved to be among the

most satisfactory for the enrichment of purple bacteria with pasteurized samples of mud and surface water have invariably failed to yield photosynthetic bacteria. The same experience has been reported to the writer by Dr. J. W. Foster (unpublished). The evidence to date therefore indicates that thermoresistant stages (endospores) are not produced by any of the organisms of this group.

h. Distribution; inocula. The previous pages have shown the simplest and, in many respects, most satisfactory approach to the methods for securing crude cultures of *Athiorhodaceae.* In a later section it will be pointed out how this method can be used to obtain strains of a definite species. Here, however, some remarks are in order concerning the best material to use for inoculation of the enrichment culture media, in connection with the distribution of the organisms in nature.

All investigators who, starting with Molisch (4), have attempted to grow crude cultures of non-sulfur purple bacteria in the laboratory, have remarked on the wide-spread occurrence of the organisms. But contrary to the oft-reported mass occurrence of the *Thiorhodaceae* (see, e.g., 34, 35), the *Athiorhodaceae* have not been encountered in macroscopically visible aggregations in nature. Buder (9) observes in this respect:

"So leicht nun die Anzucht und Kultur von Athiorhodaceen ist und so verbreitete Bürger sie nach Molischs und eigenen Erfahrungen in unseren Gewässern sind, so ist mir doch weder aus der Literatur noch aus eigener Anschauung ein ähnlich *auffallendes* Vorkommen in der freien Natur bekannt geworden wie bei den roten Schwefelbakterien. Immer waren es Aufgüsse und ähnliche Herrichtungen, in denen ihre Entwicklung eine solche Üppigkeit erreichte, dass sie sich schon dem blossen Auge durch ihre Farbe verrieten" (p. 535–536).

This passage holds equally good today. The red bacteria, so often occurring in large numbers in brines and salterns, or on salted fish or hides, have occasionally been considered as purple bacteria. But they are non-photosynthetic, and do not contain the typical pigment system of the *Thio-* and *Athiorhodaceae.* They cannot, therefore, be regarded as purple bacteria.

Nevertheless, it is difficult to collect samples of mud or surface water in which, by proper enrichment methods, one cannot demonstrate the presence of the brown or red non-sulfur purple bacteria. They often occur in considerable numbers, as shown by inoculating adequate media with progressive dilutions of the material. It may well be that they often accompany the *Thiorhodaceae* in their natural habitat, except in sulfur springs, in such numbers that they might be macroscopically visible. But the large, sulfur-containing bacteria are so much more conspicuous upon microscopic investigation; they are observably colored even as single individuals, and can thus so readily be made responsible for the coloration of the sample, that it appears superfluous to search it for other, small pigmented forms which can betray their colored nature only when viewed in masses.

The materials which seem to harbor the richest flora of *Athiorhodaceae* are, generally speaking, muds in which considerable microbial decomposition is going on, or the overlaying water. I have only on rare occasions obtained successful cultures from sand and soil samples; apparently the natural environment of the

organisms is an aqueous one. Though it is possible that rich soils actually may contain large enough numbers to yield positive results by using 1 to 5 g quantities as inoculum, it seems to me highly doubtful that a variety of the non-sulfur purple bacteria such as is readily procurable from stagnant ponds will ever be found there.

Since much of the present work is concerned with the group of *Athiorhodaceae* as a whole, it has been my purpose to acquire a varied collection of strains and types. With the aid of different media and inocula the collection thus built up comprises well over 150 strains, and the study of their characteristics seems to justify the conclusion that among them most, if not all, of the previously described representatives are found. The use of appropriate enrichment methods has made it possible to isolate practically all the different types from a single source, but various strains have been obtained from widely divergent inocula. It is, of course, quite likely that new types will be found in the future, perhaps from unusual environments, or by the application of different methods, and it is hoped that the general approach here presented may prove of assistance in further studies.

II. Pure cultures

When satisfactory enrichment cultures are available it is a relatively simple matter to proceed to the isolation of pure cultures. This might be surmised from the fact that Esmarch, as early as 1887 (36), succeeded in isolating the first representative of the group in pure culture by routine bacteriological methods. Also Molisch's account (4) of the manner in which he achieved pure cultures of *Athiorhodaceae* leaves one with the impression that the difficulties involved are not excessive.

The most important aspect is the harmful effect of oxygen on many of the purple bacteria, stressed by Molisch in the following passage: "*Die meisten Purpurbakterien wachsen nicht in ausgegossenen Platten, weil der leicht zugängliche Sauerstoff ihre Entwicklung hemmt oder ganz verhindert*, ferner ist ihr Wachstum gewöhnlich relativ sehr langsam, weshalb die Kolonien in deutlicher Form erst nach längerer Zeit erscheinen, und ausserdem tritt bei manchen, namentlich wenn sie noch relativ viel Sauerstoff empfangen, die rote Farbe erst später auf." (4, p. 11).

Since the enrichment cultures as well as pure culture studies have shown that all the members of the group, without exception, are capable of development under strictly anaerobic conditions, provided they are properly illuminated, the most certain *general* method is that of anaerobic incubation in a light cabinet, of solid media inoculated with material from enrichment cultures.

It should here be observed that not every anaerobic culture method is equally effective. In the absence of air the purple bacteria depend, for their development, on their photosynthetic activities. This will make it clear why Czurda and Maresch (20) encountered difficulties when using agar plates stacked in containers which were made oxygen-free by mixtures of pyrogallic acid and potassium hydroxide. The carbon dioxide tension in such an environment is decidedly too low for ensuring active photosynthesis on the part of the organisms.

The most satisfactory procedure is that of shake-cultures, using successive dilutions, in an agar medium, the composition of which can be varied at will. If such cultures are made in culture tubes, and the agar column after solidification is covered with a sterile, melted mixture of equal parts of paraffin and paraffin oil, they can be kept without drying out, and without becoming "aerobic" for many weeks, even months.

Concerning the composition of the medium the following may be said. A "universal" medium, i.e., one which has proved satisfactory for all strains studied, is a dilute yeast extract agar, containing 3 to 10 ml liquid yeast autolysate per 100 ml. The addition of a small amount of Na_2S (about 0.01% $Na_2S \cdot 9H_2O$), and of Na_2CO_3 or $NaHCO_3$ (0.2%) is recommended to provide strictly anaerobic conditions from the start, as well as a sufficient supply of CO_2. Although development of most of the purple bacteria can take place over a fairly wide pH range, adjustment of the reaction to pH 7 with a few drops of sterile phosphoric acid solution is preferable; at this pH the sulfide in the concentration used exerts no inhibitory effect, and yet can rapidly eliminate the last traces of oxygen.

For certain purposes the use of an agar medium of approximately the same composition as that of the enrichment culture medium may be desired. If so, it must be realized that in some media the purple bacteria, in crude cultures, occur principally as a secondary flora. Primary decompositions of the substrate under the influence of non-photosynthetic microörganisms give rise to changes in composition of the medium, and it is often the decomposition products, notably the fatty acids, which lead to the subsequent growth of purple bacteria. In general, acetate and butyrate are excellent substrates, with lactate and malate running a close second.

However, the concentration of the fatty acids should not be too high (0.2% as a maximum in the form of their Na salts), nor the pH of the medium below 7. Media of this composition, due to the complete utilization of the organic anion, tend to become extremely alkaline, however, and such cultures are therefore not as lasting as the ones in yeast extract. Whenever such "synthetic" agar media are used the addition of small amounts of yeast autolysate (1 to 5 ml per liter) is necessary to provide the required growth factors.

As a practical measure the use of soft-glass rather than of Pyrex culture tubes is recommended for isolation purposes (shake cultures). They can be easily cut at the bottom, and the agar column then blown out into a sterile Petri dish. Numerous modifications are, of course, possible, but they do not affect the principle of the method in any way. By slicing the agar column the desired colonies can be laid free for examination and transfer. In case the colonies are of considerable size, it is often expedient to bend the agar column, as soon as a large colony extends beyond the cut end of the tube, against the wall. It will then usually split in such a way that the break passes exactly by the colony.

Even with the most homogeneous enrichment cultures as an inoculum, single colonies in the first series of shake cultures do not, as a rule, represent pure cultures. This is partly due to the fact that the purple bacteria, whenever they

develop in the proximity of the culture tube wall, tend to form a film along the side of the tube which is nearest the light. Hence, the entire outer surface of the agar column is frequently covered with a thin layer of organisms which may have developed from different cells of the inoculum. It is, however, a simple matter to prepare additional series of shake cultures from well isolated colonies. The mere dilution factor generally guarantees that tubes in a second series with not more than 10 to 20 colonies actually represent pure cultures. It goes without saying that a rigorous examination of all colonies in one tube must demonstrate the identity, at least in a morphological respect, of their contents before the conclusion may be reached that a pure culture has actually been achieved.

Another method for isolating pure cultures, sometimes useful in bringing out types which are present in the enrichment cultures in small numbers, consists in streaking on aerobic yeast agar plates. Naturally, only those purple bacteria capable of development under aerobic conditions will here grow into colonies. In such cases where the large majority of the *Athiorhodaceae* in a crude culture is represented by bacteria inhibited by oxygen, isolation of the potentially aerobic minority by means of shake cultures is well-nigh impossible, whereas the latter will be the only types developing aerobically. Often one can thus readily isolate "brown bacteria" from cultures which microscopically and by shake cultures appear to contain only red forms. If aerobic culture methods are used, it is immaterial whether incubation is in the light or in the dark.

The pure cultures are kept conveniently as stab cultures in yeast extract agar, and, when necessary, covered with paraffin seals. Many strains can be grown from the moment of isolation without this precaution; others can gradually become adapted to it. Incubation, at least for a few days, should be in the light, though after the cultures have developed sufficiently they may be kept in the shade or complete darkness. When kept in the light such cultures often remain viable for a long time; I have observed actively motile cells in cultures over 2 years old. But not all strains behave in this way; and if the cultures are to be used at definite times, it is best to make transfers about once every two or three months.

Aerobic representatives may, of course, be kept on ordinary slants and grown in the dark. However, it is my impression that under such conditions the photosynthetic ability of the organisms slowly weakens. I have had cultures of a number of strains both in stabs, exposed to continuous illumination, and on slants which were regularly kept in the dark except at times when they were transferred. In the course of 10 to 15 years the "dark cultures," though still capable of slow and scanty development under anaerobic conditions in the light, were decidedly less suitable for photosynthesis experiments than the corresponding stab cultures. By a process of selection one can succeed in gradually restoring the original vigor, but this takes time, and many transfers in media where growth depends upon photosynthetic activity are required to achieve it. So far, I have not observed a permanent loss of photosynthetic activity in cultures which have been grown in the dark for many years. Whether by careful selection subcultures might be isolated which have become non-photosynthetic remains a problem for the future.

4. GENERAL MORPHOLOGY OF THE NON-SULFUR PURPLE BACTERIA

"Würde man die Purpurbakterien auf Grund ihrer morphologischen Merkmale, die ja bei der systematischen Sonderung die erste Rolle spielen müssen, allein gruppieren, so würden sie sich über das ganze Bakteriensystem verteilen."

Molisch (4), p. 26.

I. Common morphological characteristics

The numerous strains of pure cultures which have been examined have certain morphological characteristics in common, apart from those which have already been enumerated in delimiting the group as a whole. Thus, all consist of motile bacteria in which the motility is caused by polar flagella; all are gram negative and none of them is capable of forming endospores.

a. Mobility. The designation of all strains as "motile" does not mean that all or most of the cells in a culture, even in a young one, exhibit motility. Although this is true for a majority of the strains when growing under favorable conditions, there are also those which upon a cursory examination would appear as immotile. These strains regularly give rise to cultures which are conspicuously mucilaginous; the developing cells produce a slimy sediment on the bottom of the culture vessel. Frequently the entire liquid is thus transformed into a highly viscous mass which, on being poured out of the container, appears to hang together in thick strands, much like ropy milk. The flow can be reversed by tilting the container at the proper angle, and what has previously been poured out will flow back into the flask or bottle. The vast majority of the individual cells in such cultures appears non-motile, and it is only on careful and prolonged examination that one can find an occasional bacterium which moves actively about.

No doubt the extensive slime production in such cultures must be held responsible for the scant signs of motility; the bacteria are glued together, as it were, in the massive strands which can be made to whirl up from the bottom into the supernatant liquid without losing their coherence. In these strands the individual bacteria are arranged more or less regularly, and this accounts in part for the high viscosity of the cultures. If the contents are vigorously shaken with a few glass beads, the strands can be broken up, and the organisms somewhat evenly dispersed. After this treatment the "ropy" property of the culture is lost completely. This behavior is strikingly reminiscent of the structural viscosity of ropy milk caused by "*Bacillus lactis viscosis*" Adametz (*Aerobacter aerogenes*), for which Kluyver (37) has shown the relationship between ropiness and structure of mucous strands.

Microscopic examination, especially of mounts in India ink, shows the arrangement of the purple bacteria in such strands, an arrangement which exactly fits Molisch's description of his *Rhodocystis gelatinosa* (4, p. 22): "Die einzelnen Zellen sind in dem Schleimhof nicht ganz wirr durcheinander, zwar auch nicht parallel, aber doch häufig vorherrschend nach einer Richtung gelagert . . . . "

Other strains of purple bacteria, both morpholigically and physiologically different from the previous type, may form slime capsules without, however, giving rise to the formation of typical strands. Evidently the individual cap-

sules do not merge to an appreciable extent, and the bacterial cells, single or in chains, remain separate, without orderly arrangement. As a result of the mucus production, however, cultures of these organisms, too, usually contain a very small percentage of motile cells.

Although Molisch has described a number of species of *Athiorhodaceae* as "immotile," it is noteworthy that all these are characterized by slime formation. Czurda and Maresch (20) have observed motility in one of their cultures of a capsulated member of this group, but state specifically that it is of a transient nature.

Flagella stains of such cultures in which the majority of the bacteria appears motile are simple and give convincing evidence of the polar mode of insertion. It is far more difficult to obtain satisfactory preparations from the mucus-producing types. The best results have so far been obtained by placing a clump of the organisms at one edge of a large drop of water which is kept in the dark, while the opposite edge is exposed to light. The few motile cells accumulate, by phototaxis, in this region of the drop, so that after a while one can here find a much more favorable ratio of motile to immotile cells, with which a flagella stain can be attempted. In successfully stained slides these types also show clearly the presence of a single polar flagellum.

b. Gram stain. A great many slides of the various strains have, in the course of time, been stained by the Gram method. Cultures from stabs, slants, and liquid media of different composition have been used. In not a single case has any evidence been found for the presence of gram positive cells.

Molisch (4) does not mention the behavior of purple bacteria towards the Gram stain. On the other hand, Czurda and Maresch (20) report all their strains as gram negative, stating: "Gram-positive Athiorhodobakterien oder Gram-positive Inhaltsstoffe wie sie Schneider vorlagen, haben wir noch nicht angetroffen" (p. 120).

Apparently Schneider (22) is the only one who has claimed the existence of gram positive purple bacteria. In his description of the morphological characteristics of *Rhodobacillus palustris* one finds the following passage: "Dagegen sind junge in Teilung begriffene Bakterien . . . . ausgesprochen grampositiv; ihr ganzer Körper färbt sich nach Gram blauschwarz" (p. 93).

The discrepancy is due to the fact that Schneider did not, as he believed, use a pure culture of *R. palustris*. Also in a number of physiological respects the behavior of Schneider's cultures was so considerably at variance with that which I had observed with a number of strains of purple bacteria, tentatively identified as *R. palustris*, that it seemed desirable to compare the various isolates. Upon request Dr. Schneider obligingly sent me subcultures of his strain; and various experiments showed me that with these cultures his observations could be reproduced. It appeared, however, that the original cultures contained three kinds of *Athiorhodaceae*, in addition to representatives of the lactic acid bacteria, aerobic and anaerobic sporeformers, and non-photosynthetic *Pseudomonas* species. The gram positive organisms observed by Schneider, and reproduced in his fig. 2, belong to the lactic acid and aerobic sporeforming bacteria; the

purple bacteria, upon isolation in pure culture, behaved as gram negative organisms.

c. Absence of endospores. In the section dealing with the enrichment cultures it has already been remarked that inoculation of appropriate media with pasteurized materials has never yielded cultures of purple bacteria. Nevertheless, a few of the strains give, in certain media, an appearance which suggests the presence of endospores. But if such cultures are subjected to any one of the accepted methods for the specific staining of endospores (38, 46), the results are invariably negative.

It is doubtful whether the spore-like bodies, described by Schneider (22) as occurring in his cultures of *R. palustris*, are at all connected with the life history of purple bacteria. As stated before, Schneider's cultures were found to contain bacteria belonging to the groups of both aerobic and anaerobic sporeformers, so that the actual occurrence of endospores in some of his cultures would be expected.

Continued observations on strains which exhibit the above-mentioned morphological peculiarity have made it clear that the highly refractile inclusions distinctly resemble spores only under special conditions, and in fact bear no relation to reproductive structures but consist of oil or fat. This was first suggested by the appearance of the bacteria in media in which the continued growth gives rise to somewhat abnormal cell shapes ("involution forms"). In such cultures the refractile bodies showed up more numerously, while at the same time their irregular shape and size argued strongly against an endospore nature. The recent studies, especially those of I. M. Lewis (39–42), have proved the frequent occurrence of oil and fat inclusions in microörganisms, and with the aid of Sudan Black B (43) it was not difficult to ascertain that also in the purple bacteria the refractile bodies, whenever they occur, consist principally of fat.

Also, reproductive structures other than spores have never been observed in the purple bacteria.

These morphological features of the *Athiorhodaceae*, *viz.*, the occurrence of polarly inserted flagella, the negative outcome of the gram stain, and the absence of spore-formation in all strains, show that this group of organisms forms a remarkably homogeneous entity in a morphological respect. Were it not for the presence of the characteristic pigment system these properties would justify the inclusion of all *Athiorhodaceae* in the family *Pseudomonadaceae* as defined by Kluyver and van Niel (44) and adopted in the 5th edition of Bergey's Manual of Determinative Bacteriology. Thus it appears that it is no longer necessary to subscribe to the views of Molisch, quoted at the head of this section. The Viennese botanist believed that only by placing due emphasis on the occurrence of the pigment system could a haphazard distribution of the purple bacteria throughout the bacterial system be avoided. Considerations of this nature show that some definite achievements in the difficult problem of bacterial taxonomy have been attained.

How the various aspects of the morphology and physiology of the *Athiorho-*

daceae can best be evaluated at the present time for the purpose of a more or less satisfactory classification of this group will be discussed in a later chapter.

II. Differences in the morphology of different strains

While in the previous pages the common morphological characteristics of the non-sulfur purple bacteria have been stressed, it is by no means true that all the strains of this group appear very much alike. In studying them by a variety of methods, it soon became possible to recognize a number of morphologically quite distinctive groups. These are chiefly distinguishable on the basis of the shape and size of individual cells, and by the color and general appearance of the cultures.

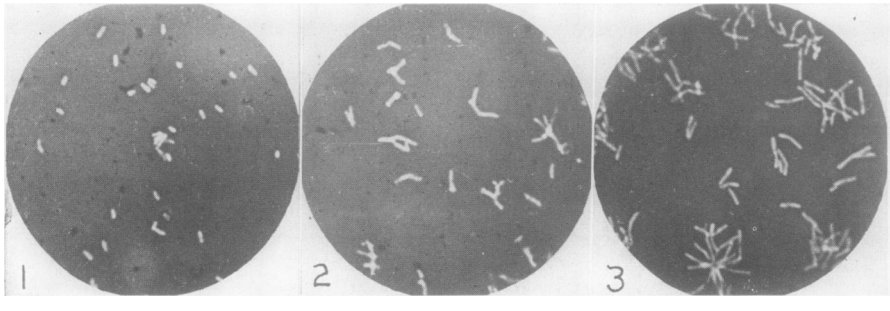


FIG. 1. *Rhodopseudomonas palustris*, Strain No. 52. Young (48 hr.). Culture in basal medium with 0.1% leucine; anaerobic; × 800.

FIG. 2. *Rhodopseudomonas palustris*, Strain No. 50. Culture in basal medium with 0.2% Na-crotonate; 7 days, anaerobic; × 800.

FIG. 3. *Rhodopseudomonas palustris*, Strain No. 18. Culture in basal medium with 0.2% glycerol; 10 days, anaerobic; × 800.

Considering the form of the cells alone, four types stand out clearly. They may be characterized as follows.

Type I. In young cultures the cells occur as small, short, slightly curved, and highly motile rods which show no tendency of becoming or remaining united in groups. As the cultures grow older, the rod-shaped cells become longer, frequently irregular, with bent or even branched forms conspicuously present. In this stage of the cultures the individual cells produce characteristic groups of somewhat star-shaped appearance. Such cultures present a close, though superficial, resemblance to *Corynebacterium* and *Mycobacterium* cultures (figs. 1, 2, 3).

Type II. Here also the individual bacteria in young cultures are small, short, and highly motile rods, but with an appearance of stretched cocci rather than vibrios. They exhibit a pronounced tendency to the formation of characteristic chains, resembling streptococci, or of irregular, long rods, the latter also frequently in strings of a zigzag arrangement (figs. 4, 5, 6).

Type III. While the microscopic aspect of young cultures is similar to that of Type II, the cells are still more spherical, and do not tend to the formation of chains. Rod-shaped structures are seldom encountered, and, when occasionally found, appear as typical "involution forms" (Figs. 7, 8).

Type IV. This is the most conspicuous morphological group, and comprises the strains in which the cells are spiral in shape (Figs. 9, 10).

Correlated with these differences in cell form are dissimilarities in the general appearance of the cultures. For example, the cultures of types I, III, and IV

are not, those of type II are usually mucilaginous. Also color differences exist which are consistent and characteristic for these groups.

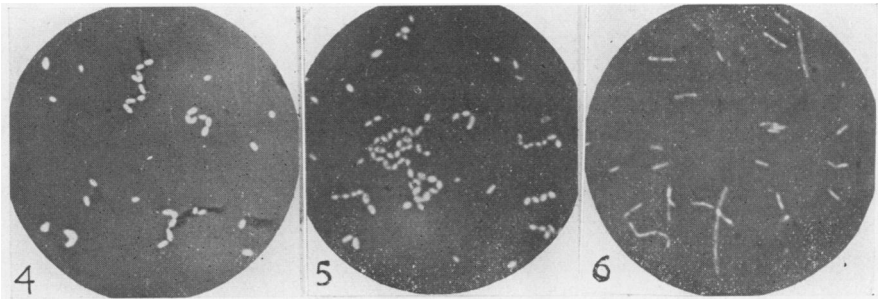


FIG. 4. *Rhodopseudomonas capsulatus*, Strain No. 42. Culture in basal medium with 0.2% Na-*iso*-butyrate; 3 days, anaerobic; × 800.
FIG. 5. *Rhodopseudomonas capsulatus*, Strain No. 32. Culture in basal medium with 0.2% Na-glutamate; 5 days, anaerobic; × 800.
FIG. 6. *Rhodopseudomonas capsulatus*, Strain No. 32. Culture in yeast extract, 0.5% phosphate, pH 7.0; 6 days, anaerobic; × 800.

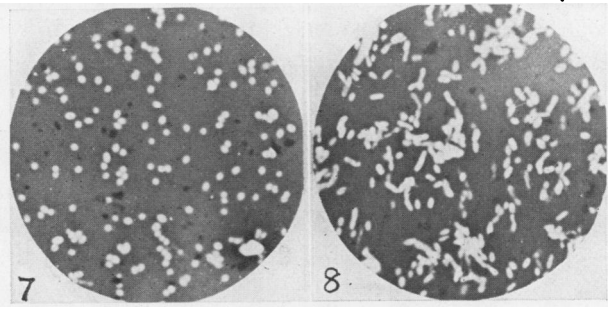


FIG. 7. *Rhodopseudomonas sphcroides*, Strain No. 36. Culture in basal medium with 0.2% ethanol; 5 days, anaerobic; × 800.
FIG. 8. *Rhodopseudomonas spheroides*, Strain No. 36. Culture in basal medium with 0.1% Na-*n*-valerate; 7 days, anaerobic; × 800.

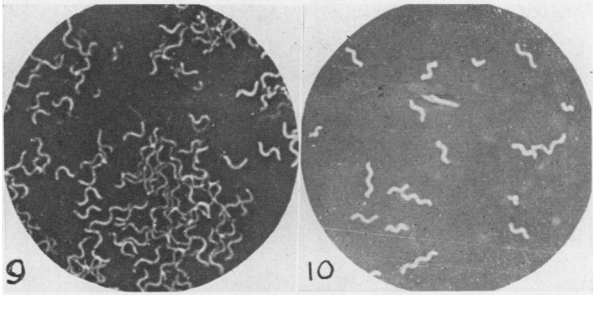


FIG. 9. *Rhodospirillum rubrum*, Strain No. 11. Culture in yeast extract, pH 7; 2 days, anaerobic; × 800.
FIG. 10. *Rhodospirillum rubrum*, Strain No. 4. Culture in yeast extract, pH 6.0; 4 days, anaerobic; × 800.

Two aspects of the coloration of the cultures must be distinguished: the pigmentation of the cell masses themselves, and the development of color in the

surrounding medium. It is often tacitly assumed that the purple bacteria are exclusively "chromophorous" in the sense of Beijerinck (47), i.e., that the pigment is intimately associated with the cell constituents, does not diffuse out of the cells, and fulfills a physiologically important function. Though in my experience this is true for the *Thiorhodaceae* which have so far been studied in pure culture, it does not hold rigorously for all the members of the group of non-sulfur purple bacteria. Both in liquid cultures and in agar stabs, one can readily observe that the development of certain strains is accompanied by a bluish-red discoloration of the medium. In sufficiently old liquid cultures, where the bacteria have settled on the bottom, it is not uncommon to find the initially colorless supernatant liquid colored a bright and perfectly transparent purplish-red. Stab cultures of the same strains show progressive diffusion of a similar coloring matter from the region of growth until, after some weeks of development, the entire medium is colored.

In the beginning I ascribed this phenomenon to a slow autolysis of the bacteria with the liberation into the environment of the protein-pigment complex first isolated and described by French (33, 45) as "photosynthin." The strains could, on this supposition, be segregated into *a.* those readily subject to autolysis, and *b.* stable ones. The possibility of the occurrence of autolysis is, especially in media which during development become strongly alkaline, far from remote, and autolysis had been demonstrated by Muller (7) in cultures of *Thiorhodaceae.* However, during the course of the investigations it became evident that the colored culture solutions cannot be explained on this basis; they are due to the production of a water-soluble pigment, very different from photosynthin, and about which more will be said later. Characteristic for these solutions is the absorption spectrum; it lacks the typical bacteriochlorophyll band at 590 mμ, but presents instead two new bands, not encountered with any of the known *Thiorhodaceae*, at 610 and 565 mμ. The strongest absorption by the solutions occurs around 535 mμ.

The excretion of this pigment occurs frequently in cultures of the morphological types I and II, whereas it has never been encountered in types III and IV. Its extent varies considerably, but seems in general related to the density of the cultures. Media which support extensive growth usually become deeper colored than those which give rise to only a faint development of the bacteria. It can, however, be detected wherever the organisms grow.

Striking also are the color differences of the cultures due to the pigmentation of the organisms themselves. But here a considerable range of shades may be observed, even with one and the same strain, depending upon the composition of the medium and the culture conditions. The extremes which I have observed with the different strains, and in a large variety of media, are a pale brownish-yellow, practically straw-colored mass of bacteria, and an intense, deep burgundy red. In between, a number of color types becomes distinguishable as more or less characteristic for certain strains. Special mention should be made of a group that normally develops with a sediment the shade of which can best be described as "peach-colored." The color is pale and very delicate; it is

reminiscent of the appearance of certain *Thiorhodaceae* in media containing sufficient sulfide, so that the individual cells are stuffed with sulfur globules, whereby the pigment system of the organisms is partially masked. Closely correlated with this type of pigmentation in the *Athiorhodaceae* is the mucilaginous nature of the growth, discussed before in connection with the motility.

On the basis of anaerobic cultures alone, two more groups of strains can be segregated. In cultures of the first group the growth appears red, varying with the medium from a deep brownish-red to a lighter shade with a faint bluish hue. The preponderance of red in these strains sets them clearly apart from that group which generally appears brown, with a dominant yellow. But this distinction is not an absolute one, as is shown by an examination of aerobic cultures of strains belonging to the latter type. If development occurs in the presence of air—and it should here be emphasized that this is not always the case; some of the strains appear to be strict anaerobes—it often presents a color which is very similar indeed to that of cultures of the first group.

While studying various representatives of the *Athiorhodaceae* Molisch (4) had noticed that not all cultures exhibit the same color. His spectroscopic investigations of extracts of the pigments led him to the conclusion that the

TABLE 2

Absorption maxima of various representatives of Athiorhodaceae in the visible region of the spectrum

	Absorption maxima, millimicrons				
Group 1	590	550		515	
Group 2	590		530		500
Group 3	590			520–510	

differences must be ascribed to the existence of two kinds of red pigment, which he designated as "bacteriopurpurin α" and "bacteriopurpurin β." The former was obtained from a pure culture of *Rhodobacillus palustris*, the latter from a *Rhodospirillum* species. The nature of these pigments will be discussed in more detail in a separate section later on; it may here be briefly stated that Molisch's contention of the existence of more than one "bacteriopurpurin" has been amply verified by more recent studies. It is now quite certain that more than two pigments of this type occur in the non-sulphur purple bacteria.

In general it can be stated that a spectroscopic examination of the intact, living cells of representatives of the group reveals three types. All three show an absorption band centering around 590 mμ which, as will be shown later, is due to the green component of the pigment system, the bacteriochlorophyll. The differences in the absorption spectra occur in the shorter wavelength region. The main characteristics of the three types appear from Table 2. The figures should be considered as approximations; a considerably greater degree of variation seems to exist. However, it is such an easy matter to observe these bands with a simple hand spectroscope that, for a general orientation, they have proved useful.

Those strains which are indubitably red all appear to contain the pigment responsible for the absorption at 550 mμ. It is of importance to note that the typical "brown bacteria" which give rise to red cultures when grown in the presence of air also show this most characteristic absorption band in the red phase, whereas anaerobic cultures of the same organisms belong spectroscopically to group 3. I wish to emphasize that the spectroscopic groups should not be confused with the four morphological types distinguished previously. The spirillum strains, for example, which have been designated as belonging morphologically to Type IV, can be separated into two groups on the basis of their spectroscopic characteristics (groups 1 and 2), while also in each of the other morphological types spectroscopic differences can be observed.

Although cultures of one strain in a variety of media may present different shades when viewed with the naked eye, the determination of the main absorption maxima shows that the color variation in such series should perhaps be ascribed to differences in the relative amounts of the various pigments, rather than to the occurrence of different pigments. The only exception to this generalization, already noted, is the production, under aerobic conditions, of a red pigment by some strains which do not appear to manufacture it in the absence of air.

III. Morphological variation in the non-sulfur purple bacteria

From the foregoing remarks concerning color variation in pure cultures exposed to different conditions, it follows that one cannot adequately characterize the color of a single strain by assigning to it a certain number from one of the existing color codes. Such a procedure would be entirely misleading unless at the same time the culture conditions were rigorously specified. The same holds true, and in some cases in a spectacularly exaggerated manner, for the morphological characteristics of any one strain. The reaction of the medium, the nature and concentration of the nutrient materials present, the culture conditions in general, exert a distinct influence upon the morphology of the organism. For this reason it is mostly impossible to interpret previous descriptions of representatives of the *Athiorhodaceae*, based largely on observations with impure cultures, in terms of identifiable species. Also here a rigid standardization or specification of environmental conditions would be required in order to furnish a brief characterization which could lay claim to accuracy and usefulness. An alternative solution consists of carrying out extensive observations on the relationship between morphology and environment, and incorporating the results in the description of the various morphological types.

Similar difficulties were encountered during the study of the *Thiorhodaceae* (3). They ultimately led me to postpone an attempt at a more satisfactory description and classification of this group until more extensive information was available. Such an attitude seemed sufficiently justified because the strains available for pure culture studies appeared to comprise only a very small number of morphologically distinctive groups, whereas collections from naturally occurring mass cultures often present a far greater abundance of morphologically recognizable

types. Of decisive importance in this respect was the fact that the most conspicuous of the species, described from observations on impure materials, had not yet been obtained in pure culture.

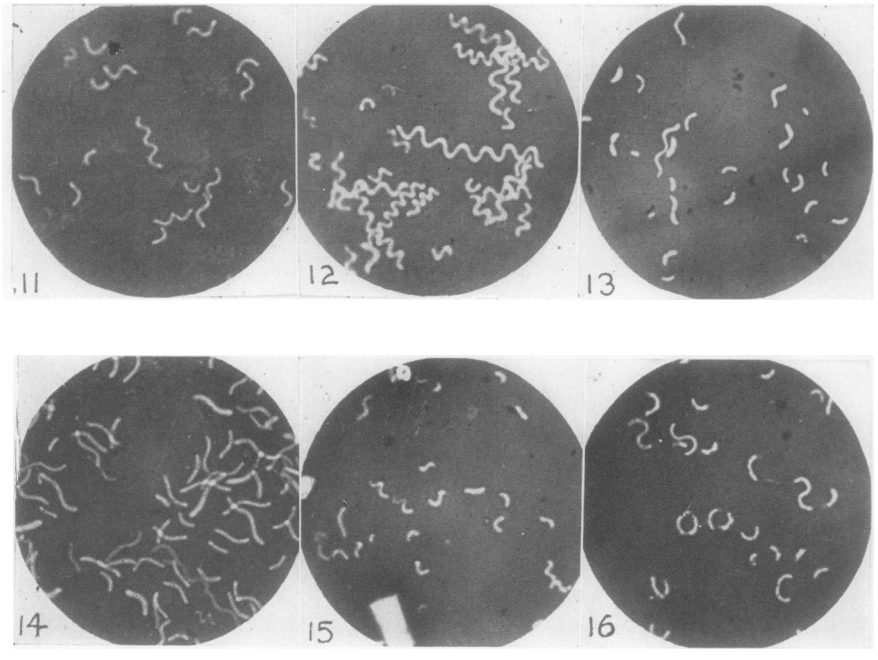
The situation in the case of the non-sulfur purple bacteria is, fortunately, quite different. Not only is the number of strains, available as pure cultures, vastly greater, but in addition it is my well-considered opinion that among these are represented all the types that have previously been more or less clearly recognized and described.

To this should be added yet another circumstance which argues strongly in favor of the contention that the general biology of the *Athiorhodaceae* is at present better understood than that of the purple sulfur bacteria. It has already been mentioned that by a judicious selection of enrichment methods it is now possible to obtain almost any one of the known representatives of the first group from a crude inoculum. Our current interpretation of the results of enrichment cultures leads to the conviction that the medium in which a certain microorganism or group of microörganisms gains predominance over the other types present in the inoculum, represents fairly accurately the conditions under which also in nature this particular group will be found in active development, in other words, corresponds to its "natural environment." The more restricted the specific microflora of an enrichment culture, and the simpler the composition of the medium, the closer will be the approximation. Hence the successful enrichment of the different members of the non-sulfur purple bacteria in media which do not support growth of other microörganisms makes one feel confident that the natural environment of these organisms has been closely approached. And this, in turn, implies that one can better evaluate the normal range of variation that each specific representative is likely to display in its natural habitat. On the other hand, since many types are found simultaneously in some of the more complex media, and their ultimate identification will have to be based on pure culture studies, it is equally necessary to pay close attention to their behavior in media which do not necessarily correspond in composition to the simplest and most specific ones.

Some of the more striking examples of morphological variability will be briefly indicated at this point; a more detailed discussion will be found with the description of the various species.

In the first place the spirilla. They are so easily recognizable, and so conspicuous that the generic diagnosis is, indeed, a simple matter. But when it comes to distinguishing or identifying species, the matter is considerably more complicated. In the relatively few studies on members of this genus (41, 48, 49), it has been customary to differentiate and describe species on the basis of the dimensions of the cells, and of the turns. The extent to which such a procedure will yield satisfactory results obviously depends upon the constancy of these characteristics. A glance at figures 11–16, illustrating the morphology of one single strain in a number of different media, will show at once how extraordinary and unexpected are the variations in this respect. Not only the length and width of the turns, and the number of turns of an individual (not necessarily a

single cell!), but also the size and shape of the bacterium itself may be so different for cultures of the same strain in various media that one would be tempted to assign the organism to a number of different species, depending upon the culture examined. And yet, it is not possible to designate any one of these morphological types as "normal," and the others as "abnormal." This is, perhaps, most adequately illustrated by the results experienced during the isolation of various strains from enrichment cultures.



FIGS. 11–16. *Rhodospirillum rubrum*, Strain No. 8; × 800; 7-day anaerobic cultures in basal medium with:

11	0.2% Na-acetate
12	0.2% Na-propionate
13	0.2% Na-*n*-butyrate
14	0.2% Na-fumarate
15	0.2% Na-malcate
16	0.2% Na-aspartate

In a variety of simple media, differing chiefly in pH or in the organic substance used, spirillum species were regularly encountered, and often constituted the majority of organisms present. The most interesting phenomenon was that the different media seemed to contain such strikingly divergent types. When pure culture isolations were attempted with the use of shake cultures in yeast agar the original expectations were, however, sadly shattered. To be sure, from all the enrichment cultures in which characteristic spirilla abounded, pure cultures of some spirillum were easily obtained. But the spectacular differences no longer appeared. The crude culture might have contained extremely large organisms, or beautifully and tightly wound corkscrews, or cells resembling

rings; the pure colonies, upon inspection, showed no observable difference from what has been known for half a century as "*Spirillum rubrum.*" Repeated transfers of the enrichment cultures to liquid media of the same composition, on the other hand, revealed that the spectacular forms persisted. And the later experiments with the isolated pure cultures of "*Spirillum rubrum*" provided the final and unambiguous answer to the problem why the unusual spirillum types were not found in the yeast-agar shake cultures; they are typical only for certain media and will reappear upon inoculation into the media in which they were first observed.

Equally characteristic, although not so spectacular changes in morphology have been observed with many of the other stains, in which the general morphology is not that of a spirillum. For example, the rather pronounced vibrio-shape of some strains can be observed clearly in some specific media only; in others these organisms grow as more or less long rods. (See figs. 1–3). Other strains, again, may occur as very short rods, resembling streptococci when they remain attached, or under different conditions, as long, irregular rods. (See figs. 4–6).

It will thus be clear that an adequate morphological description of the *Athiorhodaceae* must comprise a characterization of the organisms as they develop under a variety of environmental conditions. Naturally, the selection of such conditions as might be regarded more or less normal and significant is, in part at least, a matter of personal appreciation. In the following chapter a condensed review will be given of the general physiology of the group. From this it will become apparent what, in my opinion, should be considered as the fundamental aspects of the vital activities of the organisms. This, in turn, permits a decision as to the nature of the general habitat of the group and its representatives. Upon the criteria so developed can then be based an evaluation of the significance of the pattern presented by the morphology of the individual strains as influenced by environmental conditions.

5. GENERAL PHYSIOLOGY OF THE NON-SULFUR PURPLE BACTERIA

> "Da die Ernährungsphysiologie der isolierten Stämme noch nicht entsprechend geklärt ist, bleibt vorderhand unbekannt, welche der isolierten Stämme zusammengehören."
>
> Czurda and Maresch (20), p. 106.

I. The influence of light and of oxygen on the development of the group

In spite of the nutritional studies, carried out on this group of organisms during the past 35 years, a perusal of the literature pertaining to the subject leaves one with a feeling that the existing information is extremely fragmentary and confusing. A partial explanation for this situation is not difficult to find; the *Athiorhodaceae* comprise a relatively large group of bacteria, and the individual characteristics of various types show, in a number of respects, quite considerable differences. Even so, it should be possible to develop a more integrated and satisfactory picture of the general aspects on the basis of a clearer concept of the biochemical activities of the organisms.

A good deal of the confused state of the problem can be ascribed to the fact that for a long time the rôle of light and of oxygen in the physiology of the purple bacteria was not clearly understood. That a supply of radiant energy is not always essential in order to secure development was known to Molisch who wrote:

"Zwar ist bei Reinkulturen, soweit meine Untersuchungen reichen, das Licht nicht notwendig, denn es können manche Rhodobakterien namentlich in festen Nährböden auch im Finstern sehr gut wachsen, allein in flüssigen Nährmedien ist der Einfluss schon leicht erkennbar. Besonders auffallend macht sich die Einwirking des Lichtes im Wasser mit faulenden organischen Stoffen geltend, da ein reichliches Aufkommen oder das Auftreten der Purpurbakterien überhaupt an die Anwesenheit von Licht gebunden ist" (4, p. 72–73).

For many years thereafter the question whether light was "necessary" or merely "stimulating" was an ardently debated point. As late as 1933 Hama (50), in commenting on the matter, wrote:

"Bei meinen Versuchen wurde in der im dunklen Brutschrank bei 25°C. gehaltenen Kultur keine Entwicklung von Spirillen beobachtet. Aber natürlich sind für eine endgültige Lösung dieser Frage vollständigere Untersuchungen mit der Reinkultur erforderlich" (p. 143).

Also the problem of the effect of oxygen on the development of these purple bacteria has occupied a number of investigators, resulting in contradictory statements. Here again, the first definite pronouncements came from Molisch. Studying a variety of forms of *Athiorhodaceae*, he arrived at the conclusion that not all species react in the same manner. Some types behave as ordinary aerobic bacteria, whereas others appear to be strict anaerobes. A disregard of this divergent behavior of different strains on the part of later workers, and especially the frequent use of impure cultures, have added to the confusion.

As a more integrated concept of the fundamental physiology of the purple bacteria was developing, it became increasingly probable that there would exist a close connection between the influence of light and of oxygen on their development. The available evidence pointed strongly to a photosynthetic activity in illuminated cultures, but the failure of such cultures to produce oxygen in detectable quantities, even if Beijerinck's exceedingly sensitive "luminous bacteria method" (51) was used, indicated that the photosynthetic process was in some way different from that of the green plants. For a more detailed discussion of this problem the reader is referred to the recent reviews of the bacterial photosyntheses (8, 52, 53); suffice it to state here that these processes came to be considered as variants of a general photosynthetic reaction which can be expressed by the equation

$$CO_2 + 2H_2A \xrightarrow{\text{Light}} (CH_2O) + H_2O + 2A.$$

This equation implies an ability on the part of the organisms of synthesizing cell material (in the equation indicated as (CH_2O), a first, crude approximation) from carbon dioxide in the light, in the presence of an appropriate hydrogen donor, H_2A, the nature of which may differ for various organisms. Since oxygen will be evolved only in case the H_2A function is fulfilled by H_2O, it follows that in the presence of oxidizable substances, such as are required by the purple

bacteria for photosynthetic activity, an oxygen production will not take place in cultures of these organisms.

The non-sulfur purple bacteria seemed, according to previous investigations, to require organic substrates for growth. Supposing that these materials would play the role of H_2A in a photosynthetic reaction, it could be inferred that growth of the purple bacteria would be possible under strictly anaerobic conditions, since oxygen does not enter as a factor in the photosynthetic process. But the supply of radiant energy would be a prerequisite.

Numerous experiments have amply confirmed this. All purple bacteria are, indeed, capable of development in the complete absence of oxygen, provided the cultures are properly illuminated. On the other hand, none of the many strains of *Athiorhodaceae* has ever developed anaerobically in the "dark."

It is necessary to specify what is meant here by "dark." The photosynthetic pigment apparatus of the purple bacteria contains a bacteriochlorophyll which, though chemically closely related to green plant chlorophyll, has a distinctly different absorption spectrum. Most characteristic are the absorption maxima in the near infra-red region (800–900 mμ), and the absorbed infra-red radiation is photosynthetically functional (5, 31, 32, 33, 54). It is, consequently, possible to obtain growth of purple bacteria under anaerobic conditions if the cultures are exposed to a source of infra-red radiation in this region. From the point of view of the purple bacteria this is, however, equivalent to incubation in the light. When, therefore, in the following pages the designation "dark" is used it implies the absence of effective radiation, including the near infra-red region, rather than the visible region only.

With the demonstration that the supply of radiant energy is essential for development of the purple bacteria under anaerobic conditions, the situation has, nevertheless, been clarified only in part. For the question still remains to be answered whether any of these organisms can, as has been claimed by various investigators, develop in the dark. In view of what has been said above with respect to the effectiveness of infra-red radiation for photosynthesis, it might be assumed that positive results of non-illuminated cultures should be interpreted as resulting from the failure to eliminate this possible source of radiant energy.

Although it will not be denied that in some cases this factor may have been responsible for the observation of development, it is by no means true that growth in the dark is always caused by short-wave length, infra-red rays. The explanation is again furnished by an examination of the general equation for photosynthesis. This can be paraphrased by stating that it represents a reaction in which an oxidizable substance, H_2A, is dehydrogenated with carbon dioxide acting as the ultimate hydrogen acceptor. In this form the reaction differs from an ordinary oxidation only in the type of acceptor used. It might, therefore, be expected that those purple bacteria which are not adversely affected by oxygen should be able to carry out the dehydrogenation of H_2A also with oxygen as the final acceptor. And, because such oxidations are exergonic,[1] a supply of radiant energy would not be required for the process, in contrast to what holds for the reduction of carbon dioxide with the same substrate. Furthermore, since H_2A is present in the form of an organic substance, and so many oxidative

[1] "Exergonic" is used in preference to exothermic; see Coryell (55).

(aerobic) microörganisms are capable of development as a result of such oxidation reactions, it would be entirely in keeping with our current knowledge if the non-sulfur purple bacteria were to show the same behavior. Theoretically, then, the development of those representatives of this group which can live in the presence of air should also be possible in the dark, but under aerobic conditions.

This, again, has been amply confirmed by numerous culture experiments. The most convincing are those which demonstrate that one and the same strain, inoculated into one and the same medium, grows in darkness only if the conditions are aerobic, but not in the absence of oxygen. Such results effectively rule out the possibility that infra-red irradiation would have made development possible, for if this were the case, then growth would also have taken place in the anaerobic cultures.

The situation can therefore be summarized as follows: All members of the non-sulfur purple bacteria can grow under strictly anaerobic conditions, but only when properly illuminated. Some representatives can also develop in darkness; this is, however, possible only under aerobic conditions, and hence becomes a property restricted to representatives which are not strict anaerobes.

In the course of the studies on the *Athiorhodaceae* several observations have been made which demonstrate that the behavior of individual strains toward oxygen does not always constitute a fixed property. Especially cultures of purple spirilla show a remarkable degree of variability in this respect. It has frequently been found that a recently isolated culture of an organism, bearing a close resemblance to the well known *Spirillum rubrum* Esmarch, behaved like an obligatory anaerobe. There are, however, a number of more or less authentic strains of Esmarch's organisms which have been successfully maintained as pure cultures on slants, i.e., under aerobic conditions. The latter can consequently be grown in the dark, while the new isolates, in agreement with the previous remarks, must be cultured anaerobically, and thus develop only in the light. Stab cultures of these strains in yeast agar give rise to growth only in the lower part of the agar column, the upper part (± 2 cm) remaining devoid of growth unless the medium is covered with a seal immediately after inoculation. It may even occur that a stab culture does not develop at all unless it is sealed. After a number of transfers, particularly if made in fairly rapid succession, and using a heavy inoculum, such strains appear to become less and less adversely affected by oxygen, and ultimately transfers to aerobic slants become possible. Admittedly, the organisms behave as "micro-aerophils," but this is true also of the typical *Spirillum rubrum*. Similar experiences with initially strictly anaerobic bacteria which could gradually become adapted to live in the presence of air have been reported by Prévot in his studies on anaerobic streptococci (56).

Transitions of this kind have been observed with many other strains of non-sulfur purple bacteria. Nevertheless, it must not be inferred that it will invariably be possible to bring about such adaptations by experimental means. There are still a few strains in my collection which must be treated as obligatory anaerobes. And these, in consequence, cannot yet be grown in the dark.

The observations of Molisch concerning the effect of light on cultures of

Athiorhodaceae, quoted earlier in this chapter, can now readily be explained. Those cultures in which plant and animal remains are undergoing decomposition under a deep layer of liquid must, of course, be considered as strictly anaerobic with the exception of a very shallow surface film. Since, in such experiments, the latter is always occupied by a dense growth of aerobic microörganisms, the purple bacteria are unable to make their appearance unless the cultures are exposed to light where anaerobic development of the brown and red organisms becomes possible.

More involved is an adequate interpretation of the results obtained by Schneider (22). As has been mentioned earlier, his experiments were conducted with impure cultures, containing a variety of non-photosynthetic bacteria in addition to members of the *Athiorhodaceae*. Hence the composition of the medium and the conditions of incubation must have played a decisive rôle in determining which of the various microbes present in the inoculum would develop. A more detailed analysis of his observations will be taken up in the next section. Also the effect of light and air on the pigment production by the purple bacteria will be treated later.

II. The nutrient requirements of the non-sulfur purple and brown bacteria

a. General considerations. It need not be argued that an exact study of the nutrient requirements of any microörganism can be carried out only with pure cultures. On the other hand, it is frequently possible to derive important inferences from observations with mixed cultures which may give clues concerning the problem of nutrition, and which it would be difficult to reach otherwise, except perhaps on the basis of a systematic investigation of such a scope that it requires more than ordinary facilities and courage to carry to completion.

Such a situation is illustrated with remarkable clarity by our present knowledge of the nutritional physiology of the non-sulfur purple bacteria. When Molisch achieved pure cultures of a number of representatives of this group (4), he carried out some experiments with a view to finding a medium in which they could be grown satisfactorily. Although the number of different media employed was relatively large, their composition cannot be said to have covered a particularly wide range. Nor is it evident that in planning these media Molisch was guided by considerations of the conditions prevailing in the crude cultures where purple bacteria abounded.

Summarizing the results of experiments with two different pure cultures in media prepared from river- or sea-water by the addition of sucrose, dextrin, inulin, asparagine, glycerol, ammonium tartrate, or peptone, or simple mixtures of these compounds, Molisch concluded:

"Keine Entwicklung oder nur eine minimale findet statt in den Gefässen, die im Moldauwasser Rohrzucker, Dextrin, Asparagin, Glyzerin, weinsaures Ammoniak, Pepton oder Gemische von Asparagin mit Dextrin, Asparagin mit Glyzerin und Dextrin mit Inulin enthalten.

Hingegen zeigte sich eine reichliche Entwicklung in absteigender Reihe in den Gemischen von Pepton-Glyzerin, Pepton-Dextrin und Pepton-Inulin. Nirgends war die Vermehrung so üppig wie bei Pepton-Glyzerin, es erwies sich daher unter sämtlichen in der Tabelle

angeführten Nährlösungen diejenige, welche aus Moldauwasser mit 1 Proz. Pepton und ½ bis 1 Proz. Glyzerin bestand, unter meinen Versuchsbedingungen als die beste Nährlösung" (4, p. 66).

These pioneer investigations have been of decisive influence on the later studies of the nutritional physiology of the *Athiorhodaceae*. The "peptone-glycerin medium" of Molisch has been used by all subsequent workers, though occasionally it was modified to some extent. As late as 1937, Nakamura (57, 58) used the old formula; and Czurda and Maresch (20), the only investigators who have recently attempted to study the nutrient requirements of the non-sulfur purple bacteria in more detail, make the claim:

"Für eine erfolgreiche Fortzüchtung musste der Nährboden unbedingt 1% Pepton enthalten, eine Feststellung, die sich Mit der von Molisch, Schneider und Muller deckt. *Phaeomonas Nr. 23* bildet insofern eine Ausnahme, als es, wie noch ausgeführt werden wird, nur auf Mineralsalznährböden gedeiht. Ein Zusatz von 1% Glyzerin oder 1% Glucose hat sich bei den Kulturen unter vermindertem O_2-Druck zwar als zweckmässig erwiesen, jedoch keine auffallende Steigerung der Vermehrungsintensität oder des Ertrages herbeigeführt" (p. 107).

Obviously the complexity of such media, which may serve an excellent purpose in maintaining pure cultures, or in growing large quantities of the organisms, does not lend itself to a ready interpretation of the nutritional physiology of the organisms. Further remarks in the publication of Czurda and Maresch certainly do not ameliorate the situation. Some of their experiments, particularly with media containing peptone or yeast extract to which the sodium salts of various organic acids had been added, yielded results which apparently were not in agreement with those obtained by Gaffron (1, 5). Since the last-mentioned investigator had not used pure cultures, Czurda and Maresch, evidently unaware of the fundamental significance of Gaffron's experiments for which pure cultures were not required, concluded:

"Diese Festellungen legen neuerdings die Vermutung nahe, dass die Ergebnisse von Versuchen mit unreinen Zellgemischen durch die Mitwirkung der anderen Bakterien getrübt werden" (20, p. 108). Indeed, the impression gained from the existing literature leads to the conviction that the published reports on the nutrient requirements of the Athiorhodaceae are inadequate to lay a satisfactory foundation for a better understanding of the problem, a conviction which is emphatically supported by the following statement: "Es hat sich später gezeigt, dass das Problem der Kultur der *Athiorhodobakterien* nicht mit Hilfe einer bestimmten Nährstoffmischung gelöst werden kann" (20, p. 108).

So pessimistic an outlook is hardly justified by the facts. On the one hand it is a simple matter, as has been pointed out before, to grow the members of this group in complex media, of which I have found yeast autolysate the most satisfactory. On the other hand, a consideration of the conditions under which these organisms develop in crude cultures helps greatly in formulating a working hypothesis concerning their more exact nutrient requirements.

In the course of some early experiments with non-sulfur purple bacteria I had observed that in sugar-containing media these organisms appeared to develop much better if lactic acid bacteria were simultaneously present (3, p. 102).

Subsequently it was established that salts of various organic acids can be used readily by members of the *Thiorhodaceae*. Muller (7) then succeeded in demonstrating that this utilization is due to the occurrence of a photosynthetic process in which the hydrogen-donor function (H_2A in the equation previously given) is fulfilled by the organic substance. In the meantime Gaffron, using the manometric technique, had shown that *Athiorhodaceae* can carry out a photosynthetic reaction in the presence of various fatty acids. ((1); Gaffron did not publish his results until 1933, but he had already presented them at a meeting of the A. A. A. S. in Pasadena in 1931.) It was thus evident that, at least in the presence of organic substances, the fundamental metabolic activities of the two groups of purple bacteria are very similar.

Let us now turn our attention to the processes occurring in a satisfactory enrichment culture for these organisms, prepared according to the methods of Molisch, Buder, and others. At the outset, the organic matter is deposited on the bottom of the container, there to undergo a gradual decomposition. Except for the surface layer, the conditions in the culture vessel will very soon be anaerobic. We may, therefore, confine ourselves to a consideration of the anaerobic degradation of the substrate. The numerous experiments on such decomposition processes of complex organic materials leave no doubt as to the nature of the chief decomposition products. One may safely claim that they will consist of (*a*) gases, mainly carbon dioxide, hydrogen, and methane; (*b*) dissolved inorganic substances, among which hydrogen sulfide and ammonia are predominant; and (*c*) organic compounds, mostly belonging to the groups of simple alcohols, fatty acids, hydroxy- and dibasic acids, and amines. It is, of course, quite possible that also other groups of substances, including more complex ones, may temporarily accumulate to some extent. But their presence will be transitory.

Are the purple bacteria involved in these primary decomposition processes? At first the answer to this question would seem to be undeniably affirmative for the simple reason that they always develop in such cultures. But on second thought this becomes very doubtful. There are three important observations that clearly do not conform with this view. The first one is concerned with the time at which the purple bacteria appear in macroscopically visible numbers. In the experiments of Molisch, Buder, and others, in short, before continuous illumination was used for securing enrichment cultures of purple bacteria, these organisms usually were found, in sufficient numbers to color the liquid, only after one to two weeks. Now it is a well-known fact that the decomposition of the plant and animal remains sets in considerably earlier, and in the course of a few days has already reached an appreciable extent. Hence the fact that the purple bacteria are not immediately found indicates strongly that they are not concerned in the primary decomposition of the complex materials. This is supported by the distributions of the red organisms in the vessels. They do not occur in close contact with the organic matter originally introduced, but in the liquid above, sometimes coloring the entire contents of the container up to the surface. In the early stages one frequently finds them as delicate clouds,

several centimeters above the deposit on the bottom. Since most of the recommended materials for securing enrichment cultures of purple bacteria are insoluble in water, this observation leads directly to the conclusion that these organisms develop by utilizing substances in solution, rather than by decomposing the initial substrate. And, thirdly, microscopic examination of such cultures shows that representatives of the *Thiorhodaceae* generally develop side by side with members of the *Athiorhodaceae*. The appearance of the former, stuffed with sulfur globules, shows convincingly that they, at least, are growing at the expense of hydrogen sulfide. For this group, then, it must be admitted that its development constitutes a secondary flora and that its appearance is made possible by the occurrence of decomposition processes which give rise to the formation of hydrogen sulfide, a thesis which was eloquently defended by Winogradsky (10) as early as 1887!

The above considerations make it seem inevitable to deduce that also the non-sulfur purple bacteria occur in the enrichment cultures as a secondary flora, depending for their growth on the production of simple breakdown products formed by a primary, and varied, microflora from the raw material used. In this connection the experiments of Gaffron, demonstrating the rapid utilization of a large number of fatty acids by *Athiorhodaceae*, gain an added significance because this class of substances would undoubtedly constitute an important fraction of the primary decomposition products. In particular must this be true in decomposing oils and fats where Seliber (65) and Pigulewski and Charik (66) had found purple bacteria growing in profusion. The development of the non-sulfur purple bacteria in the enrichment cultures could thus be ascribed to the gradual accumulation of various primary decomposition products, simple in nature, which would be utilized extremely efficiently by the photosynthetic mechanism previously described. It should then be possible to culture these organisms in media of a very simple composition, analogous to those used for growing the purple sulfur bacteria, but containing organic substances instead of sulfide.

Various attempts have been made in the course of the past several years to verify this. Although it has thus far been impossible to secure satisfactory development with any one of the numerous strains except in the presence of complex substances, such as peptone or yeast extract, this does not imply that the large quantities of these substances, used since Molisch's studies, are required. Under proper conditions surprisingly small amounts suffice.

Prior to 1935 it was generally believed that a bacterium, unable to develop in a medium containing an ammonium salt as the only nitrogen source, but which would grow profusely in media with peptone or meat extract, thereby demonstrated that the organism could not synthesize the various amino acids which are part of its protein structures. Since the beautiful investigations of Knight *et al.*, the Lwoffs, W. H. Peterson and co-workers, and many others, it has become clear that this is not necessarily the case. The ability of even such fastidious organisms as the propionic and lactic acid bacteria to utilize ammonia nitrogen to a large extent for synthesizing many of their proteinaceous components from this simple nitrogen source has been firmly established. This, together with the demonstration that the special growth factors required by such organisms are often identifiable with a

particular amino acid or with the prosthetic groups of various enzymes has given rise to the idea that, in microbes as in the metazoa, special growth factors or vitamins fulfill the same function, *i.e.* that of supplying essential constituents which the organism in question cannot itself manufacture from ammonium salts in the presence of an otherwise suitable substrate. If this special constituent contains nitrogen, it may still be maintained that the organism requires "complex nitrogen compounds"; but the meaning of this statement has lately undergone a considerable change.

These recent developments in our understanding of the complex nutrient requirements of microörganisms make it readily conceivable that yeast extract and similar materials contribute one or more growth factors which the *Athiorhodaceae* are unable to synthesize themselves. It is easy to understand that such compounds would regularly be present in the natural environment of the organisms, which always live together with a host of non-photosynthetic bacteria of different types and nutrient requirements. Hence, even if the raw material undergoing decomposition through the activities of the primary flora does not contain the specific factors required by the purple bacteria, it is safe to assume that the accompanying microflora will synthesize them. It is, therefore, possible that the *Athiorhodaceae*, limited in their natural distribution to localities where growth factors always occur, have lost the property to synthesize one or more of them.

A comparison with the nutrition of the *Thiorhodaceae* is of interest in this connection. The few members of that group which have so far been studied in pure culture do not appear to require any such growth factors. But it must also be remembered that the natural habitat of these bacteria is not restricted to muds and similar places where anaerobic decomposition processes go on, liberating the hydrogen sulfide necessary for growth of the sulfur bacteria. They also occur in sulfur spring waters, and in this environment they are not likely to encounter a supply of organic growth factors. These ecological reflections are, of course, adapted from the ideas so ingeniously propounded by Lwoff (60, 61), as early as 1936, in connection with the parasitic nature of certain bacteria.

The reason for this excursion into the realm of growth factors is that the available evidence rather strongly supports the belief that the yeast extract, etc., found to be necessary in order to secure development of the *Athiorhodaceae*, serves mainly as a source of one or more such substances. Firstly, it has been demonstrated that the quantity of yeast extract needed for full development is very small indeed. Furthermore, several experiments have proved that with low concentrations of yeast extract a maximum development is shown only by cultures in media which contain ammonia-nitrogen as well. These will be discussed presently; let us first return to the problem of the general nutritional requirements.

b. Carbon requirements. In media prepared with the standard inorganic salts, including a sufficient supply of carbon dioxide in the form of sodium bicarbonate, and varying quantities of yeast extract, the development of the non-sulfur purple bacteria is, as would be expected, very nearly proportional to the amount of yeast extract as long as this is used in low concentration. If the complex organic substrate is not in excess of 1 ml liquid yeast autolysate per liter of inorganic medium, the growth is practically negligible. But if to such a culture solution any one of a number of simple organic substances is added, the *Athiorhodaceae* develop profusely. This, then, demonstrates conclusively that such substances can be and are used by these organisms as the main nutrients.

Thousands of cultures of this kind have been set up and examined during the past several years. The results have made possible an interpretation which is

in full agreement with the previous theoretical deductions. Furthermore, it has become clear why different investigators, using different methods for the study of physiological problems of the non-sulfur purple and brown bacteria, have reported such widely divergent conclusions.

In general, it can be stated that the various strains belonging to this group can utilize a considerable number of simple organic compounds. In complete agreement with Gaffron's manometric experiments with cell suspensions, it has been demonstrated that fatty acids represent excellent substrates for growth of the organisms. This, however, appears conclusively only from such experiments in which the culture medium does not contain a large enough amount of peptone or yeast extract to result in dense growth in the absence of a fatty acid salt.

Under such conditions the growth is rigorously proportional to the amount of organic acid present in the medium, as long as the latter is added in small amounts. Table 3 summarizes the results of one representative experiment in which a mineral medium, containing 1 ml per liter of liquid yeast autolysate was used with increasing amounts of sodium acetate. These data show not only the strict proportionality between available acetate and cell yield, but they permit

TABLE 3

*Cell yield of purple bacteria grown in the presence of sodium acetate**

ACETATE CONC. (PER CENT)	0	0.05	0.10	0.20	0.30
μl cells per 10 ml	<0.5	9	18	35	56

* Medium contained 2 ml per liter yeast autolysate; see below.

some further computations which reveal important features of the growth of purple bacteria in such media.

The cell yield was determined by centrifugation for 1 hour; the cells were tightly packed. The dry weight of the sediment is approximately 20%, and from previous chemical determinations it is known that the dry cell material contains 55–56% C and 11–12% N (16). Disregarding the very small amount of organic material contributed by the 0.1% yeast autolysate, which itself does not allow measurable growth in the absence of acetate, the figures of table 3 can then be converted so as to show how much of the carbon, available as acetate, has been converted into cell material (table 4). From the fact that these approximate calculations show that the conversion of acetate-carbon into cell material proceeds with an efficiency of about 70% we must now also conclude that the acetate is used in growing cultures of *Athiorhodaceae* in much the same manner as in anaerobic, illuminated cultures of *Thiorhodaceae*. For the latter organisms, Muller (7) determined by direct chemical measurements a conversion of 63–82% of acetate-carbon into cell substance. The efficiency is, furthermore, of the same order of magnitude as that observed by Gaffron with cell suspensions under conditions where growth was excluded. Such determinations (1, 5, 8) have demonstrated that around 80% of the acetate-carbon used was converted into assimilatory products.

This magnitude of the efficiency is, of course, possible only on account of the photosynthetic nature of the metabolic process. Considering that the primary assimilatory product must subsequently be converted into the multitude of cell constituents, it is understandable that during the secondary conversions losses, mainly if not exclusively in the form of carbon dioxide, occur.

If one continues to increase the acetate concentration above 0.3% the growth of purple bacteria soon ceases to increase in direct proportion to the amount of substrate. It seems that this is due to the fact that a maximum population is reached which cannot, under ordinary circumstances, be surpassed. Here again, a surprisingly close agreement is revealed between Gaffron's experimental results and my own. Using yeast extract media, corresponding in my experience to about 10% by volume of yeast autolysate, enriched with sodium citrate and potassium butyrate, and an inoculum of an impure culture of purple bacteria, Gaffron reports a yield of about 1 gram dry bacteria per liter (1). The same amounts of cell material were obtained with pure cultures of *Spirillum rubrum* in yeast extract-glycerin media (62), and, later, with various strains of *Athiorhodaceae* in a mineral medium with yeast autolysate and sodium malate (unpubl.).

TABLE 4

Relationship of carbon in cell material and in medium

CONC. OF $NaC_2H_3O_2$, PER CENT	0.05	0.10	0.20	0.30
Cell material from 10 ml				
a. mg dry weight	1.8	3.6	7.0	11.2
b. mg carbon	1.0	2.0	3.9	6.25
Acetate available in 10 ml as mg carbon	1.44	2.9	5.8	8.7
Per cent conversion	70	69	67	72

From these data one may infer that the yield of 56 μl of cells per 10 ml of medium, obtained in the above-discussed experiments with 0.3% acetate, approaches quite closely the maximum yields obtained with what at first sight would seem to be vastly more satisfactory media.

These results also explain why Czurda and Maresch (20) failed to observe better growth of purple bacteria upon the addition of fatty acids to their medium, since the organisms can develop to the maximum extent in ordinary yeast extract or 1% peptone media without any further additions. Hence it is clear that experiments in which such concentrated solutions of complex materials are used cannot be expected to yield information concerning the nutritional value of added substances. It still remains possible that even in the experiments of Czurda and Maresch the fatty acids were utilized by the bacteria, leaving more of the peptone or yeast extract untouched. But this could have been decided only on the basis of chemical determinations at the end of the incubation period.

What has so far been remarked concerning the growth of the *Athiorhodaceae* in the presence of acetate can be applied, with a few provisions, to development with other fatty acids as well. However, two factors must be borne in mind in any attempt to extrapolate the results of the acetate cultures. The first is that

the higher fatty acids are toxic in relatively low concentrations. Since the toxicity is primarily due to undissociated acid molecules, their inhibitory effect is enhanced in acid media (See also (5, 16)). Whereas concentrations of sodium acetate of 0.3–0.5% can be tolerated by the bacteria in a neutral medium, a considerably lower concentration of the higher fatty acids must be used in order to insure growth. For some strains even 0.05% of the valeric acids and higher members of the series does not permit growth; other strains can tolerate these substances up to 0.1%.

This toxic effect of the higher fatty acids must be held responsible for some anomalies in Nakamura's publication (57). From his experiments on photosynthesis by *Rhodobacillus palustris* in the presence of various fatty acids it would appear that *n*-caproic acid and the higher homologs cannot be utilized. The same results were obtained in respiration experiments with the Thunberg technique. In the former series the final substrate concentration in the suspension was M/120; in the latter it appears to have been as high as M/40. Gaffron, using final concentrations of M/400, had found that substrates which were ineffective in Nakamura's experiments, were readily metabolized (1).

The above-mentioned results were secured in experiments with dense suspensions of non-proliferating bacteria. But also in culture experiments, it has been established that growth of many, if not all, members of the *Athiorhodaceae* is possible in media with heptylic, caprylic, and pelargonic acids, provided these substances are used in concentrations of not over 0.03% (M/500–M/1000). Higher members of the fatty acid series were not investigated because of their relative insolubility. From Gaffron's results one might expect growth to occur also in their presence, at least up to stearic acid.

A priori one might expect that the toxicity of a fatty acid can be represented as a simple function of its concentration and the pH of the medium. Some culture experiments, designed to establish this relationship for a number of fatty acids on a quantitative basis have been conducted. But the results cannot yet lay claim to the required accuracy for making them significant. This is in part due to the fact that, as soon as metabolism starts, the alkalinity of the culture solution increases as a result of the simultaneous assimilation of carbon dioxide and the practically complete conversion of the anion or acid molecule into cell substance with the cations remaining in solution. Thus a rise of one pH unit, even in media containing 0.3% (M/30) phosphate buffer, is of common occurrence if the initial pH is below 6.8. Added to this is the difficulty that media on the acid side of neutrality in glass-stoppered bottles tend to lose carbon dioxide. These difficulties can, of course, be overcome by such devices as constant equilibration with gas mixtures containing the requisite amount of carbon dioxide; with such refined methods the experiments have, however, not yet been carried out. The preliminary results obtained so far corroborate the expectation that the lower the pH, the lower is also the total fatty acid concentration which will still permit development. That this is not due to a direct pH effect on the organisms was clearly demonstrated by the inclusion of cultures with utilizable substrates of a non-acidic nature. Such cultures have shown that many of the

non-sulfur purple bacteria can grow satisfactorily in media at pH 6.0, *i.e.* far below that which in combination with low concentrations of fatty acids inhibits development.

For growth experiments with higher fatty acids one is thus compelled to use increasingly low substrate concentrations for the higher homologs. Ordinarily this might imply a decrease in cell yield to such an extent that it would become difficult to evaluate the difference in growth in the media with and without added substrate. However, the metabolism of the purple bacteria in the presence of higher fatty acids results not only in a complete conversion of the organic substrate into cell substance, but is accompanied by the actual assimilation of carbon dioxide, as shown by the experiments of Gaffron and others (1, 5, 7, 16). The carbon dioxide uptake is proportional to the length of the carbon chain of the fatty acid, so that for every additional CH_2 group about 0.4 mol of CO_2 is fixed. This means that a maximum yield of bacterial cells can be obtained with far smaller concentrations of the higher fatty acids than with acetate. Again, the

TABLE 5

Relation between yield of cell material and fatty acid used as substrate

FATTY ACID	MOL. WT. OF Na-SALT	MG CARBON PER MILLIMOL	CO_2-UPTAKE PER MILLIMOL		MG CARBON IN CELL MATERIAL FROM 1 GM SUBSTRATE
			millimol	mg Carbon	
Formic acid	68	12	−0.6	−7.2	75
Acetic acid	82	24	−0.2	−2.4	230
Propionic acid	96	36	0.3	3.6	350
Butyric acid	110	48	0.65	7.8	435
Valeric acid	124	60	1.0	12.0	500
Caproic acid	138	72	1.4	16.8	550
Heptylic acid	152	84	1.7	20.4	600
Caprylic acid	166	96	2.0	24.0	635
Pelargonic acid	180	108	2.4	28.8	665

proportionality of CO_2-uptake and length of carbon chain was first established by manometric measurements of the metabolism of cell suspensions of *Athiorhodaceae*. These results have now been fully confirmed by growth experiments. The average cell yield per gram of sodium salt of various fatty acids is presented in table 5. It will be seen that the amount of cell material obtainable with, *e.g.*, pelargonic acid is almost three times as large as with acetic acid per gram of substrate. The latter substrate yields a maximum crop when used in concentrations of about 0.3%, amounting to around 55–60 μl of cells per 10 ml of medium. Since as much as 25 μl of bacteria may be produced in media containing only 0.05% sodium pelargonate, an evaluation of the growth in media containing very small amounts of the higher fatty acids as the chief substrate presents no difficulties.

From a theoretical consideration of the conditions in enrichment cultures of *Athiorhodaceae* it was deduced that various substances resulting from the primary decompositions of the chemically complex plant and animal materials might

logically be expected to serve as the substrates for the purple bacteria. The experiments with the various fatty acids have thus fully confirmed the expectations for this one group of substances. But the same holds true for a variety of other organic compounds which normally occur as products of the anaerobic decomposition of complex substrates. This has been shown by using the same general technique as was employed with the fatty acids, *viz.*, the inoculation of a mineral medium containing a small amount of yeast autolysate as a source of growth factors, and various simple carbon compounds as the chief substrate, with pure cultures of non-sulphur purple bacteria. Since growth at the expense of the yeast autolysate alone is negligible, the availability of the organic compounds tested can be judged by comparing the development of the strains in the different media. In this manner it has been found that also various unsaturated acids, hydroxy acids, dibasic acids, etc., as well as a number of amino acids can be used instead of fatty acids.

Of particular interest are the results obtained with non-acidic compounds. Gaffron had concluded from his manometric experiments that only substances with a carboxyl group can be used as assimilation substrates by the *Athiorhodaceae*. He wrote in 1935: "Das Vorhandensein einer Carboxylgruppe ist Grundbedingung dafür, dass ein Körper als Substrat der Assimilation dienen kann" (5, p. 308). Since, however, alcohols undoubtedly occur among the primary decomposition products of the complex materials used in enrichment cultures, the previous ecological considerations would lead one to expect that these simple substances could be used at least by some of the non-sulfur purple bacteria. And this all the more because many microörganisms, capable of growing at the expense of fatty acids, are known to be endowed with the ability to use the corresponding alcohols as well.

Foster (6) was the first to clarify the situation. He showed, both by growth experiments and by manometric measurements, that certain strains of *Athiorhodaceae* are indeed able to utilize several simple alcohols, both primary and secondary. The former are usually converted entirely into cell material, whereas the latter are oxidized to the corresponding ketones with a concomitant reduction of carbon dioxide. His experiments also indicated the reasons for Gaffron's failure to observe the utilization of alcohols by the purple bacteria, the rate of alcohol oxidation being considerably lower than that of the decomposition of fatty acids, even under the best conditions. Furthermore, the latter are realized only when cells are used which have been grown with alcohols as the chief organic compounds of the medium. Foster found that cells from cultures in the more common, alcohol-free media will not metabolize alcohols at a measurable rate except after a prolonged period of "adaptation," a process which requires incubation of the organisms in the presence of alcohol for many hours. (Personal communication). Consequently it is evident why Gaffron, using concentrated yeast extract media for growing the bacteria, and measuring their metabolism over short periods of time only, reached his conclusion.

Apart from the simple alcohols, studied by Foster, also polyvalent alcohols (glycerol, mannitol, sorbitol) and carbohydrates can be used by some repre-

sentatives of the *Athiorhodaceae*. But it should be emphasized that not all members of this group have the same physiological characteristics. While a more systematic survey of the results obtained will be presented further on, it may here be pointed out that there exist definite correlations in this respect between the morphological features of the various representatives and their ability to grow in the presence of special organic compounds. A few examples may serve to illustrate this point.

It may be recalled that the experiments of Molisch had indicated that glycerol and certain carbohydrates should be considered as the best substrates for the non-sulfur purple bacteria in the presence of peptone. A complete survey of the nutritional requirements of the large number of pure cultures has convinced me that this is not the case. Although there are types which can utilize these substances in the mineral medium with 0.1% yeast extract, many strains fail to grow in the presence of glycerol, and a large number appear unable to use carbohydrates. Still fewer develop in media in which mannitol or sorbitol are the major carbon sources.

Most surprising, especially in view of the previously reported results obtained by other investigators with dextrin and inulin, was the observation that the numerous strains which correspond closely to Molisch's description of *Rhodobacillus palustris* appear unable to use the simple hexoses, glucose, mannose, and fructose. It must, however, be realized that the polysaccharides recommended by Molisch are usually very far from chemically pure. It is, therefore, logical to believe that the satisfactory growth resulting from the use of the latter substances must be ascribed to the presence of impurities.

This conclusion is supported by two independent facts. Firstly, Nakamura (57) found that of seven carbohydrates, tested as substrates for *Rhodobacillus palustris*, only dextrin appeared to be used. At present there is less reason than ever to believe that polysaccharides can be used without undergoing a preliminary depolymerisation; the putative experimental support for the opposite contention has, in each case that has been carefully examined, been shown to rest on a misinterpretation or faulty technique.[2] Nakamura's, as well as Molisch's results strongly indicate, therefore, that the impurities in the dextrin used caused the observed effects.

The second line of evidence for this interpretation is furnished by the results obtained with two different fructose preparations. With the first I invariably observed good growth of all strains of purple bacteria, while several of them failed to develop in the corresponding glucose media. A second, better grade batch of fructose, as also the product obtained from the first by repeated recrystallization, yielded negative results wherever glucose did. These experiments demonstrate conclusively that impurities in commercial carbohydrate preparations do occasionally cause anomalous results.

In connection with the established inability of *Rhodobacillus palustris* to utilize sugars special mention must be made of Schneider's claim (22) that this organism does not grow in peptone-glucose media. From my own experiments it has

[2] See especially Kluyver and Custers (63), and Stanier (64).

appeared that development occurs in peptone solutions without sugar, and, furthermore, I had never noticed an inhibitory effect of glucose on my strains. A repetition of the experiments with Schneider's isolates confirmed his results. But the interpretation is not that glucose itself is toxic to some strains of *Rhodobacillus palustris*. The cultures, kindly furnished by Dr. Schneider, were contaminated with lactic acid bacteria, and the rapid development of these organisms in sugar-containing media caused an increase in acidity sufficient to prevent the growth of the purple bacteria. Also the production of acid in glycerin media, reported by Schneider, is not due to the metabolic activities of *Rhodobacillus palustris*, but to contaminating microörganisms.

Having thus disposed of the conflicting reports on the effects of sugars on the non-sulfur purple bacteria, it may be concluded that these substances can be used to differentiate between various representatives of the group. But not only the carbohydrates and related substances are valuable for this purpose; equally characteristic differences in the behavior of the various strains are exhibited with respect to other substrates, such as propionate, tartrate, citrate, certain amino acids, and alcohols. Other compounds, especially succinic and glutaric acids, although utilizable by all strains, yield only meager cultures in some cases, sharply contrasted with the profuse development of other strains in the same medium. The only substrates which appear to be equally satisfactory for all strains are acetate, butyrate, crotonate, lactate, malate, and a few others.

These results necessitate a complete revision of the prevalent ideas concerning the carbon nutrition of the *Athiorhodaceae*. At the same time they can and will be used for a more complete characterization of the different species of this group, and ultimately, have served the purpose of devising enrichment culture methods for several of the species that can be recognized at present.

c. Nitrogen requirements; growth factors; minerals. Since the foregoing experiments were made possible only after it was found that a low concentration of yeast autolysate could be substituted for the commonly used 1% peptone or yeast extract media, a brief discussion of the effect of different concentrations of the latter material on the development of the purple bacteria is in order. The experiments carried out in connection with the problem have been conducted mostly with media in which acetate was used as the chief substrate. Occasionally, however, they have been repeated with different compounds, and, because the results have always been in complete agreement with those of the former series, they need not be dealt with separately. I believe that it is safe to assume that more extensive future investigations will not materially change the fundamental aspects of the nutrient requirements which have been developed with the aid of acetate cultures.

As usual, the general results will be illustrated by a representative experiment.

To a sterile mineral medium, prepared with distilled water containing 1 gram $(NH_4)_2SO_4$ and 0.2 g $MgCl_2$ per liter, were added measured amounts of sterile Na_2S and $NaHCO_3$ solutions, the latter sterilized by filtration through Seitz filters under pressure, so that the final concentrations were 0.005% and 0.3% respectively. The reaction was adjusted to pH 7.0 with sterile H_3PO_4 solution, after which 30 ml of a M/1.5 phosphate buffer (pH 7.0) were added per liter of medium. This basal medium was enriched with varying amounts of

sterile yeast autolysate and sodium acetate, inoculated with a pure culture of a *Rhodospirillum* strain, and dispensed aseptically into sterile, glass-stoppered bottles, the latter being completely filled and stoppered. The cultures were incubated in a light cabinet

TABLE 6

Development of Rhodospirillum sp. in the presence of different amounts of yeast autolysate and sodium acetate

EXPT. NO.	YEAST AUTOLYSATE ML PER LITER	SODIUM ACETATE G PER LITER	CELL VOLUME IN μL PER 10 ML
1	0	0	0
2	0.1	0	0
3	0.2	0	0
4	0.5	0	0
5	1.0	0	0.5
6	2.0	0	0.5
7	3.0	0	0.5
8	0	0.5	0
9	0.1	0.5	4
10	0.2	0.5	6
11	0.5	0.5	8
12	1.0	0.5	9
13	2.0	0.5	8
14	3.0	0.5	9
15	0	1.0	0
16	0.1	1.0	7
17	0.2	1.0	16
18	0.5	1.0	17
19	1.0	1.0	18
20	2.0	1.0	18
21	3.0	1.0	19
22	0	2.0	0
23	0.1	2.0	10
24	0.2	2.0	20
25	0.5	2.0	33
26	1.0	2.0	36
27	2.0	2.0	35
28	3.0	2.0	39
29	0	3.0	0
30	0.1	3.0	10
31	0.2	3.0	21
32	0.5	3.0	*
33	1.0	3.0	57
34	2.0	3.0	56
35	3.0	3.0	58

* Not determined.

with constant illumination at a temperature of 30°C. After they had reached full development, the contents of the bottles were thoroughly mixed, and aliquots centrifuged for the determination of the total cell volume. The results are presented in table 6.

It is clear that it is not only the acetate concentration which determines the final cell yield, but that the latter is profoundly influenced by the amount of yeast autolysate as well. Although it is true that the development is proportional to the amount of acetate in the presence of enough yeast autolysate, nevertheless, this proportionality is reached only with increasingly large amounts of the latter substrate. In figure 17 the interrelations are shown graphically.

Evidently, the medium with the lower yeast extract concentrations is deficient in some material needed for development; with 0.1 ml per l the maximum cell yield amounts to 10 μl per 10 ml. A two-fold increase in the amount of yeast extract will allow of a corresponding rise in the number of cells, provided, of

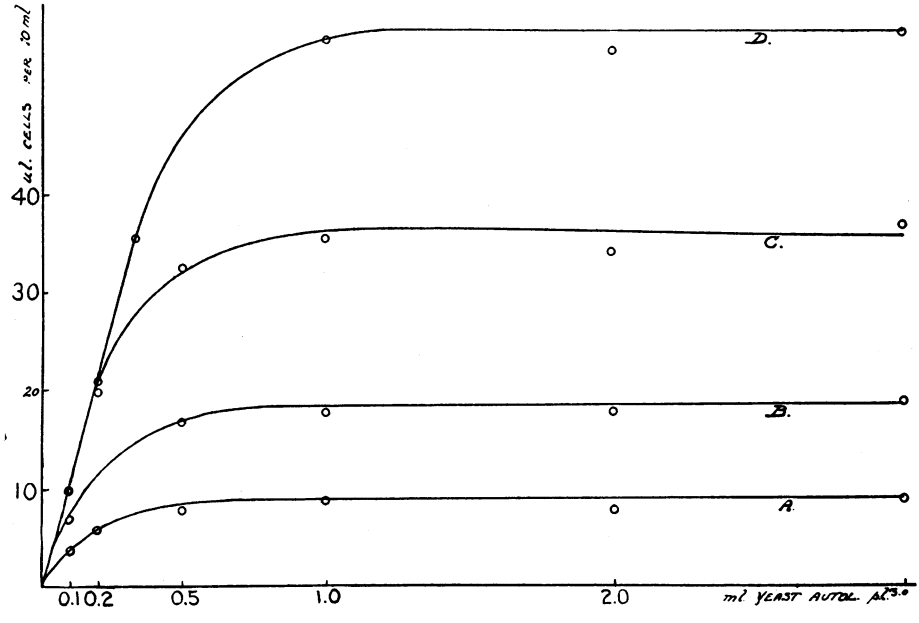


FIG. 17. Relation between cell yield and yeast extract concentration in cultures of *Rhodospirillum rubrum*, Strain No. 6, in basal medium with Na-acetate.
Curve A—0.05% acetate; Curve B—0.10% acetate; Curve C—0.20% acetate; Curve D—0.30% acetate.

course, the medium contains enough acetate. With 1 ml or more yeast autolysate per liter the growth appears to be limited by the acetate.

About the nature of the special material which is supplied by the yeast extract not much can yet be said. It is, however, certain that the autolysate does not supply the bacteria with more than a small fraction of their nitrogen requirements. This follows from the simple computation of the total quantity of nitrogen present in the yeast extract and in the bacteria in densely populated cultures. The extract contains around 1% total nitrogen; the nitrogen content of the organisms is about 11–12% of the dry weight (16). Hence 1 ml of yeast autolysate contains about 10 mg total nitrogen. Yet this small amount is adequate to produce, in the presence of enough acetate, a quantity of cell material

as large as 5.5 ml, representing a dry weight of approximately 1 gram, with 110–120 mg organic nitrogen. Even if all the nitrogen of the yeast extract were utilizable by the bacteria for conversion into cell materials, the total supply would be not more than one tenth of the amount required. It is, therefore, necessary to conclude that the ammonium salts in the medium are used as the main nitrogen source.

This has been further supported by experiments in which a standard medium with 1 ml of yeast autolysate per liter and 0.2–0.3% acetate was used, containing varying amounts of $(NH_4)_2SO_4$. They have shown that the last-mentioned substance, if present in very low concentrations, can become a limiting factor for growth. Above 0.03% $(NH_4)_2SO_4$ no effect is observable; at lower concentrations the development of the bacteria remains below the possible maximum in proportion to the amount of $(NH_4)_2SO_4$ supplied.

So far I have not carried out more than a few preliminary experiments in an attempt to identify the substance or substances in the yeast extract which are necessary for growth of the *Athiorhodaceae*. The data are not yet interpretable in terms of distinct chemical entities. It is, nevertheless, clear from the experimental results that different members of this group of organisms appear to have different requirements. Generally, growth is possible with as little as 1 ml of yeast autolysate per liter in the presence of a suitable, nitrogen-free organic substrate. But a number of strains show a vastly increased rate of development, as well as greater final yields of organisms with considerably higher concentrations.

An interesting effect pertaining to the mineral nutrition of the organisms may also be mentioned at this point. The medium usually employed contains only the elements H, O, C, N, P, S, K, Na, and Mg in appreciable quantities. Special precautions for avoiding the presence of other elements were never taken, and in view of the necessary addition of yeast extract it is reasonable to assume that many more elements were, indeed, present, and in sufficient quantities to meet the requirements. One of these plays an important role at least in the nutrition of the purple spirilla.

During his investigations on the oxidation of alcohols by members of the *Athiorhodaceae*, Foster made the observation that *Rhodospirillum* strains often develop as a flocculent precipitate instead of producing the more typical homogeneous, deep-red cultures. This abnormal mode of growth he traced to a lack of sufficient calcium in the medium; the addition of 0.01% $CaCl_2$ sufficed to prevent the formation of the tangled masses of sprilla which constitute the precipitate. (Personal communication.) Such a relatively high calcium concentration does not, however, influence the amount of growth, the latter being essentially the same with and without added calcium salt. It is thus likely that its peculiar effect must be attributed to special ionic interrelations or balances, determining the behavior of surfaces (See, in this connection, Heilbrunn, (67)).

As long as the chemical nature of the essential substances supplied by the yeast extract is not known, it is clearly impossible to study effectively the exact mineral requirements of the *Athiorhodaceae*, and the possible role of "trace elements" in their nutrition. Although the bacteria generally do not lend themselves particularly well to an experimental approach to this problem, the abun-

dant development of the non-sulfur purple bacteria in media with very low concentrations of the different ingredients may render these organisms valuable for the study of mineral nutrition once it becomes possible to add the as yet unknown factors in a chemically pure form.

d. Inorganic substances as substrates for photosynthesis and growth of the non-sulfur purple bacteria. The foregoing sections have shown that a large variety of simple organic compounds can be used by the representatives of the non-sulfur purple bacteria for photosynthesis as well as for growth. But those are not the only possible substrates for organisms of this group.

In 1933 Gaffron published the important observation that his (impure) cultures of *Rhodobacillus* could also bring about an oxidation of hydrogen sulfide, coupled with a photochemical reduction of carbon dioxide (1). Similar results were later obtained by Nakamura (57) with pure cultures. And when, in 1934, Roelofsen (54, 68) established that molecular hydrogen can be used for photosynthesis by *Thiorhodaceae*, Gaffron soon afterwards reported the same reaction for representatives of the *Athiorhodaceae* (5). Since then various investigators have corroborated the occurrence of these reactions (16, 32, 57, 69–73). It must, however, be pointed out that not all strains of this group are characterized by the ability to use sulfide or hydrogen; in 1930 I had conducted a number of experiments on photosynthesis in the presence of sulfide and of hydrogen with *Rhodospirillum* strains, obtaining completely negative results.

The above-mentioned investigations had all been carried out with the aid of the manometric technique, and hence with cells previously grown in organic media. It is, however, obvious that those purple bacteria which can utilize oxidizable inorganic substances for photosynthesis should also be able to grow, in the proper media, chiefly on the basis of these reactions. This has been verified by numerous culture experiments.

The first of these were conducted in 1930, in an attempt to grow *Athiorhodaceae* in sulfide media. By using an inorganic medium with very small amounts of peptone and various sulfide concentrations it was demonstrated that the presence of sulfide results in increased growth of the organisms in anaerobic, illuminated cultures. It was also observed that, at the end of an experiment, the sulfide had become oxidized, mainly to free sulfur. This result was reported in 1935 (52) and attention drawn to the apparent discrepancy with Gaffron's observations from which a complete oxidation of sulfide to sulfate could be inferred. It is now clear that the incomplete oxidation in the culture experiments was due to the use of media which allowed only a very scanty development. Since the further oxidation of elementary sulfur proceeds extremely slowly by the small number of cells present—the development being limited by the lack of sufficient growth factors—it may easily be overlooked! In manometric experiments, on the other hand, where a large number of organisms is used along with a very small quantity of sulfide, a complete oxidation can be readily demonstrated. From the culture experiments it follows, however, that the oxidation of sulfide to sulfur takes place preferentially to a further oxidation of the latter. This is also substantiated by the statement of Nakamura (57) that *Rhodobacillus* cells from a sulfide-containing medium are covered with sulfur globules.

All sulfur purple bacteria can utilize thiosulfate as well as sulfide. Hence it was reasonable to expect that those *Athiorhodaceae* capable of oxidizing sulfide could also attack thiosulfate, and even grow at the expense of this oxidation. This has been tested experimentally, and the results have been unambiguous. In a medium with only a small amount of yeast extract, which by itself yields no more than a barely perceptible growth, several strains produce good cultures upon the addition of 0.1–0.2% $Na_2S_2O_3$. That the latter is converted into sulfate has been shown by chemical determinations of the sulfate content of blanks and inoculated media. Without yeast extract or other complex materials growth has not been observed in thiosulfate solutions.

Under similar conditions the development of certain strains of non-sulfur purple bacteria has been shown to proceed at the expense of molecular hydrogen.

Thus the experimental evidence, demonstrating the ability of *Athiorhodaceae* species to grow by virtue of their utilization of sulfide, thiosulfate, and molecular hydrogen, adds still another argument in favor of the previous ecological considerations. It has now been shown that the various products resulting from the primary decomposition of plant and animal remains can, one and all, serve as major substrates for the development of the non-sulfur purple bacteria.

At this point I regret to announce that so far one group of substances, the lower amines, has been omitted from the list of substrates investigated. This omission is particularly deplorable because these compounds will undoubtedly be formed during the anaerobic decomposition of proteinaceous materials. It is therefore *a priori* to be expected that the amines, too, can be used for photosynthesis and growth by some members of the group. During his extensive studies on the aerobic decomposition of various groups of carbon compounds den Dooren de Jong (74, 75) found the lower aliphatic amines to be attacked principally by a small group of apparently highly specialized bacteria, the *Protaminobacter* species.[3] In view of the existence of this specialized group of aerobic bacteria it is quite conceivable that also among the purple bacteria typical "specialists" for the oxidation of these amines would be encountered. It is hoped that this gap in our present knowledge will soon be filled.

e. The nutrition of the non-sulfur purple bacteria in the dark. While the foregoing discussion of the physiological characteristics of the non-sulfur purple bacteria has dealt chiefly with the nutrient requirements of the organisms when grown under anaerobic conditions and in the light, a few remarks should be added concerning the development under aerobic conditions, and in the dark.

On a number of occasions it has already been pointed out that those strains which can develop in the presence of air, can also be grown in the dark, but only aerobically. The media which will permit development are essentially the same as those in which growth can occur in the light. It must, however, be remembered that in the latter case carbon dioxide appears to be used as the only final hydrogen acceptor for the oxidation of the substrate. Furthermore, this carbon dioxide reduction results exclusively in the production of cell materials. Hence

[3] den Dooren de Jong proposed the generic name *Protaminobacter* for these organisms. Janke (76) has, however, pointed out that this is a misnomer, and substituted the designation *primoraminophagous* microbes for the members of den Dooren de Jong's genus.

the amount of cell material produced is of the same order of magnitude as the amount of substrate decomposed. This is not the case if the organisms must depend on oxygen as an ultimate hydrogen acceptor, *i.e.*, when they develop in the dark. Under these circumstances a large proportion of the organic substrate is decomposed with the liberation of CO_2—and NH_3 if the substrate contains amino acids which can be utilized—so that the yield of cell material from the same amount of substrate is naturally much smaller in aerobic, dark cultures than in illuminated anaerobic ones.

This difference in yield becomes particularly striking if an inorganic oxidizable substrate, such as hydrogen or thiosulfate is used. It is evident, and in the next section this point will be elaborated, that under these circumstances the growth of the purple bacteria becomes virtually autotrophic. Now, it is well known that the "efficiency" of autotrophic, non-photosynthetic bacteria, as reflected in the relation between the amounts of cell material formed and of the substrate oxidized, is usually quite low. This means that most of the available hydrogen is ultimately transferred to oxygen, and that only a small fraction is used for the production of cell substance from carbon dioxide. The elimination of the substrate oxidation with molecular oxygen by culturing the organisms anaerobically in the light must, therefore, of necessity increase the cell yield many-fold.

Nevertheless, from the point of view of the physiological properties of the purple bacteria the important point is not the crop of organisms obtainable under aerobic and anaerobic conditions, but the fact that any medium which will allow the development of the *Athiorhodaceae* by photosynthesis can also serve as a satisfactory substrate for aerobic growth in the dark.

For the ecology of the purple bacteria this fact has little or no importance. While one can thereby understand how the purple bacteria are able to live in nature during periods of darkness, it should by no means be interpreted to mean that in nature these organisms will ever be found thriving under competitive conditions when light is not periodically available to them. The ability to grow aerobically in the absence of light is a physiological potentiality, demonstrable with pure cultures, but one without ecological significance.

Special emphasis should be placed on the fact that none of the *Athiorhodaceae* thus far studied develops in the dark in the absence of oxygen. This is in complete agreement with the observations of Gaffron (1), demonstrating that suspensions of *Rhodovibrio* under these conditions exhibit no measurable metabolic activities, both in the absence and in the presence of a suitable substrate. Later studies (5) have shown, it is true, that suspensions of other members of the group may produce acidic substances when incubated anaerobically in the dark. But the small magnitude of this metabolism on the one hand, and the failure to influence its extent by the addition of substrates, indicate that the phenomenon cannot be considered as comparable with those fermentation processes which enable certain types of microörganisms to develop in the absence of air.

Both Gaffron (5) and Nakamura (57) have found that *Athiorhodaceae* can carry out a nitrate reduction. While the former showed this process to occur in the light, where it is comparable with the photochemical nitrate reduction

observed by Warburg with *Chlorella*, the latter demonstrated that it can also take place in the dark. This makes it possible that non-sulfur purple bacteria, like some common aerobic bacteria, might be grown anaerobically in the dark in nitrate-containing media. Conclusive experiments in this respect have not yet been carried out. But even if this were the case, it would not by any means invalidate the conclusion that the metabolism of the *Athiorhodaceae* is primarily oxidative.

III. A brief characterization of the nutritional physiology of the Athiorhodaceae

a. General aspects. Recapitulating, we may now conclude that the various angles of the nutrition of the non-sulfur purple bacteria, presented in the previous sections, have furnished ecological as well as direct experimental evidence which permits a relatively simple and unified concept of their general physiology.

Like most representatives of the *Pseudomonadaceae*, which they morphologically resemble, the purple bacteria carry out a typically oxidative metabolism. A great variety of simple organic substances—alcohols, fatty acids, hydroxy- and dibasic acids, etc.—can be used as substrates, and these are oxidized during metabolism with the use of an extraneous hydrogen acceptor. Although oxygen is the most common one, other acceptors, notably methylene blue and nitrate (57) can be substituted for it.

Furthermore, the oxidation of organic substances by ordinary aerobic microorganisms results in the production of carbon dioxide, coupled with a "primary assimilation" in the sense of Barker (77), in which a considerable amount of the substrate-carbon is converted into cell material. In a number of carefully studied examples the extent of this assimilation has been shown to be of a high order of magnitude; a conversion of one-half or even two-thirds of the substrate-carbon into assimilation products is common (77–83; 48). The same phenomenon has been observed with cultures of *Athiorhodaceae* oxidizing acetate in the dark in the presence of oxygen (8); even quantitatively the results are in agreement with those obtained with colorless algae, yeasts, *Pseudomonas* and *Spirillum* species.

Again, just as various members of the *Pseudomonadaceae* are capable of oxidizing inorganic compounds, such as thiosulfate and molecular hydrogen, as well as organic substrates, so do certain members of the non-sulfur purple bacteria possess this property.

All these similarities justify the characterization of the metabolism of this group of organisms as typically oxidative, and as essentially similar to that of other oxidative organisms. But in spite of the fact that the above derivation seems eminently logical, and the final conclusion well-nigh unavoidable, this characterisation is, nevertheless, unsatisfactory because it does not include the most outstanding feature of the physiology of the purple bacteria.

The reason for this situation is not hard to discover. The designation "oxidative" of necessity covers only one side of the metabolic activities; it describes the fate of the oxidizable substrate, but not the nature of the final oxidant. Biological oxidations share with the purely chemical ones the requirement that they must be accompanied by concomitant

reductions. And, although the oxidizability of a substance by a variety of oxidizing agents has long been recognized in chemical reactions, the term "oxidative" in biological processes has often been unconsciously construed to imply that the oxidant is molecular oxygen. It is true that this restriction was eliminated by Wieland's demonstrations that other substances, such as quinone and methylene blue, could function as hydrogen acceptors (*i.e.* oxidizing agents); yet the tendency has been to consider such systems more as interesting potentialities, induced by artificial laboratory conditions, than as biologically important realities.

Yet, it is especially among the microörganisms that one meets with cases in which the implication of a typically "oxidative" metabolism should not be that molecular oxygen enters into the reaction as final hydrogen acceptor. The most clear-cut examples are the processes of nitrate reduction, of sulfate reduction, and of carbonate reduction. The first, it is true, is ordinarily carried out by organisms which are essentially aerobic, and which can, therefore, normally use molecular oxygen for the oxidation of the substrate. It is thus possible to consider this process as an "aberrant" type of oxidative metabolism, imposed by the environmental conditions, and on a par with the reduction of methylene blue under anaerobic conditions. This line of reasoning cannot, however, be applied to the other two processes. The sulfate-reducing bacteria are anaerobes; they cannot live in the presence of air. At the same time, the fate of the oxidizable substrate is in no way different from that which typifies its degradation under the influence of an aerobic, oxidative organism. The important distinction is that, as far as is known, the sulfate-reducing bacteria can use only one specific group of substances, *viz.* the oxidized sulfur compounds sulfate, sulfite, thiosulfate, etc., as the ultimate hydrogen acceptors. The same appears to be true for the bacteria causing the methane fermentation; the only known acceptor for these organisms is carbonate or carbon dioxide (See Barker (84–87)). Consequently it appears that the term "oxidative metabolism" comprises a number of processes, similar or identical with respect to the fate of the oxidizable substrate, but characteristically different and readily distinguishable on account of the final oxidizing agent.

By taking this more comprehensive view of oxidative metabolism into consideration, it becomes possible to characterize the physiological properties of the purple bacteria more adequately. This group of organisms possesses the ability, unique among the bacteria, of carrying out an oxidative degradation of various substrates with carbon dioxide as the only final hydrogen acceptor, *but dependent upon proper illumination.* What this implies concerning our concepts of the process of photosynthesis has been discussed in more detail elsewhere (8).

Of great importance for an evaluation of the relative physiological importance of the photosynthetic and the "dark" oxidation of a substrate are some experiments of which an account has been rendered previously (8). It has repeatedly been pointed out that the *Athiorhodaceae* are capable of oxidizing one and the same substance either in the dark or in the light. In the former case oxygen is normally used as the ultimate oxygen accepter, while in the latter the oxidation can proceed under anaerobic conditions because here carbon dioxide fulfills this function. Now it has been shown conclusively (8) that suspensions of non-sulfur purple bacteria, in the presence of oxygen, carbon dioxide, and an appropriate substrate, fail to consume oxygen if the suspensions are illuminated. In spite of the presence of oxygen, the carbon dioxide utilization is exactly the same as in comparable experiments carried out under anaerobic conditions. Consequently in illuminated cultures the photosynthetic mechanism appears to be the only functional one, and the complete suppression of oxygen consumption by

illumination demonstrates convincingly that the purple bacteria must be considered primarily as photosynthetic organisms.

b. Thio- and Athiorhodaceae. Physiologically, the non-sulfur purple bacteria are thus related on the one hand to the purple and green sulfur bacteria by virtue of the photosynthetic nature of their metabolism, and on the other to the various types of non-photosynthetic organisms with a typically oxidative metabolism. With regard to the first-mentioned relationship, which has already been discussed to some extent in connection with the delimitation of the group of *Athiorhodaceae*, a few additional remarks are here in order.

While both *Thio-* and *Athiorhodaceae* can carry out a photosynthetic process in the presence of various oxidizable substances, a satisfactory differentiation seemed possible on the basis of the ecologically important fact that in nature the sulfur purple bacteria appear to be restricted to those localities where sulfide is present, while the other group develops primarily in organic media. Now that it has been shown, however, that organisms which have for many years been considered as typical *Athiorhodaceae* are also capable of utilizing oxidizable sulfur compounds instead of organic substances, this distinction becomes much more difficult.

An additional feature has been brought out by the investigation of the nutrition of the non-sulfur purple bacteria which can be used to separate the two groups. This is the recognition that the various members of the *Athiorhodaceae* require special, as yet unknown, growth factors of an organic nature. Whereas, therefore, *Thiorhodaceae* may develop in strictly inorganic media, this is not possible for representatives of the other group. It is well to remember, however, that only a very few of the typical sulfur purple bacteria have so far been obtained and studied in pure culture, so that it is conceivable that growth of some types may yet be shown to depend upon the presence of specific organic materials. With this in mind I have, during the past several years, made a number of attempts to culture some of the large *Thiorhodaceae*, particularly *Chromatium okenii*, by using sulfide-containing media enriched with complex organic materials (yeast extract, etc.), but the results have not been encouraging. Also, one must admit the possibility that certain species of *Athiorhodaceae* will be discovered which can develop in the absence of special organic growth-factors. If such organisms were simultaneously endowed with the ability to oxidize sulfur compounds, they would physiologically become indistinguishable from the *Thiorhodaceae* on the basis of our present knowledge. And the small pseudomonads, described in 1931 as typical sulfur purple bacteria (3), may be regarded with some justification as a case in point. This is all the more true since I have obtained enrichment cultures of these organisms with organic media inoculated with marine mud samples.

Thus the oft-repeated complaint of the systematist that nature knows of no sharp distinctions, and thereby renders his attempts arbitrary, may again be reiterated. While there is every reason to favor a subdivision of the purple bacteria into two separate groups, and while it is clear enough that this can readily be achieved if we consider only the most characteristic representatives

of the two entities, it remains a difficult task to select and define the criteria which appear to be the most logical and useful. We shall face this problem in a later section on the classification of the *Athiorhodaceae*.

c. Auto- and heterotrophic bacteria. The physiological similarities between the photosynthetic non-sulfur purple and brown bacteria and the organisms with an oxidative, but not photosynthetic, metabolism are less pronounced than those between *Thio-* and *Athiorhodaceae*. Yet, if one compares the metabolism of the purple bacteria in the dark with that of non-photosynthetic organisms, it is impossible to point to even one small but characteristic difference.

Here one can, of course, argue that the division between purple bacteria and the *Eubacteriales* along physiological lines is simple and sharp, because it can be based upon the ability of the purple bacteria to metabolize by means of a photosynthetic process. This, moreover, fits the ecological facts; enrichment cultures of purple bacteria have never been achieved except under conditions of proper illumination, so that it is reasonable to accept the proposition that the photosynthetic mode of life represents the most important physiological characteristic of the group. The experiments discussed at the end of section *a* (p. 48) bear this out convincingly.

But this, in turn, raises another problem, to which Czurda and Maresch (20) have called attention. The normal requirements for growth of the *Athiorhodaceae* include the presence of organic substances. For a long time it has been customary to refer to such organisms as heterotrophs, in contrast to the autotrophic living beings whose nutritional needs can be satisfied entirely by inorganic compounds. Now it is obvious that an autotrophic bacterium must necessarily synthesize all its cell constituents exclusively from carbon dioxide and other minerals, whereas such a complete synthesis would be superfluous for organisms living in the presence of organic matter. But if it were demonstrated that carbon dioxide plays an important role also in the synthetic processes of organisms living in organic media it would be difficult to maintain the distinction.

In this connection Czurda and Maresch have reported an experiment from which they concluded that in a peptone medium the *Athiorhodaceae* utilize only carbon dioxide as a carbon source. This is a far-reaching conclusion, and certainly not justified by the experimental evidence. The experiment was planned so as to enable them to compare the development of seven strains of *Athiorhodaceae*, in the light and in the dark, under aerobic and anaerobic conditions, and in the presence and absence of carbon dioxide. The medium used was a 1% peptone "Vaillant" solution at pH 5.2 for the CO_2-free cultures; in a corresponding series the presence of carbon dioxide was insured by the addition of 0.25% $NaHCO_3$. In this series the pH of the medium was, however, raised to about 7.2, so that the conditions included an important extra variable apart from the presence or absence of CO_2. Two of the strains did not develop in any of the media used; the remaining five all grew in the medium with $NaHCO_3$, though only in the light; while only two yielded positive cultures in the illuminated but CO_2-free peptone solution. In table 7 the results are summarized.

I have already remarked that an acid medium either impedes or completely suppresses the development of the non-sulfur purple bacteria. It is, therefore, not surprising to find that the neutralized medium is superior to the acid one. The two strains which yielded positive cultures in the initially CO_2-free, acid solution developed considerably more slowly than in the bicarbonate-containing, neutral environment; also, the pH in the former had risen to 7.9 and 8.1. This is understandable since in the breakdown of the peptone ammonia is produced. The results indicate that these two strains were less sensitive to acid than

the others, or that the inoculum was large enough so that the organisms introduced could gradually create a more satisfactory environment as the result of their metabolism, or that a combination of these factors was operative. The delayed development is also logically accounted for by these possibilities. The failure of two strains—among which was one of *Rhodospirillum rubrum*—to grow under any conditions is not explained; it indicates that the experiment cannot be considered as having been conducted under satisfactory conditions. The same holds good for the negative results of all the cultures kept in the dark In the absence of comparable data on the development of the seven strains in neutral media in the absence of CO_2 it is hard to understand how the results obtained can be used to derive the conclusion that the three strains which developed in the medium neutralized with $NaHCO_3$, but not in the acid, CO_2-free solution, are obligatory carbon-autotrophs, and utilize only CO_2 and not the peptone as a carbon source.

It is not only the fact that the requisite controls are not represented in the experiment which makes this conclusion untenable. During the past 30 years it has been repeatedly shown that many microörganisms—indeed all that have been carefully tested—fail to grow in media which are kept rigorously CO_2-free. (See, for example, Gladstone *et al.* (88)). May one, then, deduce from such observations that all these organisms, molds, yeast, protozoa, and a large variety of bacteria, "are obligatory carbon autotrophs and utilize only carbon dioxide as a carbon source"? Certainly not; and yet this is what Czurda and Maresch did for the purple bacteria on the basis of experimental results which are in no way different!

TABLE 7

Résumé of Czurda and Maresch's results

	OXYGEN PRESENT			OXYGEN ABSENT		
	Light		Dark	Light		Dark
	CO_2-free	0.25% $NaHCO_3$	0.25% $NaHCO_3$	CO_2-free	0.25% $NaHCO_3$	
Number of positive cultures.....	2	5	0	0	5	0
Initial pH.....	5.2	7.2	7.2	5.2	7.2	5.2; 7.2

We must, consequently, conclude that Czurda and Maresch have not contributed satisfactory experimental evidence for their contention. Nevertheless, the theoretical problem raised by them is one which deserves attention. By Gaffron's experiments (1, 5) it was firmly established that illuminated suspensions of *Athiorhodaceae* utilize carbon dioxide during their metabolism of the higher fatty acids. It would thus be possible to infer that the organic substrates serve exclusively as hydrogen donors, and that the synthesis of cell material proceeds entirely from carbon dioxide through its photochemical reduction. But this is not necessarily the case. Gaffron's experiments do not exclude the possibility that "during the breakdown of these organic compounds there may be formed intermediate products which can serve immediately as raw material for some of the anabolic reactions" (52, p. 140–141). Foster, in 1940, expressed the same opinion: "It is far more likely that in the course of the oxidation of . . . organic substrates there may be formed intermediate products which can be directly converted into cell materials. The recent studies on oxidative assimilation by colorless organisms furnish a very strong support for this view" (6, p. 134). Elsewhere (8) I have presented experimental evidence in favor of the concept that the organic substrate is, in fact, used by photosynthesizing *Athiorhodaceae*

in a manner analogous to, if not identical with, that in which it serves as a carbon source for non-photosynthetic organisms.

The fact remains, however, that in the light the non-sulfur purple bacteria can and do utilize carbon dioxide at least partly for the synthesis of cell materials. Furthermore, Foster's experiments have shown convincingly that special organic substances, such as secondary alcohols, are used exclusively as hydrogen donors, and not as building materials, since these substrates are quantitatively converted into the corresponding ketones which are left in the medium as metabolic end products. In addition to this we must consider the cases in which growth of *Athiorhodaceae* results from the oxidation of sulfide, thiosulfate, and hydrogen as incontrovertible evidence for a synthesis of cell substance from carbon dioxide as carbon source.

In view of the above considerations, it would thus appear possible to consider at least some members of the group as potentially autotrophic, if it were not for the fact that all the typical representatives require additional growth factors. The problem of proper terminology is hereby rendered much more difficult of solution. The strictest interpretation of the designation "autotrophic" implies an utter independence of other living organisms, *i.e.*, a completely mineral nutrition. In this sense the *Athiorhodaceae*, with the possible exception of a few types (see discussion in the preceding section), are not autotrophs. But the term has often—and not without justification—been used with reference to organisms capable of utilizing carbon dioxide for the synthesis of cell constituents. On this basis it is, however, most unsatisfactory to describe a bacterium which can grow in a strictly mineral medium by oxidizing molecular hydrogen as autotrophic, and a closely related organism as heterotrophic merely because for growth it requires the presence of, for example, 1 μg per liter of biotin. It will be conceded that the metabolism of the two organisms must be very similar indeed, and that it would, therefore, be undesirable to characterize their nutrition by terms which carry a connotation of profound differences. But if in this case an organism is admitted among the autotrophs because it requires only a very small amount of one single organic substance, and can produce all its other cell materials from CO_2 as the only carbon source, it becomes difficult to draw a sharp line of demarcation, because this would have to be settled on a quantitative basis. And just how much synthesis from CO_2 should be required to tip the balance in favor of one or the other term? The experience of the past five years has shown convincingly that all living organisms possess the ability to use carbon dioxide for some syntheses. As Werkman and Wood state it, "the close relationship between the heterotroph and the autotroph is becoming increasingly clear" (28, p. 12).

All this reflects the ever recurrent difficulty of carefully defining terms for use in scientific writing. Pirie's cogent remarks in a discussion of the terms "life" and "living" are worth quoting in this connection:

"Now, however, systems are being discovered and studied which are neither obviously living nor obviously dead, and it is necessary to define these words or else give up using them and coin others. When one is asked whether a filter-passing virus is living or dead the only sensible answer is: 'I don't know; we know a number of things it will do and a number

of things it won't and if some commission will define the word 'living' I will try to see how the virus fits into the definition.' This answer does not as a rule satisfy the questioner, who generally has strong but unfortunate opinions about what he means by the words living and dead" (89, p. 12).

In the present instance it seems to me preferable not to attempt a rigorous definition of the terms auto- and heterotrophic which may satisfy some, and upset others, but, after having called attention to the difficulties involved in the use of these words (see also 59), abstain from using them in order to characterize the metabolic properties of the *Athiorhodaceae*. What has so far been said concerning the general physiology of the group should, at any rate, give a far more complete picture of the behavior of the organisms than can ever be rendered by one single word.

6. THE PIGMENTS OF THE NON-SULFUR PURPLE AND BROWN BACTERIA

I. Introduction; physiological effects of the pigments

The characteristic colors of the non-sulfur purple and brown bacteria are chiefly due to the occurrence of two types of pigments. As far as has been ascertained, they appear to be distributed more or less evenly throughout the cytoplasm. By various methods it has been possible to extract and separate the main components. Since the pigment system is of fundamental importance for the physiological behavior of the organisms inasmuch as it is operative in the photosynthetic processes, a discussion of its properties appears pertinent.

Not until 1935 were definite chemical studies on the pigments reported. The earlier studies were primarily concerned with determinations of the optical properties of the bacteria themselves and of crude extracts, and with problems in terminology, although it is only fair to record that Engelmann's fundamental investigations on the relations between the physiological effects of light of different wavelengths and the absorption spectrum of the purple bacteria, dating from 1883–1888, laid the foundation for an interpretation of their physiology (90, 91).

It is a curious phenomenon that Molisch is so often credited with having supplied the first important contributions to our knowledge of these pigments. As Buder (9) has pointed out, all students of the purple bacteria, from Ray Lankester in 1873 on, have agreed on the complexity of the "bacteriopurpurin," the name used by Lankester to refer to the entire pigment system of the organisms. In a little-known publication Ewart (92), as early as 1897, clearly demonstrated that the purple bacteria contain both a green and a red pigment which can be separated by the use of proper solvents. Nadson (93) subsequently paid especial attention to the green, and his student Arcichovskij (94) to the red component, for which he proposed the name "bacterioerythrin," reserving the designation "bacteriopurpurin" for the complex in the sense of Lankester. Three years later Molisch (4), apparently rediscovering the presence of both green and red pigments in the purple bacteria, introduced the name bacteriochlorin for the green pigment, while limiting the term bacteriopurpurin to the red component. As a new contribution he produced evidence for the existence of two different red pigments, bacteriopurpurin α and β. In order to avoid confusion Buder (9) later proposed to standardize the terminology by accepting bacteriopurpurin to designate the complex pigment, bacteriochlorin the green, and bacterioerythrin the red components. At present these names have no more than an historical interest. The recent

investigations on the chemical nature have made it logical to coin terms more in keeping with the structure of the substances, about which more anon.

While the gradual development of methods for extracting and separating the green and red pigments led to their ultimate isolation as chemically pure compounds, and thus contributed greatly to the elucidation of their composition, a few reports were published which indicated that in the cells these components might be present in the form of a chemical combination which was destroyed by the treatment with various solvents. This attitude is particularly pronounced in the papers of Ljubimenko (21) and of Lévy, Teissier and Wurmser (95). Actually, the evidence presented by these workers is not convincing, although the latter investigators made it probable that the pigments occur as protein compounds, and that the protein component appeared to consist of globulin. The recent work of French (33, 45) has dispelled all doubt in this respect; it must now be accepted as firmly established that the organisms contain the pigments in the form of chromoproteins. It has not been definitively settled whether both red and green pigments are combined with the same or with different proteins.

That these pigments have an important physiological function was shown by Engelmann's observations on the behavior of purple bacteria in a spectrum (90, 91). Under special conditions the direction of their movements is governed by light; moving from a strongly illuminated section of the field into one more dimly lit, they rapidly reverse the direction of progression ("Schreckbewegung" in Engelmann's terminology). Since light of different wavelengths is absorbed to a different extent, and thus exerts the same influence as areas of different intensity, the result of exposing a suspension of purple bacteria to a spectrum is that after a while the organisms have accumulated in certain, rather narrow regions of the spectrum. From the behavior of the bacteria in and around these regions it is clear that the latter are experienced as "light," while the parts of the spectrum from which the cells gradually disappear, correspond to "dark" bands with respect to the sensitivity of the organisms. Engelmann proved that the accumulations occur in exact correspondence with the absorption spectrum of the bacteria. In view of the concept that only absorbed radiation can be physiologically active (law of Grotthus-Draper) this is, of course, not surprising. But special significance attaches to the fact that the aggregations were found to be most pronounced in the near infra-red region and least in the blue where, nonetheless, a strong absorption by the bacteria can be recognized. Concerning this last accumulation Engelmann wrote: ". . . und in günstigem Falle auch die Andeutung eines verwaschenen breiten Bandes zwischen etwa 0.55 und 0.52 (μ) kenntlich sind." (91, p. 162). The more extensive and careful experiments of Buder (9) have fully confirmed this behavior; the phototactic responses of the purple bacteria are most pronounced in the infra-red region though still perceptible in the blue.

This point is of interest because the separation of the green and red pigments has made it possible to ascribe the various absorption bands of the organisms to either one of the two types of pigments. Thus the work of Nadson, Molisch, Buder, and all later students has shown beyond a doubt that the green component is responsible for the infra-red absorption bands and for the one around 590 mμ, while the bands in the shorter wavelength region are exclusively due to absorption by the red pigments.

The occurrence of bacterial accumulations in the range below 590 mμ consequently suggests that the red pigments may be weakly functional in causing phototactic reactions, albeit the green component is far more active in this respect. Now, the close connection of the latter with the photosynthetic metabolism of the purple bacteria has been conclusively established by the measurements of French (32) of the rate of photosynthesis in light of different wavelengths from which it not only follows that the green pigment is photosynthetically functional, but also that light absorbed by the red components is completely lost for the photosynthetic process. The important study of Schrammeck (24) on the quantitative aspects of phototaxis by purple bacteria has demonstrated how extraordinarily sensitive the organisms are to differences in light intensity. But because his work was done with unfiltered light from electric bulbs it is as yet impossible to reach any more definite conclusions as to the relative effects of light absorbed by the green and the red pigments on phototaxis. It is not hard to believe in the existence of a carotenoid-sensitized photobiological response, especially in view of the intimate relation of phototropic responses of green plants with carotenoid pigments as established in many instances. The anomalous situation in the case under discussion is that here two fundamentally different pigments, one of which fulfills a specific function in metabolism which the other is known not to possess, should cause qualitatively the same physiological effect, though different in intensity. French (32) has hinted at a possible explanation on the basis of the assumption that the phototactic reaction might be complicated by secondary, chemotactic effects. Similar complications could also arise from superimposed thermotactic phenomena. Much careful work will, however, be necessary before this problem can be more satisfactorily interpreted.

If there be some doubt concerning the existence of a direct phototaxis of the purple bacteria caused by the red pigments, no hesitation is justified in accepting the view that for photosynthesis these organisms can use only light absorbed by the green pigment. First and foremost this statement is supported by French's exact studies (see above). Additional evidence has been furnished by experiments on growth of various purple bacteria in a spectrum. (Eymers and Wassink, mentioned in (96); many unpublished experiments of Dr. W. Arnold). The growth regions coincide accurately with the absorption bands of the organisms except that at wavelengths below 570 mμ growth does not occur.

For a more detailed treatment of the photosynthetic reaction of the purple bacteria the reader should consult other sources (8, 97, 98).

II. The green pigments

When Ewart had found (92) that a green pigment could be extracted from purple bacteria by alcohol, he carried out some further simple experiments with the solution. The pigment was easily transferable to ether or benzene; the solutions showed a red fluorescence, faded rapidly when exposed to light in the presence of oxygen, and turned brown on treatment with acid or alkali. From these observations he concluded cautiously that "a green dye apparently identical with chlorophyll" was present in the organisms.

The green pigment which Nadson (13) later studied was, however, distinctly different from chlorophyll with regard to its absorption spectrum, which showed a pronounced band around the Fraunhofer D-line (600–580 mμ). Molisch arrived at the conclusion that the green component could not be chlorophyll, at the same time admitting that some characteristics of the extracted substance were strongly reminiscent of the typical plant pigment: "Die schöne grüne Farbe, die Ausschüttelungsversuche und die schwache, rote Fluoreszenz könnten auf die Vermutung führen, dass man es beim Bacteriochlorin eigentlich mit Chlorophyll zu tun habe. Die spektroskopische Prüfung und manches andere spricht aber ganz dagegen . . ." (4, p. 79). Apart from the absorption spectrum of the green solutions, which is totally different from that of chlorophyll, a clear distinction was indicated by the treatment of the solutions with strong alkali. Molisch had observed that chlorophyll solutions at first turn brown, and later become green again when subjected to the action of alkali. (Molisch's chlorophyll test, later known as the "phase test"). The green extracts from purple bacteria responded to the addition of alkali by turning brown, but never resumed a green color. The same difference was exhibited by the effect of acid. While recognizing the profound differences in the behavior of solutions of "bacteriochlorin" and of chlorophyll, he nevertheless stated: "womit aber nicht gesagt sein soll, dass zwischen Bacteriochlorin und Chlorophyll nicht auch verwandschaftliche Beziehungen bestehen könnten" (4, p. 80).

One finds the same reasoning in Buder's discussion of the green bacterial pigment. He, however, recognized another important problem which Nadson and Molisch had not raised. Much more aware of the importance of Engelmann's phototaxis experiments, which Buder repeated and expanded, the latter realized that it was still uncertain whether the green or the red component of the purple bacteria pigment system was responsible for the marked and spectacular absorption in the infra-red region. Direct measurements of the infra-red absorption by the green solutions were not carried out, however. Buder tried to solve the problem by studying the phototactic accumulation of the purple bacteria in light filtered through solutions of the green and red components respectively, expecting that the solution of the particular pigment which was characterized by the infra-red absorption bands would prevent the organisms from exhibiting the corresponding phototactic bands. Much to his surprise he found that the accumulations in the infra-red region occurred behind both the solution of bacteriochlorin and that of bacterioerythrin, but were eliminated if a filter of living bacteria or a ferrous sulfate solution was used. As an interesting possibility Buder points out that perhaps a third pigment is present in purple bacteria which absorbs mainly in the infra-red:

"Mit Sicherheit lässt sich aus diesem unerwarteten Ausfall der Versuche nicht allzuviel schliessen. Er legt aber eine Möglichkeit, die man bisher zu berücksichtigen überhaupt keine Ursache hatte, als Gegenstand weiterer Prüfung nahe. Es wäre nämlich zu untersuchen, ob etwa eine dritte Komponente des Bakteriopurpurinkomplexes vorhanden ist, die sich durch starke Absorption der fraglichen Gebiete des Infrarots auszeichnet, in Alkohol und Schwefelkohlenstoff aber nicht oder nur sehr schwer löslich ist. Da das

sichtbare Absorptionsspektrum des Bakteriopurpurins sich durch die Übereinanderlegung der Spektra des Bakterioerythrins und -chlorins, wie es scheint, ziemlich restlos erklären lässt, bliebe für ein drittes "Pigment" keine wesentliche Absorption im sichbaren Bereiche mehr übrig. Es müsste also ein nahezu farbloser Körper sein" (9 p. 549).

It may here be remarked that later studies have shown that actually the green pigment is the one which is responsible for all infra-red absorption bands, but that its absorption characteristics undergo considerable changes upon extraction with alcohol. In this connection special attention should be given to the extensive and careful studies of Wassink *et al.* (96, 99, 100). They demonstrated that the green alcoholic extracts of a variety of pure cultures of purple bacteria exhibit only one absorption maximum in the infra-red, situated at 774 mμ. The living cells, on the other hand, show two or more distinct maxima in this region, and their positions vary with the species. The differences may be considerable, as shown by the data summarized in table 8.

TABLE 8

Infra-red absorption bands of various species of purple bacteria (after Wassink et al.)

STRAIN	ABSORPTION MAXIMA, mμ.						
Thiorhodaceae							
Type 1	895			855–850		796	
Type 2	895		865		804		
Athiorhodaceae							
Type 1		892–885		850		799	
Type 2			880–863		802		
Type 3			875			800	
Alcoholic extract of all types							774

Aqueous extracts of the purple bacteria pigments, prepared by supersonic disintegration or by grinding of the cells (French 33, 45, 101; Katz and Wassink (99, 100)), display absorption spectra which are in excellent agreement with those of the intact cells from which they are obtained. Since the 774 mμ absorption maximum of the alcoholic extracts is not found by spectroscopic examination of any of the living purple bacteria, it is reasonable to believe that the alcoholic solutions contain a "decomposition product" of the pigment complex.

This is essentially the same conclusion at which Ljubimenko (21) had arrived in 1921. His studies were undertaken in the hope of establishing a relationship between the pigments of the purple bacteria on the one hand and the green plant chlorophylls and the green bacteria pigment on the other hand. The results obtained led him to express his views as follows: "Il est probable que le bactériopourpourine, matière colorante des bactéries pourpres, est intimement liée aux substances albuminoides du protoplasma. Sous l'action de divers dissolvants qui rompent cette liaison par une simple coagulation de ces substances, la bactériopourpourine se décompose en une série de dérivés de couleur bleu et rouges" (21, p. 119).

A closely similar situation exists with respect to the absorption spectra of chlorophylls a and b in green plants and in alcoholic solutions. In the 1920's Ljubimenko derived from the existing discrepancies the conclusion that chlorophyll occurs in the plant in the form of a protein complex. The later careful studies of Mestre (105–107) have contributed important experimental support for this concept. Furthermore, the investigations of a number of enzymes by Warburg and his school have demonstrated the occurrence of a pronounced shift towards longer wavelengths in the position of the absorption maxima of the prosthetic groups following their recombination with the protein carrier. All this tends to lend conviction to the idea that the disappearance of the characteristic infra-red absorption maxima of intact purple bacteria or their aqueous extracts upon extraction with alcohol, coincident with the appearance of a new band in the green alcoholic solution, is a result of the severance of the pigment from a protein-dye complex.

These investigations on the absorption spectra of living organisms, aqueous extracts, and alcoholic solutions furnish a complete explanation of the results which Buder obtained in his studies on the "accumulation bands" of purple bacteria in the infra-red region behind an alcoholic bacteriochlorin solution. The latter is, namely, transparent to wavelengths which are absorbed by the living cells, so that the typical phototactic aggregations at wavelengths longer than 780 mμ can still occur in light that has passed through the alcoholic extract. And, although it is practically certain that the infra-red absorption in the organisms is principally due to the green component, one might still admit that Buder's idea of the existence in the cells of a third component, responsible for the absorption maxima in the infra-red only, was essentially correct. With the possibility of identifying this "third component" with proteinaceous constituents, and of envisaging the pigment system of the organisms as a protein-pigment complex, the experimental evidence of various investigators, and the concepts of Buder, Ljubimenko, and others have thus been harmoniously correlated.

While in most of the foregoing work similarities as well as differences between the green pigment of the purple bacteria and chlorophyll had been stressed, an approach to the chemical nature of the "bacteriochlorin" was not made until 1934.

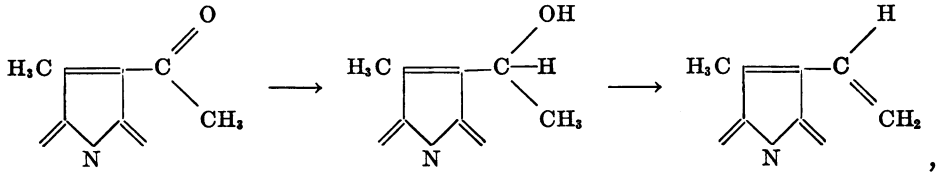
It is true that in 1925 Lévy *et al.* (95) had carried out some studies on the green component, obtained by extraction of purple bacteria with methanol, but the tests were crude, and the results difficult to interpret. They concluded: "D'après ses propriétés chimiques, il nous semble que la bactériochlorine doit probablement être considérée comme un pigment parent des carotinoides, si l'on veut comme un 'lipochrome' qui ne serait pas un carotinoide proprement dit" (95, p. 304). But it remained for Schneider to establish the close similarity of the green pigment with the plant chlorophylls on the basis of chemical analyses of the purified product (108, 109), at the same time proposing a change of name to bacteriochlorophyll: "Die Untersuchung hat ergeben, dass der grüne Farbstoff der Purpurbakterien dem Chlorophyll ausserordentlich nahe verwandt ist. Er ist demnach richtiger als 'Bakteriochlorophyll' zu bezeichnen, da der Name Chlorin schon für andere Körper der Chlorophyllreihe vergeben ist" (109, p. 222).

The most important results of Schneider's investigation are the demonstration that the green pigment is a pyrrol dye with porphin nucleus, containing mag-

nesium in complex combination. The combustion analysis led to the formula $C_{55}H_{72}O_6N_4Mg \cdot 1H_2O$. From the presence of 6 oxygen atoms Schneider inferred that bacteriochlorophyll is more closely related to the green plant chlorophyll b than to chlorophyll a. By preparing a large number of conversion products along lines which had previously been worked out (especially by Willstätter and Stoll, Stoll and Wiedemann, and H. Fischer *et al.*, during their studies on chlorophyll), Schneider substantially supported the contention that the green component of the purple bacteria pigment is a chlorophyll. For example, treatment of bacteriochlorophyll with dilute acid results in the loss of the magnesium atom from the molecule with the formation of bacteriopheophytin; the latter, still a wax, can be converted into the readily crystallizable bacteriopheophorbid, and this, in turn, into its methyl ester; while from these porphin compounds various porphyrins can be obtained by the usual methods.

At about the same time H. Fischer and co-workers commenced the publication of their investigations on the chemistry of bacteriochlorophyll, through which not only important details were added, but also misconceptions were cleared up, and ultimately the exact chemical structure of the molecule became fairly well established (110–116). Thus they demonstrated the presence of a phytyl group which can be split off by "chlorophyllase," and succeeded in carrying out the degradation of bacteriochlorophyll to oxo-pheoporphyrin a_5, the latter a typical derivative of the chlorophyll a series. The last-mentioned conversion can be achieved without having recourse to the "oxo-reaction," whereby the vinyl group of chlorophyll a is converted into an acetyl group. This transformation of bacteriochlorophyll into a substance identical with one obtained from chlorophyll a established that the bacterial pigment should be regarded as related to chlorophyll a rather than to b. The sixth oxygen atom in the bacteriochlorophyll is not present in a formyl group, as it is in chlorophyll b, but in an acetyl group which occupies the same position in the molecule as does the vinyl group in the green plant chlorophylls. Also, it was found that bacteriochlorophyll contains two hydrogen atoms in excess of chlorophyll a which can be very easily removed. The various compounds of the bacteriochlorophyll series then give rise to dehydro-derivatives, still more closely related to similar members of the chlorophyll a series. How close this relationship is was shown by the recent syntheses of dehydro-bacteriopheophorbid and dehydro-bacteriochlorin from derivatives of chlorophyll a, and by the beautiful experiments in which the acetyl group of dehydro-bacteriopheophorbid was transformed into a vinyl group, thus practically achieving its conversion into chlorophyll a.

In connection with the successful interconversion of the acetyl compound via the α-hydroxy stage to the vinyl derivative, according to the reaction:



Fischer, Mittenzwei, and Hevér wrote:

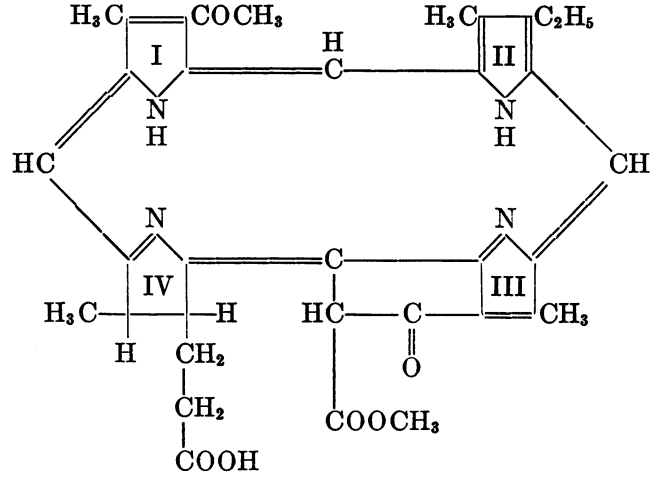
"Die α-Oxy-körper bilden somit neben den Acetylderivaten eine neue Brücke zwischen Chlorophyll a and Bacterio-chlorophyll. Durch Wasserabspaltung der 'analytischen' α-Oxyverbindungen im Hochvakuum zu Chlorin e_6-triester konnte eine weitere wichtige Zwischenstufe in der nun folgenden Übersicht der bestehenden Übergänge dargestellt werden:

Phäophorbid a ⇄ Chlorin e_6 ⇄ 2,α-Oxychlorin e_6 ⇄

⇄ 2-Acetyl-chlorin e_6 ⇄ Dehydro-bacterio-phäophorbid a.

Da Phäophorbid a bereits von uns reversibel in Chlorophyllid a übergeführt ist, und von Phäophorbid aus auch in Phäophytin, bzw. Chlorophyll a, ist damit der endgültige Beweis geliefert für die vollständige Übereinstimmung der Konstitution von Dehydro-bacteriophäophorbid a und Chlorophyll a. Noch steht der definitive Beweis aus dass bei der Dehydrierung von Bacterio-chlorophyll keine Konstitutionsänderung eintritt. Angesichts der grossen Labilität von Bacterio-chlorophyll halten wir dies nicht für gänzlich ausgeschlossen" (116, p. 162).

The most probable formula for the dehydrobacteriopheophorbid, according to Fischer *et al.*, thus is:



With a phytyl group substituting for the acidic hydrogen of the propionic acid attached to pyrrol ring IV, and the two hydrogens in rings I and II replaced by Mg, this structure should also closely represent the formula of the bacterio-chlorophyll itself, with the position of the two extra hydrogen atoms still in doubt.

Relevant to these views are also the spectroscopic investigations of various derivatives of chlorophyll a and bacteriochlorophyll by Stern and Pruckner (162). Although so far restricted to measurements in the visible region, the position of the absorption bands supports the chemical evidence for the occurrence of an acetyl group in bacteriochlorophyll, located in the same place where chlorophyll a carries a vinyl group.

Schneider, considering bacteriochlorophyll as more closely related to chlorophyll b than to a, looked for a second green component in the purple bacteria, and presented some evidence for its existence. According to the studies of

Fischer *et al.* this substance would, however, be a decomposition product of genuine bacteriochlorophyll and not a regular constituent of the pigment system. The latter investigators could find no indications for the occurrence of more than one bacteriochlorophyll in *Thiocystis violacea*, one of *Thiorhodaceae*. Since Schneider had investigated the pigment of *Athiorhodaceae* cultures, which undoubtedly represented a mixture of different species, it was entirely possible that the conflicting evidence might have been due to the fact that the various members of the groups of *Thio-* and *Athiorhodaceae* do not all contain identically the same bacteriochlorophyll. The marked variations in the infra-red absorption spectrum of different organisms, commented upon before, could readily be considered as experimental support for this assumption.

Nevertheless, the evidence obtained to date does not bear this out. In the first place, chromatographic analyses of bacteriochlorophyll extracts from pure cultures of several species of non-sulfur purple bacteria have made it appear most unlikely that any one species contains more than one bacteriochlorophyll. Furthermore, the absorption spectra of the alcoholic solutions are, as far as has been ascertained, identical. This also includes the alcohol extracts from members of the *Thiorhodaceae* (See also (99)). Thirdly, the various bacteriochlorophyll extracts have been used for the preparation of pure bacteriopheophytin, bacteriopheophorbid, and bacteriomethylpheophorbid. A careful comparison of the absorption spectra, melting points, mixed melting points, and analytical data of these products again has yielded no indication for the existence of different bacteriochlorophylls.[4] Although it is true that until now such comparative studies have not been carried out with pure bacteriochlorophyll preparations obtained from different species, the present information thus suggests that the various purple bacteria contain the same bacteriochlorophyll. (Many unpublished results, in part with Drs. E. Wiedemann and W. A. Arnold, and referred to in (104) and (117).) In view of the fact that the combination of the pigment with protein radically alters the absorption characteristics, it seems most reasonable to assume for the present that the differences in infra-red absorption exhibited by various species must be ascribed to compounds of one bacteriochlorophyll with different proteins, a conclusion with which French (33) and Wassink *et al.* (100) concur.

III. The red and yellow pigments

If a mass of purple bacteria is treated with concentrated sulfuric acid, the material soon turns blue. This reaction, first reported by Winogradsky (11), became the basis for the belief that the purple bacteria pigment would be a lipochrome. Nadson, while admitting the validity of the observations, nevertheless pointed out that the inference was at best premature: "From this it may be deduced that there are, indeed, lipochromes in the cells of these bacteria. But one should not conclude that bacteriopurpurine is itself a lipochrome"

[4] This statement is correct only for the green pigment of the purple bacteria. The green sulfur bacteria contain a chlorophyll which is most certainly not identical with either bacteriochlorophyll or chlorophylls a and b.

(93, p. 112). Both the solubility and absorption spectrum characteristics argued strongly against it. This state of affairs led to Arcichovskij's study (94) of the lipochrome constituent, "bacterio-erythrin," which he obtained in crystalline form and characterized so well spectroscopically that even to-day it is possible to identify this pigment with one of the various carotenoids now known to occur in purple bacteria. This is of importance because it has become clear that, contrary to what appears to be true for the green component of their pigment system, the purple bacteria contain a variety of carotenoids. Molisch (4) distinguished two, but to-day it is certain that many more exist.

Again, the early studies on the red pigment components dealt with their properties chiefly as far as their behavior towards various solvents and their absorption spectra were concerned. More recently investigations have been carried out with a view to establishing the chemical nature of the substances. The first of these (62, 118) led to the isolation in a crystalline form of a carotenoid pigment from *Rhodospirillum rubrum*. On account of its origin and chemical properties the compound was named spirilloxanthin; it is a dark purple, beautifully crystalline substance, easily decomposed by light and oxygen, and its absorption spectrum agrees with that of Arcichovskij's bacterio-erythrin, and with Molisch's "bacteriopurpurine α."

Soon afterwards Karrer and Solmssen (119, 120) obtained a similar substance, which they named rhodoviolascin, from mass cultures of *Rhodovibrio*. The melting points, solubility characteristics, and absorption spectra of spirilloxanthin and rhodoviolascin show a striking resemblance. The analytical data, especially the results of carbon and hydrogen determinations, are not in such good agreement, however. This is the reason why different formulas have been proposed for spirilloxanthin (62) and rhodoviolascin (119–123). According to the analyses of van Niel and Smith the former compound can best be represented by $C_{48}H_{66}O_3$ with 15 double bonds per molecule, while Karrer and Solmssen's results suggest $C_{42}H_{60}O_2$, containing 13 double bonds, for rhodoviolascin. It should be pointed out that the presence of two methoxyl groups in the latter substance (120) has been corroborated also for spirilloxanthin (van Niel and Wiedemann, unpublished). The number of double bonds is computed from hydrogenation data, and the result naturally depends upon the assumed molecular weight of the substance. Thus, the values of van Niel and Smith are reduced to 12.8 and 12.9 if the molecular weight of spirilloxanthin were 596 instead of 690.5, while Karrer and Solmssen's data, upon recalculation for a substance with the higher molecular weight, would indicate the presence of 15 double bonds per molecule.

There are a few observations which, for the time being, favor a skeptical attitude to the most recent formulation of the structure of rhodoviolascin by Karrer and Koenig (123). One is that the position of the absorption bands would be in better agreement with a larger number of double bonds (see also (62)). Karrer and Solmssen remark in this connection: "... man wird bis auf weiteres die Annahme machen müssen, dass die langwelligere Lichtabsorption durch die Methoxylgruppen mitbestimmt ist" (120, p. 4). A second difficulty lies in the fact that spirilloxanthin appears to contain one active hydrogen atom per

molecule (van Niel and Smith (62); van Niel and Wiedemann, unpubl.) which could be readily understood if the molecule contained one hydroxyl group, but for which there is no place in Karrer's formulation. Thirdly, the oxidation of rhodoviolascin by ozone yields acetone (120), which is contrary to the expectation if the formula proposed (123) were correct. It is true that the analysis of the products resulting from the oxidation of rhodoviolascin by permanganate (122, 123) makes it highly probable that the pigment contains a bixin structure, but this does not necessitate a structure of the rest of the molecule analogous to that of the better known carotenoids.

It is, of course, possible that spirilloxanthin and rhodoviolascin are not identical. In 1936 I presented Prof. Karrer with about 500 mg of pure spirilloxanthin, and asked him to compare this substance with rhodoviolascin. The determination of a mixed melting point would have gone far to settle this problem, but Karrer has not yet reported the results.

So much for the best known of the red pigments. In the extracts of *Rhodospirillum rubrum* five additional yellow and red pigments have been found (62), but until now no one has been obtained in sufficient quantity to allow its closer study. From spectroscopic observations one seems to bear a resemblance to spirilloxanthin, with all three absorption maxima shifted toward shorter wavelengths.

Karrer and Solmssen have obtained several other red pigments from *Rhodovibrio* mass cultures. The main distinctions are still based on the position of absorption maxima, and it seems premature to consider them all as chemically distinct individuals. This is the more true because the melting points of some of the fractions, as well as the position of the major absorption bands are very similar. Only one, rhodopin, has been obtained in a pure enough state to make the analyses significant; they indicate an empirical formula $C_{40}H_{58}O$ or $C_{40}H_{56}O$. Originally (119) Karrer and Solmssen claimed one methoxyl group per molecule of this pigment; in a later publication (121) the "small" methoxyl content of the original sample is ascribed to the admixture of rhodoviolascin. Since a methoxyl-free rhodopin would require approximately a half-and-half mixture with rhodoviolascin in order to account for the earlier found methoxyl content, the explanation seems extremely far-fetched. In addition, van Niel and Wiedemann (unpubl.) have isolated a red pigment with the characteristics of rhodopin from certain cultures of *Athiorhodaceae* which do not produce spirilloxanthin (rhodoviolascin); and in spite of the absence of the di-methoxy compound the beautifully crystalline substance appears to contain one methoxyl group.

The studies of Schneider (124) on the carotenoids of *Athiorhodaceae* may also be mentioned as providing further evidence for the complexity of the pigment system. Isolation of individual components was not attempted; the chromatographic analysis of extracts showed, however, the presence of a mixture of various colored substances.

Summarizing the present status of our knowledge of the red components of the purple bacteria pigments it may be said that the information is as yet conflicting and indefinite. None of the formulas so far proposed has been suffi-

ciently well substantiated, and it would be uncritical to go beyond the assertion that several different individual components have been met with. For the sake of convenience I have collected the information on absorption spectra in table 9.

Perhaps the most significant general conclusion that can be drawn from an inspection of table 9 is that by a spectroscopic examination three groups of red pigments can be distinguished. The first, corresponding to Arcichovskij's bacterioerythrin and Molisch's bacteriopurpurin α, appears to be represented by spirilloxanthin and rhodoviolascin. The second comprises the pigments of the bacteriopurpurin β type. Among these Karrer's rhodopin probably constitutes the quantitatively predominant type; but the group, spectroscopically characterized by the position of its long-wavelength absorption band at 545–550 mμ (in CS_2), is likely to contain a number of chemically distinct pigments. The third group is composed of orange and yellow pigments such as reported by

TABLE 9

Absorption spectra of the "red" constituents of the pigments from purple bacteria

PIGMENT	MELTING POINT	WAVELENGTHS AT ABSORPTION MAXIMA IN VARIOUS SOLVENTS												REFER.
		CS_2			$CHCl_3$			C_6H_6			EtOH			Petroleum ether			
Bacterio-erythrin		570	530	490							527	493					94
Bacteriopurpurin α		565	530		540	510											4
Spirilloxanthin	219	569	531	501	540	506	479	545	508	479	529	494		526	493	465	62
Rhodoviolascin	218	573	534	496	544	507	476	548	511	482	526	491	(465)				119
Bacteriopurpurin β		545	500		520	490											4
Bacterioerythrin β		545	505	485													9
Rhodopurpurin	161–162	550	511	479	523	487		527	490					502	472		119
Rhodopin	171	547	508	478	521	486	453				505	474		501	?	440	122
Rhodovibrin	168	556	517														121
2nd pigment from *Rhodospirillum rubrum*		551	518	485													62
Flavorhodin	111–113	502	472	441	482	453					472	443		470	442		122

van Niel and Smith (62) and by Schneider (124). Flavorhodin (122) is the only constituent which has so far been obtained in crystalline form. This must be ascribed to the fact that in the pigment extracts so far studied the red and purple components were predominant.

It is, however, to be expected that a continuation of the study of the carotenoids produced by different species of *Athiorhodaceae* will reveal cases in which the orange and yellow pigments are produced in far greater quantity than the red ones. In general, it seems likely that various strains differ markedly in the composition of their pigment system as far as the red components are concerned. This is substantiated by a number of observations. Firstly, Molisch obtained extracts from pure cultures of *Rhodobacillus palustris* which must have contained chiefly spirilloxanthin, and not more than small amounts of other carotenoids because the absorption spectra reveal only the characteristic bacteriopurpurin α bands. Similarly, Molisch's discovery of bacteriopurpurin β was possible by

extracting pure cultures of a *Rhodospirillum* species which obviously contained little or no spirilloxanthin. Furthermore, the study of the red pigments of *Rhodospirillum rubrum* (62) showed convincingly the vast preponderance of spirilloxanthin among its carotenoids.

Such observations fit in well with the results of an examination of the cultures of different types of non-sulfur purple bacteria. In the section on the morphological characteristics of this group, it has already been remarked that they can in part be distinguished by the color of the growth. Especially the careful spectroscopic studies of French (33, 45, 101, 125) on pure cultures of a number of species have shown that three types can be differentiated by the position of the long-wavelength absorption bands of the carotenoids, respectively at 550, 530, and 500–510 mμ. I have spectroscopically examined thousands of cultures, though much more cursorily, and believe that French's measurements can be used to characterize three distinctive groups of *Athiorhodaceae*.

While these various observations are strongly indicative of significant differences in the composition of the pigments of various *Athiorhodaceae* species, they do not rule out the possibility that in addition to one strongly predominant type of pigment a number of other carotenoids would be formed as well by many or all strains, though in considerably smaller amounts. From a perusal of the publications of Karrer and Solmssen it would, however, appear as if both rhodoviolascin and rhodopin were produced in more or less equal quantity by some representatives of the purple bacteria, and that the composition of the pigment complex of any one species is extremely variable. The following quotations bear this out.

"Auch die Zusammensetzung der den Rhodovibriokulturen entzogenen Carotinoidmischungen ist bei verschiedenen Ansätzen in qualitativer und quantitativer Hinsicht Schwankungen unterworfen; so konnte, worauf wir früher schon verwiesen, Flavorhodin öfters nicht festgestellt werden. Gelegentlich beobachteten wir eine Carotinoidfraktion, die mit β-Carotin identisch zu sein scheint, in anderen Ansätzen aber nicht gefunden wurde" (121, p. 1019).

"Seither wurde ein Thiocystisstamm in grösserem Masstab bei uns weitergezüchtet und dessen Carotinoidgemisch untersucht. Dieses erwies sich als ebenso kompliziert zusammengesetzt wie jenes der Rhodovibriobakterien und schien letzterem auch in der Zusammensetzung nahezukommen" (121, p. 1020).

"Mengenmässig am stärksten vertreten sind Rhodoviolascin und Rhodopin, die somit in erster Linie für die Färbung der Bakterien verantwortlich sind. Die Ausbeuten betragen für diese beiden gereinigten Pigmente je 20–30 mg pro 300 Liter reifer Nährlösung" (121, p. 1019).

Nevertheless, these remarks are decidedly misleading. When Karrer and Solmssen base their observations on results obtained with mass cultures of *Rhodovibrio*, it must be kept in mind that their cultures did not in any way represent pure cultures of a *Rhodovibrio* species. Undoubtedly the major part of the organisms that developed in these mass cultures consisted of purple bacteria. But small and uncontrolled variations in the culture conditions must have had a pronounced effect on the specific composition of the bacterial flora. In fact, some statements in their papers make it obvious that *Thio-* as well as

Athiorhodaceae were present in these "*Rhodovibrio*" cultures. Hence their investigations cannot be interpreted in terms of the composition of the pigment complex of any one species of non-sulfur purple bacteria. The same holds true for their study of the red pigments of the sulfur purple bacterium *Thiocystis*. Here again, the mass cultures must have contained various representatives of *Thio-* and *Athiorhodaceae*. One should, therefore, expect that they found a mixture of carotenoids, produced by various species, and in unpredictable proportions.

It is significant that in a later paper they report: "Herr Peter R. O. Bally hatte die Freundlichkeit, uns "roten" Schlamm vom Nakura-See in Kenya Colony zu senden. Dieser lieferte mit Chloroform oder Schwefelkohlenstoff einen roten Extrakt, dessen scharfes Absorptionsspektrum mit demjenigen des Rhodoviolascins genau übereinstimmte. Andere Carotinoidbanden liessen sich nicht nachweisen. Es wird zu prüfen sein, ob hier andere Purpurbakterien vorliegen oder ob die Umweltsbedingunen die ausschliessliche Bildung von Rhodoviolascin begünstigt haben" (123, p. 462–463). From the fact that the material investigated consisted of a natural mass culture of sulfur purple bacteria it is safe to infer that it consisted mostly of *Thiorhodaceae*, not contaminated to a quantitatively appreciable extent with non-sulfur purple and brown bacteria. Consequently, the extracts also did not show the variety of pigments represented in the above-mentioned mixed cultures.

It is obviously futile to attempt a satisfactory description of the pigments of different *Athiorhodaceae* species until the individual carotenoids have been adequately characterized. And it appears to me that the most expedient approach to this problem will be a study of the pigments produced by different species when grown in pure culture.

So far, the red pigments have chiefly been characterized by the absorption spectra of their solutions in organic solvents. Yet this is not the form in which these substances occur in the bacterial cells. That the absorption bands of living bacteria can be correlated with the occurrence of specific pigments follows from the observations of Molisch, Buder, and French. However, here again one meets with a certain complexity which it is necessary to indicate.

In connection with the discussion of the absorption spectra of bacteriopurpurin α and β, Molisch stated: "Bringt man die Spektren des Bakteriochlorins und des Bakteriopurpurins (in Schwefelkohlenstoff) zur Deckung, so resultiert dann so ziemlich des Spektrum der lebenden Bakterien.... Untersucht man anstatt des Rhodospirillum eine Aufschwemmung des Rhodobacillus palustris in Reinkultur, der, die wir wissen, nicht wie das rote Spirillum Bakteriopurpurin β, sondern die Modifikation α enthält, so erscheint das Spektrum dem des Rhodospirillums im wesentlichen gleich, man findet nur die Absorptionsbänder des Bakteriopurpurins, entsprechend der hier vorkommenden Modifikation α etwas gegen Rot verschoben" (4, p. 83–84).

Now, the only absorption spectrum of a bacterial suspension which is reproduced in Molisch's treatise is one of a *Rhodospirillum* species; no data are presented with respect to the position of the bands of a *Rhodobacillus palustris* suspension beyond the rather vague reference to a "slight shift towards longer wavelengths." The longest wavelength absorption band due to the red pigments of Molisch's *Rhodospirillum* culture is clearly around 545–550 mμ, which is in good agreement with French's determinations both for the intact bacteria

and for the proteinaceous, aqueous extracts obtained by grinding cells of *Rhodospirillum rubrum* (550 mμ). But here the first inconsistency appears. For Molisch's *Rhodospirillum* is alleged to contain bacteriopurpurin β, while the *Rhodospirillum rubrum* culture used by French is the identical strain which had previously (62) been shown to contain principally spirilloxanthin, and the latter is spectroscopically identical with Molisch's bacteriopurpurin α! It is thus easy to understand why Molisch contended that the carotenoid absorption bands in carbon bisulfide should be used in order to obtain the agreement between the spectral characteristics of the bacteria themselves and of the superimposed green and red extracts, because the long wavelength absorption maximum for bacteriopurpurin β in carbon bisulfide is situated at 545 mμ. But such a procedure fails to account for French's measurements; his *Rhodospirillum rubrum* strain should accordingly exhibit an absorption band around 570 mμ, since this is the position of the corresponding maximum of spirilloxanthin (bacteriopurpurin α) in carbon bisulfide. The absorption spectrum of *Rhodospirillum rubrum* would, therefore, show a far better correspondence with that of the combined extracts if the carotenoid fraction were dissolved in benzene (maximum for spirilloxanthin 545–550 mμ) instead of in carbon bisulfide.

Furthermore, a band characteristic for spirilloxanthin in carbon bisulfide (570 mμ) has never been encountered in the examination of living cultures or of aqueous cell extracts of any of the non-sulfur purple bacteria. There is also disagreement in the data on the absorption bands of *Rhodobacillus palustris*. Spectroscopic examination of several of my strains, closely resembling this species, reveals an absorption maximum at 530 mμ rather than at 550 mμ, just as do the aqueous extracts of *Rhodovibro* cultures, reported by French (33) who used a strain of *Rhodovibrio* which has, in my experience, proved almost indistinguishable from *Rhodobacillus palustris*. Extracts of the carotenoid pigments of these representatives appear to contain rhodopin (bacteriopurpurin β) rather than spirilloxanthin. Here again a reasonable agreement in the position of the maximum is obtained with benzene solutions of the carotenoids; the long wavelength absorption maximum of rhodopin in this solvent is situated at about 530 mμ.

Had Molisch inadvertently interchanged the legends for the designation of the absorption spectra of the red pigments extracted from *Rhodospirillum* species and *Rhodobacillus* respectively these discrepancies would automatically have resulted. It cannot now be decided whether this may have occurred. However, in that case another and independent observation by Buder presents new difficulties. In his studies on the accumulation of purple bacteria in a spectrum Buder found a pronounced aggregation of a *Rhodospirillum* species around 530 mμ.

For a long time these various inconsistencies have greatly puzzled me. As far as the conflicting data on the *Rhodospirillum* species are concerned, the probable solution is, nevertheless, rather simple. It is possible to reproduce all the observations so far mentioned, *viz.* the absorption spectrum found by Molisch and French, the extraction with carbon bisulfide of a red pigment which has the bacteriopurpurin β characteristics, the isolation of spirilloxanthin, and the

accumulation in a spectrum at 530 mμ, with purple spirilla. But this requires the use of two distinctly different *Rhodospirillum* species. One of these produces spirilloxanthin, has, in the living state, an absorption maximum at 550 mμ; and accordingly aggregates phototactically at this wavelength. The other, brown rather than red in cultures, does not produce spirilloxanthin, but a pigment with rhodopin-like properties. Its cultures consequently display a maximum at 530 mμ, and phototactic accumulations likewise occur at this wavelength.

It would thus be possible that Molisch also made his observations on the absorption spectrum of living bacteria with one species of *Rhodospirillum*, while using the other type for preparing the carotenoid extracts. In that case it would be understandable that he felt the need for using carbon bisulfide solutions of the red pigment in order to make the absorption spectra of living cells coincide with their extracts. This would also account for the omission in the publication of an absorption spectrum of *Rhodobacillus palustris* cells, and for the vagueness of the statement concerning the exact position of the bands.

TABLE 10

Approximate position of long-wavelength carotenoid absorption bands of different types of non-sulfur purple and brown bacteria, and of the major red and yellow pigments

GROUP OF BACTERIA	ABSORPTION BAND OF INTACT CELLS	LONG-WAVELENGTH ABSORPTION MAXIMUM OF PRINCIPAL PIGMENT IN			
		CS_2	C_6H_6	$CHCl_3$	EtOH
1	550	570	550	540	525–530
2	530	540–550	530	520	505
3	505	520	500	495	485

However this may be, the available information tends to favor the conclusion that the carotenoid absorption bands of the bacteria can be more nearly reproduced with benzene extracts than with carbon bisulfide extracts. It is, moreover, clear that different species of *Athiorhodaceae* which show certain well-marked absorption characteristics contain different carotenoids as principal pigment constituents. At present it is convenient to distinguish three main groups, characterized as indicated in table 10.

The pigments themselves appear to belong to the spirilloxanthin (rhodoviolascin) type for group 1, and to the rhodopin type for group 2. Those of the third group have not yet been sufficiently studied; the absorption maxima do not bear a close resemblance to flavorhodin for which the long-wavelength bands are around 500–505 mμ, 480 mμ, and 470 mμ in carbon bisulfide, chloroform, and ethanol solutions, respectively.

While in the foregoing discussion an attempt has been made to correlate the color of non-sulfur purple and brown bacteria cultures with the occurrence of certain carotenoid pigments, it should be borne in mind that it is by no means necessary that all the red and yellow pigments found in purple bacteria belong to the carotenoids. It has previously been mentioned that some strains of *Athiorhodaceae* produce a diffusible, purplish-red pigment. That this bears no

relation to the water-soluble pigment complex of the purple bacteria which French investigated and refers to as "photosynthin"[5] is shown by the following observations. In the first place, the strongly colored, bacteria-free supernatant solutions of such cultures do not, as a rule, contain proteinaceous, autolytic products which can be precipitated by the addition of acid or by heating. Even if a slight flocculent precipitate does result from such treatment, this is never more than faintly colored; by far the larger portion of the pigment remains in solution. The addition of acid does not produce the color change from red to green, described by French for "photosynthin," but a barely perceptible change to a more purplish tinge, which is entirely reversible. The solution is heat-stable, both at an alkaline and acid reaction; it can be evaporated to dryness on a water-bath without any apparent change in the pigment. Furthermore, the coloring matter is not precipitated by ammonium sulfate, even to saturation, or by trichloroacetic acid. Completely different and characteristic is also the absorption spectrum. It lacks the bacteriochlorophyll band at 590 mμ, and shows three bands, at 610, 565 and 535 mμ, of which the last is the most pronounced. These bands are not exhibited by the cells themselves.

From an alkaline solution the pigment is not extracted by a variety of organic solvents. However, upon acidification it can be readily transferred to amyl alcohol, from which it can again be extracted with an aqueous sodium bicarbonate solution. This appears to be the simplest method for purification of the dye. I have obtained it in the form of crystals but not yet in sufficient quantity to permit of further chemical tests. In old cultures of strains which produce this diffusible pigment one can frequently observe among the deposit of cells a colored precipitate which somewhat resembles these crystals, but its nature has yet to be proved.

Whatever this substance may be, it evidently is not a carotenoid. Hence one might also find other non-carotenoid pigments as products of the metabolism of the *Athiorhodaceae*. The brown to yellow pigments which are responsible for the absorption bands at 505–510 mμ of cell suspensions of certain strains thus need not necessarily be extractable with carbon bisulfide or chloroform. In this connection it is of interest to call attention to the pronounced color change, almost amounting to a bleaching, which the addition of acid produces in suspensions of these brown organisms. Such a color change is not likely to be due to a carotenoid.

Then there is the red pigment which some of the brown strains produce when growing in the presence of air. Shake cultures of such bacteria in culture tubes not covered with paraffin illustrate this phenomenon most spectacularly; the colonies in the upper few millimeters of the agar column appear a deep red, while further down light-brown to yellow colonies develop. It is apparently not necessary that the bacteria be growing in order to effect the development of the red color. A brown, liquid culture in which growth has come to a standstill will, when aerated, turn red in the course of a few hours. French (125) has shown that

[5] See also the remarks of MacKinney (126) in connection with the terminology of the pigment-protein complexes in leaves, algae, and bacteria.

the "photosynthin" extracts of brown cells do not change color upon aeration: hence the red pigment is not produced from a precursor by an auto-oxidation process. Active cell metabolism seems to be a prerequisite for the change to occur. From the absorption spectra of the aqueous extracts of brown and red cell suspensions it is evident that a new absorption band around 540 mμ characterizes the latter. Although this might suggest the formation of a pigment of the spirilloxanthin or rhodopin type, it would be premature to accept this without supporting chemical evidence. French even concludes: "Its absorption spectrum would suggest that it [is] not a carotenoid" (125, p. 408). This conclusion is based on the curve resulting from a computation of the relative absorption at different wavelengths of a brown and a red "photosynthin" extract. If, however, the yellow pigments of the brown form are involved in chemical transformations during the change from brown into red, this curve does not represent the absorption spectrum of the newly formed red component. For the present, then, this remains a problem.

The above remarks also show that the environmental conditions may considerably influence the composition of the pigment system of the purple bacteria. The most striking effects are no doubt exerted by light and by oxygen. As a rule pure cultures grown aerobically in the dark produce only little pigment, an observation made by all previous investigators. The more restricted the oxygen supply in such cases, the more pronounced is also the pigmentation. This can be seen particularly clearly on slant cultures which have been inoculated while the agar surface is yet moist. Many strains then tend to develop both on the surface of the slant and in the region between the agar and the wall of the tube, where conditions favor growth of micro-aerophilic organisms. On such slants the pigment production appears inversely proportional to the exposure of the bacteria to oxygen. The most abundant pigmentation occurs, however, in illuminated cultures, where it seems little, if at all, influenced by oxygen with the previously noted exception where a distinct color change results. Schneider's claims to the contrary must again be ascribed to his use of impure cultures resulting in a change of flora with changes in oxygen pressure and medium.

In view of the fact that oxygen does not participate in the metabolism of suspensions of non-sulfur purple bacteria in the light (p. 48) the relative independence of pigment production from oxygen tension is not surprising, although admittedly conclusions drawn from experiments with non-growing bacteria need not hold true for cultures in which multiplication takes place.

Finally, what has here been remarked concerning the effects of environmental conditions on pigment formation is based entirely on visual observations. No careful quantitative studies have yet been made on the pigments of the *Athiorhodaceae*. And, until the various components are better known, such investigations cannot well be attempted.

7. THE CLASSIFICATION OF THE NON-SULFUR PURPLE BACTERIA

"Für die Systematik lässt sich das Verhalten zum Licht bis jetzt wenigstens kaum verwerten; nur bezüglich der Farbstoffproduktion scheint es hin und wieder .... eine Rolle zu spielen."

Migula, (128), p. 362.

I. The taxonomic position of the group

The first official recognition which purple bacteria received as a systematic group came with the creation of the order *Thiobacteria* by Migula. Though Winogradsky on the basis of his morphological studies (11) had subdivided the sulfur bacteria into several genera, among which the greater number were composed of purple sulfur bacteria, he had not attempted to group the genera into larger units, or to clarify the systematic position of his units with respect to other bacteria.

Migula did not propose the new order till 1900. In the section on the classification of the bacteria which he wrote for Engler-Prantl's handbook (127) Winogradsky's genera of the purple sulfur bacteria occur scattered over the various families. Nor is the order *Thiobacteria* mentioned in the first volume of his masterly "System der Bakterien" (128). Between 1897, the date of publication of this volume, and 1900, when the second volume appeared (129), it must have occurred to him that the separation of the morphologically rather conspicuous sulfur bacteria from the other bacterial species was desirable. As a result the second volume of the great treatise contains the sulfur bacteria in a new order, contrasted with the order *Eubacteria* by the following definitions:

Order I. Eubacteria. "Zellen ohne Centralkörper, Schwefel und Bacteriopurpurin, farblos oder schwach gefärbt, auch chlorophyllgrün" (129, p. 1).

Order II. Thiobacteria. "Zellen ohne Centralkörper, aber Schwefeleinschlüsse enthaltend, farblos oder durch Bacteriopurpurin rosa, rot oder violett gefärbt, niemals grün (129, p. 1039).

One looks in vain, however, for an exposition of the reasons which had induced Migula to create the new orders. No doubt this omission, coupled with the great influence which Migula's "System" has exerted, is largely responsible for the perpetuation of the order *Thiobacteria* without much of an attempt on the part of later systematists to clarify the issue. The name has been changed to *Thiobacteriales* (135–137), and the order has been generally accepted. But just which organisms should constitute this group, and on what basis its members should be systematically united, these have become issues which are in dire need of careful consideration. The now existing confusion shows a regrettable lack of the application of acceptable taxonomic principles, and often a lack of interest in, or familiarity with the organisms themselves.

Yet, there is also an obvious reason for these developments which it is well to consider in some detail because a clearer view of the situation is apt to point the way to a more satisfactory solution.

In 1900 the only purple bacteria that had received special attention were the red sulfur bacteria. Winogradsky's studies on the physiology of these organisms (10, 11) had revealed a similarity to that of the colorless sulfur bacteria, both groups being capable of oxidizing hydrogen sulfide to sulfuric acid. The hitherto strictly ecological group of the sulfur bacteria had thereby been characterized more adequately on a physiological basis. Migula had used the morphological adjunct of the metabolic activity, the storage of sulfur droplets, as a criterion for his new order, and then proceeded to subdivide the *Thiobacteria* into two families.

The *Beggiatoaceae* were composed of the colorless, the *Rhodobacteriaceae* of the red or purple sulfur bacteria. What cannot be too strongly emphasized is that, at this time, all the known sulfur bacteria, both red and colorless forms, presented morphological features which seemed to set them clearly apart from the members of the Eubacteria.

Thus Molisch, in 1907, found that a family status had already been assigned to those purple bacteria which are at the same time sulfur bacteria. And in his opinion this group was closely related to the organisms of which he had made a special study. The relationship appeared, in fact, so distinct that he felt justified in combining all the purple bacteria into one new order, and in separating the colorless and the purple sulfur bacteria. His reasoning is apt, and, if later systematists had taken better notice of it, a more satisfactory development might have been expected. On account of the importance of the passage, it is here cited:

"Als wesentlicher Charakter würde den Thiobakterien die merkwürdige Eigenschaft zugeschrieben, in ihrem Innern Schwefel einzulagern, oder wenn man die von Nathansohn neu entdeckten auch dazu zählt, wenigstens ausserhalb des Zellenleibes reinen Schwefel zur Abscheidung zu bringen. Die von mir entdeckte Gruppe von Purpurbakterien scheidet aber überhaupt nicht Schwefel ab, diese Fähigkeit geht ihnen, obwohl sie sonst in den physiologischen und morphologischen Eigenschaften mit den schwefelhaltigen Purpurbakterien vielfach übereinstimmen, gänzlich ab. Ich wäre daher dafür, dass man die Purpurbakterien von den farblosen Schwefelbakterien als eigene Ordnung abtrennt, hauptsächlich deshalb, weil sich auf Grund meiner Untersuchungen herausgestellt hat, dass eine grosse Zahl von Purpurbakterien Schwefel überhaupt nicht einlagern kann, also dem wesentlichen Charaktermerkmal der Thiobakterien gar nicht entspricht.

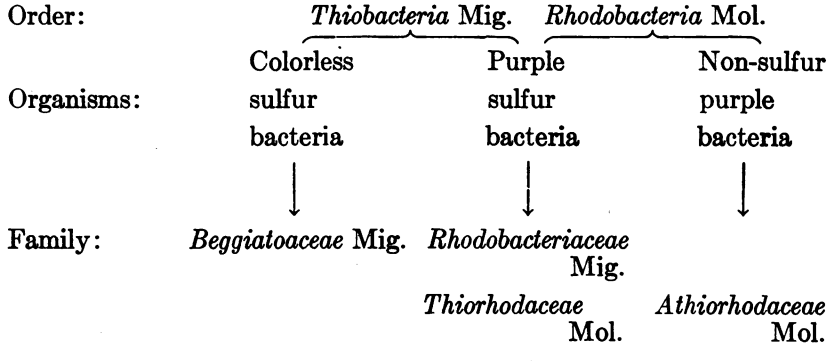
Die Purpurbakterien enthalten .... zwei ungemein charakteristische Farbstoffe, das rote Bakteriopurpurin und das grüne Bakteriochlorin. Und so wie man bei der systematischen Gliederung der Algen den Farbstoffen eine grosse Wichtigkeit zuerkennt, ich erinnere nur an die Phykochromaceen, Florideen und andere, so erscheint es mir auch speziell bei den Purpurbakterien, die infolge der Lebensweise, ihres Vorkommens und ihrer Farbstoffe eine ziemlich gut umschriebene physiologische Gruppe bilden, zweckmässig, sie alle wegen ihrer eigentümlichen Farbstoffe zusammenzufassen. Ich bin mir wohl bewusst, dass sich gegen eine solche Abgrenzung vom systematischen Standpunkt Bedenken erheben lassen, zumal da ja die Rhodobakterien morphologisch vielfach voneinander abweichen und mit anderen bekannten farblosen Gattungen, abgesehen von dem Farbstoff, übereinstimmen.

Würde man die Purpurbakterien auf Grund ihrer morphologischen Merkmale, die ja bei der systematischen Sonderung die erste Rolle spielen müssen, allein gruppieren, so würden sie sich über das ganze Bakteriensystem verteilen. Vorläufig scheint mir eine physiologische Gruppierung ungemein zweckmässig, ich stehe also, in dem ich unter Purpurbakterien nicht eine natürliche Gruppe von Organismen verstehe, sondern eine physiologische, auf demselben Standpunkte wie Winogradsky bezüglich der Schwefelbakterien.

Von diesem Gesichtspunkte ausgehend, fasse ich sämtliche bisher bekannten Purpurbakterien zusammen zur Ordnung der Rhodobacteria, die sich wieder in zwei Familien gliedert, 1. in die Familie der Thiorhodaceae und 2. in die der Athiorhodaceae. Die erste umfasst diejenigen Purpurbakterien, die in ihrem Innern freien Schwefel in sichtbaren Kügelchen einzulagern vermögen, die 2. umgreift hingegen alle die Purpurbakterien, denen die erwähnte Fähigkeit vollständig abgeht" (4, p. 25–27).

With this second introduction of a large systematic unit, again created exclusively on physiological grounds, a peculiar situation arose. It is understandable

that investigators who took notice of Molisch's publication felt inclined to agree that the highly characteristic pigmentation of the purple bacteria might be as good a criterion for the foundation of an order as is the production of the special pigments which typify the large groups of the red and brown algae. But was there any good reason for disregarding the physiological similarities between the colorless and the red sulfur bacteria as a strong token of the relation between these two groups? At the time the purple sulfur bacteria appeared as a link between two utterly distinct groups of bacteria, and in the two proposals for the orders *Thiobacteria* Migula and *Rhodobacteria* Molisch these organisms formed a bone of contention. The following diagram brings this out.



But while Migula, unaware of the existence of a group of non-sulfur purple bacteria, could conveniently omit a consideration of its systematic position, Molisch, in stressing the obvious relationship between the two kinds of purple bacteria, might have indicated the fate of the colorless sulfur bacteria in a system of classification in which only the purple bacteria were united into an order. This he failed to do.

Since both propositions could be defended, it is only natural that systematic attempts in later years have favored a grouping of the organisms either on the basis of the sulfur metabolism with maintainance of Migula's order (especially Bavendamm (34)), or on the basis of pigment production (Richter (130); Orla Jensen (131); Benecke (132)).

The prototype of a new approach is the rudimentary classification proposed by Kruse (133), who suggested the combination of various kinds of bacteria in a separate unit "Phycobacteria."

"Manche gewöhnlich zu den Bakterien gestellte Wesen, die Beggiatoen and andere farblose Schwefelbakterien, ferner die roten Schwefel- und Purpurbakterien, die Leptothrix, Cladothrix, Phragmidiothrix, Gallionella usw., ... stehen den Spaltalgen . . . . noch näher. Wir möchten vorschlagen, sie geradezu Phykobakterien (Algenbakterien) zu nennen, von ihnen aber als besondere Unterordnungen die farblosen Schwefelbakterien und Purpurbakterien abzutrennen" (133, p. 1160).

The colorless sulfur bacteria and all the purple bacteria here appear as equivalent suborders. It is a little difficult to understand why the non-sulfur purple bacteria are included with other groups as providing evidence for the closer

relation with the algae; they do not possess any of the special characteristics which Kruse enumerates in support of this thesis, such as size, pleomorphism, the presence of a central body, and motility without flagella. Vahle's studies (134) had shown that, apart from the pigment production, an essential difference between the purple bacterium *Spirillum rubrum* and the colorless *Spirillum volutans* cannot be detected. It is probable, however, that Kruse was, more than Vahle, inclined to adopt Molisch's view of the relation between sulfur and non-sulfur purple bacteria, so that any argument which was built upon a consideration of the sulfur bacteria would necessitate the inclusion of the *Athiorhodaceae*.

For classification purposes Kruse's proposal was, however, too vague to be more than suggestive. Its influence may be apparent in later attempts, but since Kruse himself never introduced definite names for orders, families, etc., it is hard to trace. Nevertheless, the idea of establishing a large systematic group on the basis of general characteristics indicative of a relation of certain bacteria to algae, molds, protozoa, etc., was to be developed with a distinct appreciation for its applicability to a better system of classification, especially in the United States. It was Buchanan (135) who, after a survey of the fundamental characteristics of numerous bacterial species, concluded that as many as six major groups could be recognized for each of which he proposed an ordinal rank. The second of these orders, named *Thiobacteriales* in accordance with the rules adopted for botanical nomenclature, corresponds in part with Migula's *Thiobacteria*, and was based essentially on the work of Winogradsky:

"The work of Winogradsky and others on the sulfur bacteria has led some authors to recognize the true bacteria (*Eubacteria*) and the sulfur bacteria (*Thiobacteria*), as primary coordinate groups" (135, p. 160).

Yet, the delimitation of the group differs in one respect quite markedly from that of Migula. Buchanan wrote:

"The thiobacteria [are] characterized by certain relationships to sulphur. They all grow best in the presence of hydrogen sulphid, and always contain sulphur granules or bacteriopurpurin or both" (135, p. 161).

In the publication here referred to Buchanan did not mention Molisch's publications on the purple bacteria. This is included in the bibliography to a later paper of the series (136) which contains a definition of the order:

"*Thiobacteriales* Ordo nov.

Cells various, typically containing either granules of free sulphur, or bacteriopurpurin, or both, usually growing best in the presence of hydrogen sulphide. The cells are plant-like, not protozoan-like, not producing a pseudoplasmodium or a highly developed encysted resting stage. Spores are rarely or never formed" (136, p. 461).

Hence, whereas Migula had considered the presence of sulfur globules as an obligatory criterion for all members of his order *Thiobacteria*, this now has become a facultative characteristic, equivalent to, and apparently replaceable by the presence of bacteriopurpurin. It seems obvious that this emendation was inspired by the recognition of the group of non-sulfur purple bacteria, and by Molisch's contention of the close relationship of *Thio-* and *Athiorhodaceae*. Since Molisch had also demonstrated that the last-mentioned organisms are not sulfur bacteria in Winogradsky's sense, the earlier description ("they all grow best in the presence of hydrogen sulphid") had to be modified accordingly.

Here, then, we see the three groups, the colorless sulfur bacteria, the purple sulfur bacteria, and the non-sulfur purple bacteria, formerly in part combined into two overlapping orders by Migula and Molisch, merged into one systematic unit. Buchanan's suggestion was adopted by the committee of the Society of American Bacteriologists (138), commented upon favorably by Breed, Conn, and Baker (139), and it has been incorporated in the past 5 editions of Bergey's Manual of Determinative Bacteriology. Also Pribram (140) accepted the order in the above sense. Very similar is, furthermore, the system proposed by Bavendamm (151) in 1936. The points of difference are, however, worth a brief discussion because they emphasize a difficulty which will presently be taken up.

In introducing his classification Bavendamm states: "In Anlehnung an den verdienstvollen Erforscher der Schwefelbakterien Winogradsky, der diesen Namen zuerst aufgestellt hat und durch van Niel insofern gerechtfertigt ist, als tatsächlich die Verwertung des Schwefels und seiner Verbindungen als einziger Energiequelle das feste Bindeglied zwischen allen Thiobakterien ist, bin ich nicht dem Vorschlag von Engelmann und Molisch gefolgt, den farblosen Schwefelbakterien die Purpurbakterien entgegenzusetzen. Ich habe vielmehr den Leuco- und Chlorothiobakterien die Rhodothiobakterien zugestellt und die schwefelfreien Purpurbakterien zunächst den schwefelspeichernden anhangsweise zugeordnet" (151, p. 48).

So definite is here the stress on the sulfur metabolism that this becomes the sole criterion of the group of Thiobacteria:

"Bakterien, die Sulfide, Sulfite, Thiosulfate und elementaren Schwefel dehydrieren. Es sind entweder farblose oder gefärbte, obligate oder fakultative Anorgoxydanten, die Schwefel und seine Verbindungen als einzige Energiequelle verwerten können" (151, p. 49).

The subdivision of this group is then carried out as follows:

A. Colorless sulfur bacteria, chemoautotrophic (*Leucothiobacteria*).
B. Colored sulfur bacteria, photoautotrophic (*Chromothiobacteria*).
   I. Purple sulfur bacteria (*Rhodothiobacteria*); with addendum: non-sulfur purple bacteria (*Athiorhodobacteria*).
   II. Green sulfur bacteria (*Chlorothiobacteria*).

The *Athiorhodobacteria* do not, apparently, constitute a group of equivalent rank with the *Leuco-*, *Rhodo-*, and *Chlorothiobacteria;* they form an appendix to the second, with the following characterization:

"Durch Bakteriopurpurin rot gefärbte Bakterien, meist von üblicher Bakteriengrösse, die offenbar Übergangsformen der Purpurschwefelbakterien darstellen. Sie sind nicht in der Lage, Schwefel und seine Verbindungen als einzige Energiequelle zu verwerten. Sie sind obligat heterotroph, aerob im Dunkeln und anaerob im Licht" (151, p. 49).

This passage shows that Bavendamm cannot but admit the very close relationship between sulfur and non-sulfur purple bacteria; in fact, the latter are considered as transition stages. But how can one ever hope to properly place one of these organisms, specifically described as obligatory heterotrophic, unable to use sulfur and its compounds as sole energy sources, in a larger group for which the autotrophic nature and the oxidation of inorganic sulfur compounds is a prerequisite?

Obviously, in so emphasizing the "sulfur bacteria" nature of the *Thiorhodaceae*, the sulfur-free counterparts must be either left out, or smuggled in. If the two groups of purple bacteria are considered as taxonomically related, this should come out in the definition of the larger unit of which they both form a part. This Bavendamm failed to do, and hence his method of treatment is clearly inferior to that of Buchanan, although the latter's diagnosis of the *Thiobacteriales* is

open to the criticism that it is difficult to understand why the presence of bacteriopurpurin is somehow equivalent to the occurrence of sulfur globules.

Admittedly, the synthesis accomplished by the establishment of Buchanan's order appears fully justifiable since the purple sulfur bacteria seem to form such an obvious link with the colorless sulfur bacteria on the one hand, and with the non-sulfur purple bacteria on the other. But there is a fallacy in this argument which has not been adequately realized. This is the exclusive use of two completely independent characters, occurring together in one type, for establishing relationships between groups which each possess only one of them. In this way it would be argued that because there are organisms which cause both an alcoholic fermentation and a plant disease, the group of bacteria which produce alcohol must be related to those which have a different metabolism but are plant pathogens. It is, of course, not restricted to the use of physiological characteristics; any two independent characters combined in one, and also found separately in other groups would lead to the same conclusion. The argument is logically inadmissable.

Consequently it becomes necessary to re-evaluate the evidence for the affinities between the colorless sulfur bacteria, the purple sulfur bacteria, and the non-sulfur purple bacteria, so that the order *Thiobacteriales* as now defined may be replaced by a more acceptable grouping of the organisms that comprise it.

Firstly we should face the question whether the facts justify the maintenance of a systematic unit based upon a specialized sulfur metabolism. Winogradsky's investigations (10, 11), restricted to the larger forms, gave the impetus to its creation, both in view of the peculiar morphology of the "sulfur bacteria," and of the important new principle in biochemistry (chemo-autotrophism) which he derived from his physiological studies. But the work of Nathansohn (141), Jacobsen (142, 143), Beijerinck (144, 145), Lieske (146), Waksman and Joffe, (147), Starkey (148), and others made it evident that the same fundamental type of metabolism, the oxidation of hydrogen sulfide, sulfur, and thiosulfate, is shared by many organisms which are morphologically typical *Eubacteriales*. To be sure, these do not, as a rule, deposit sulfur droplets inside their cells, and hence do not belong to the "sulfur bacteria" as long as the latter are defined as organisms internally containing recognizable globules of elementary sulfur. Such a definition is, however, quite unsatisfactory, as has been pointed out before. (Cf. p. 3; also (3, p. 56–57)). Those who have examined the typical microflora of sulfur springs are familiar with the organism which Miyoshi (149) named *Leptothrix sulfurea*, and which differs from *Thiothrix* only in that the filaments do not seem to store sulfur inside, but deposit it externally. Since there are good reasons for believing that the cell diameter determines the location of the sulfur deposition, it is much more reasonable to base the definition of a sulfur bacterium on its characteristic metabolism. In that case one would, however, have to include the *Thiobacillus* species, etc., whereby the group would become so heterogeneous that its usefulness seems problematical. Such an assemblage certainly could no longer be considered as even remotely "natural."

This must have been responsible for the attempts at formulating the group

of "sulfur bacteria" in such a manner as to avoid the need for including the genus *Thiobacillus*. But despite the fact that this has been achieved, however artificial and unsatisfactory the manner, the present group of "genuine" colorless sulfur bacteria is, upon closer inspection, no more than a conglomerate of species whose only claims to inclusion in a common order seem to be that they contain sulfur globules, and are difficult to fit into any of the remaining orders. It hardly needs pointing out that a group which contains the colorless counterparts of *Oscillatoria*, *Phormidium*, and *Schizothrix*, as well as the large, non-flagellated *Achromatium*, the flagellate *Thiophysa*, and the vibrio-like *Thiospira*, corresponds so little to a systematic entity that even the inclusion of *Thiobacillus* would not make it less satisfactory.

This leads to the conclusion that the colorless sulfur bacteria had best be abandoned as a group, except perhaps in a physiological sense. In the latter interpretation there is no reason to exclude the pseudomonas-like *Thiobacillus* species. For taxonomic purposes the various members should be distributed over a number of quite distinct and separate groups.

If this be accepted as a logical procedure, then the present order of the *Thiobacteriales* becomes limited to the purple bacteria, in fact corresponds to Molisch's order *Rhodobacteria*, or Pringsheim's *Rhodobacteriales* (150). Once regarded as a physiological group, characterized by the presence of bacteriopurpurin, it can today be defined more adequately since it has been shown that this pigment—or at least its green component—permits of a photosynthetic metabolism. In the previous sections it has been observed repeatedly that this metabolism is fundamentally similar in both the sulfur and the non-sulfur purple bacteria, and that the photosynthetic aspects mark it as quite distinct from that of other bacteria, including the colorless sulfur bacteria. Is this a strong enough argument to maintain a systematic unit for all the purple bacteria?

It appears that different investigators have been inclined to answer this question in different ways. Schneider, the first to take up this matter after the photosynthetic nature of the metabolism of the purple bacteria had been established, wrote:

"Im Gegensatz zu Bavendamm (1924) glaube ich, dass man bei einer Beurteilung der Verwandtschaft dieser Bakteriengruppen nach phylogenetischen Gesichtspunkten die Thiorhodaceen mit den Athiorhodaceen zusammenfassen und sie nicht wegen ihrer Fähigkeit zur Speicherung von Schwefel den farblosen Schwefelbakterien zuordnen sollte" (22, p. 83).

As we have seen, Bavendamm later did decide to adopt a system of classification in which the two groups of purple bacteria were kept close together. But because he still clung to the idea of a major group of "sulfur bacteria," the attempt led to considerable difficulties.

Fundamentally different was the solution which Pribram (152) proposed in 1933. It represents one of the few modern attempts to develop a classification which is consistently based upon the use of morphological characteristics only. Hence the various purple bacteria, both *Thio-* and *Athiorhodaceae*, are found scattered over three of the four orders of the subclass *Algobacteria*, along with

colorless species of *Micrococcus*, *Pseudomonas*, etc. This is not the place to enter into a detailed discussion of Pribram's system. It may suffice to state that, on account of the very limited number of morphological features used, it contains much that seems of doubtful value. Few microbiologists to-day would, I believe, want to take too seriously an arrangement in which *Chromatium*, *Pseudomonas*, and *Myxococcus* are placed in Winslow's family *Pseudomonadaceae*, while *Serratia* and *Hillhousia* together form a second family of the *Pseudomonadales*, and in which the sulfur purple bacterium *Rhodonostoc* is considered as being linked with the *Streptococcus* species by *Leuconostoc mesenteroides*.

The primary use of morphological criteria was also adopted by Kluyver and van Niel (153) in developing an outline for the classification of the *Eubacteriales*. Here, however, physiological characteristics were considered as equally indispensable, though they were employed only for the definition of genera. Since morphologically the various purple bacteria do not exhibit features which are different from those of the "true bacteria," they were all included in this order, forming the genera with a photosynthetic metabolism (153, table 1). In this way a clear-cut separation of the purple bacteria from the nonphotosynthetic *Eubacteriales* is achieved. The only drawback is that the group as a whole is not represented by a single taxonomic unit. This is due to the fact that the purple bacteria cannot be defined as a group except on the basis of their unique photosynthetic metabolism. As long as certain orders, families, and tribes of bacteria are segregated on morphological grounds, then obviously these customary taxonomic terms cannot be used to designate groups of organisms with the same general morphology, but with special physiological properties.

Perhaps this is not too serious a disadvantage. By combining morphological and physiological characters for the delimitation of smaller systematic units (genera) a system is developed in which similarities in one or the other aspect can easily be visualized. Table 11, a greatly condensed version of the one published by Kluyver and van Niel (153), makes this clear.

Such a tabulation shows that the use of important morphological characters as the guiding principle in bacterial classification results in a columnar arrangement of genera which may together be regarded as higher taxonomic units (e.g. families), whereas the primary use of physiological properties leads to rows of genera possessing this attribute. The decision as to which of the two shall be used for the creation of larger systematic entities may seem quite arbitrary. And, in fact, the two alternatives have been used in the past without much discrimination. One fact stands out clearly; wherever they have been employed side by side in the same system the outcome has been confusing. Only by a consistent use of one or the other can this be avoided.

But is the selection merely a matter of personal preference, of a desire to stress the aspects in which the particular investigator has been most interested? I believe that there are good reasons for thinking otherwise. Stanier and van Niel (154) have shown that at the present time one can clearly distinguish three primary classes among the bacteria on the basis of their general morphology. These, the *Eubacteriae*, the *Myxobacteriae*, and the *Spirochaetae*, represent groups

without any clearly recognizable interrelations, but in themselves they appear to be quite homogeneous. Again, in the *Eubacteria* definite assemblages stand out more or less clearly as morphologically characterized entities (153). It is true that there exist a number of organisms whose affinities are not yet very distinct, but this does not affect the main thesis.

On the other hand, some of the outstanding physiological properties that have been deemed of a sufficiently essential nature to be used for the purpose of combining larger groups of bacteria occur scattered throughout a number of morphological "families." In many cases it is quite obvious that such physiological groups are far less uniform than the morphological ones. Good examples of this kind are the sulfur bacteria, the hydrogen bacteria, the iron bacteria, the urea bacteria, etc.

TABLE 11

Morphological and physiological characteristics of some of the genera of the Eubacteriales

PHYSIOLOGICAL TYPE OF METABOLISM	MORPHOLOGICAL		
	Polarly flagellated rods	Spherical organisms	Peritrichously flagellated rods
Photosynthetic	*Chromatium*, *Rhodovibrio*, *Rhodospirillum*, etc.	*Thiopedia*, *Thiosarcina*, etc.	
Chemoautotrophic	*Sulfomonas*, *Nitrosomonas*, etc.	*Nitrosococcus*, *Siderocapsa*, etc.	
Heterotrophic oxidative	*Pseudomonas*, *Vibrio*, *Spirillum*, etc.	*Micrococcus*, *Sarcina*, etc.	*Kurthia*, *Bacillus*, etc.
Fermentative	*Zymomonas*, etc.	*Streptococcus*, *Betacoccus*, etc.	*Aerobacter*, *Aerobacillus*, *Clostridium*, etc.

These considerations tend to favor the view that a physiological classification is less apt to lead to a rational system as far as the larger units are concerned than a strictly morphological one. The columns rather than the rows in table 11 would thus appear the more logical for consolidation into groups of higher taxonomic rank. Naturally, special names for physiological groups will continue to be used. However, they should not be converted into family or order designations. Sometimes a physiological character may be associated with a morphologically restricted group. The "lactic acid bacteria" are a good case in point. Nevertheless, one must not lose sight of the fact that the term "lactic acid bacterium" generally implies a morphological as well as a physiological characterization. If all the bacterial types capable of fermenting sugars with the formation of lactic acid as the chief metabolic product were included, the group would certainly not represent a taxonomically acceptable unit.

The above arguments for a primarily morphological classification apply, of course, to the purple bacteria. Nevertheless, in this instance one could make out a good case for the use of a physiological property. The photosynthetic ability of this group of organisms could well be considered as a characteristic of such far-reaching importance that it would seem adequate as the basis for a major differentiation in the bacterial kingdom. It may here be reiterated that a separation of the bacteria from the bluegreen algae is at present impossible in any other way (See 154). It could even be contended that such a segregation of the purple bacteria would rest on a morphologically detectable difference provided by the occurrence of a special pigment system. This, however, would be valid only on account of its established function; past attempts to differentiate bacterial groups larger than genera by pigmentation have invariably led to difficulties.

In any case, even the use of the photosynthetic metabolism for the creation of an order for the purple bacteria in the class *Eubacteriae*, as was proposed by Stanier and van Niel, is not free from objections. While for the moment photosynthesis may appear to be an utterly distinctive process, future developments in our understanding of the photosynthetic process may well obliterate this. When first conceived, the distinction between auto- and heterotrophic metabolism seemed quite sharp indeed; during the past several years it has become increasingly evident that a clear line of demarcation cannot be drawn (59).

The purple bacteria so far known form a group in which there is but a limited morphological diversification. More in particular is this true for the non-sulfur purple bacteria; they all fall, as has been pointed out, in the morphological family of the *Pseudomonadaceae*, and represent the pseudomonas, vibrio, and spirillum types, resembling completely the non-photosynthetic members of these tribes. If, under certain conditions, one of the non-sulfur purple bacteria would fail to produce its prominent pigment system, it would thereby become indistinguishable from a typical *Pseudomonas*, *Vibrio*, or *Spirillum* species. The loss of chlorophyll formation in certain genera of algae is well known, and the parallel genera *Chlorella-Prototheca*, *Euglena-Astasia*, *Chlamydomonas-Polytoma* among the *Chlorophyta*, as well as *Oscillatoria-Beggiatoa*, *Phormidium-Thiothrix*, *Schizothrix-Thioploca* in the *Myxophyta* show convincingly the derivation of the colorless forms. Since the transformation from pigmented to non-pigmented forms, but not the opposite, has been experimentally achieved, it appears more logical to consider the purple bacteria as the progenitors of the corresponding non-photosynthetic bacteria.

This does not imply that each and every one of the representatives of the latter group should have an immediate counterpart among the purple bacteria. It is to be expected that various modifications of colorless forms would have arisen secondarily. But an independent derivation of the purple bacteria and the bacteriochlorophyll-free representatives of the spherical and polarly flagellated *Eubacteriae* would necessitate the assumption of a fully parallel development in the two groups. The segregation of the purple bacteria as an order in the

Eubacteriae, as proposed by Stanier and van Niel (154), would suggest this less probable relationship.

For these reasons it seems to me preferable to abandon also the physiological group of the purple bacteria as a taxonomic unit, and to incorporate the various members as genera in the corresponding morphological families of the *Eubacteriae*. The primary subdivision of this large assemblage can then be carried out much more consistently; the order *Rhodobacteriales* could profitably be replaced by an order *Pseudomonadales*, comprising all polarly flagellated, gram negative bacteria.

By thus proposing a taxonomic revision in which neither the order *Thiobacteriales* Buchanan, nor the order *Rhodobacteriales* Pringsheim is maintained, a grave inconvenience from the point of view of determinative bacteriology is likely to result. Heretofore the purple bacteria as well as the colorless sulfur bacteria, both possessing rather outstanding characteristics which made their allocation to a restricted group a simple matter, could be identified much more readily than would be possible on the basis of a rigidly morphological system of classification. This situation can be greatly improved by realizing that the restriction of Latin names for the columns in Kluyver and van Niel's diagram pertains exclusively to the problem of taxonomy. This does not mean, however, that additional sets of determinative keys could not be prepared which would allow of the rapid differentiation of bacteria on the basis of physiological characteristics. This, in fact, seems a most desirable elaboration of the systems now in use which are practically without exception based upon a confused utilization of morphological and physiological criteria for the differentiation of orders and families. If this were done, the accumulated experience of many workers in different branches, and with varied methods of approach could be made available in a much more accessible manner than is possible by the present compromises. Complications can be avoided by the use of common, non-Latin names for such physiological groups, i.e., for the conglomerates of organisms which are represented by the rows in the before-mentioned diagram.

Past criticism of both "scientific" and "utilitarian" systems have frequently failed to recognize the need for both approaches. There has been an unfortunate tendency to identify a proposed system of classification with a set of determinative keys. The adoption of a number of accessory keys for determinative purposes would, I believe, materially simplify the problem of achieving a more consistent system of classification without sacrificing its usefulness from the determinative standpoint.

If, for example, a key were available by which various "sulfur bacteria"—but not *Thiobacteriales* or *Rhodobacteriales*—could be identified as far as the genus, then such an ecological-physiological group, comprising *Thiobacillus*, *Beggiatoa*, *Chromatium* species, etc., would not be in the least objectionable, since this group does not now constitute a larger, real or pretended, *taxonomic* unit. Yet it would be of considerable help to the student of an ecological community of microörganisms who has had no opportunity or desire to familiarize himself with representatives of other groups. Also certain families, e.g. the *Nitrobacteriaceae*, *Lactobacteriaceae*, etc., could more logically be replaced by common designations (chemo-autotrophs, acetic acid and lactic acid bacteria), and thus become more recognizably what they, indeed, are: physiological groups.

Since a determinative system, at least as far as the keys are concerned, is aimed at being avowedly utilitarian, an approach from as many angles as are now represented by specialized interests could in this manner be consolidated. At the same time, the final "system of classification" would not need to be a compromise. Even organisms of uncertain systematic position, temporarily placed in one or more appendices to the "system," would still be "determinable" with the aid of one or more of the various keys.

The allocation of the non-sulfur purple bacteria to an order *Pseudomonadales* of the *Eubacteriae* appears to me taxonomically desirable, as pointed out before. The genera, discussed in detail in the next section, belong morphologically in the families *Pseudomonadaceae* and *Spirillaceae*. Hence the once united group of the "*Athiorhodaceae*" becomes scattered, though, as will shortly appear, not to the extent which Molisch feared as a result of abandoning an attempt at creating a "physiological order" for the purple bacteria. With the aid of determinative keys, including aspects of the physiology and ecology of the organisms, the purple and brown bacteria can, however, still be admitted as a special group on the basis of their pigment system and photosynthetic metabolism. But the characterization of this group will then be divorced from implications of primary taxonomic significance.

II. Detailed classification of the non-sulfur purple and brown bacteria: the genera

There exists only one detailed systematic treatment of the non-sulfur purple and brown bacteria based upon original observations. This is the system developed by Molisch (4) in which the family *Athiorhodaceae* is subdivided into seven genera, each one morphologically defined. Since a very small number of morphological characteristics appeared sufficient for a differentiation, the resulting system has the irresistable appeal of simplicity. This can best be appreciated by an examination of Molisch's arrangement which follows.

Family *Athiorhodaceae*.

a. Cells combined into families.
   Division in one direction only.
   1. Cells rod-shaped, many embedded together in a common mass of slime..... *Rhodocystis*.
   2. Cells spheres or short rods, arranged like strings of beads, each string surrounded by a slime capsule..... *Rhodonostoc*.

b. Cells single.
   Division in one direction.
   1. Cells spherical, immotile..... *Rhodococcus*.
   2. Cells straight rods, immotile..... *Rhodobacterium*.
   3. Cells rod-shaped, motile..... *Rhodobacillus*.
   4. Cells short, curved, bean- or comma-shaped, with a polar flagellum, very motile ..... *Rhodovibrio*.
   5. Cells spirally wound, with polar flagellum or tuft of flagella..... *Rhodospirillum*.

This system has stood the test of time remarkably well. For 35 years it has been copied without a single essential modification by every one who has written on this group of organisms. The only change has been based on nomenclatorial considerations. Since the generic name *Rhodococcus* appeared to be a homonym, Buchanan, in 1918 (136), proposed the substitution of *Rhodosphaera* for *Rhodo-*

coccus. Seven years later the same author (137) pointed out that also the new name "is invalid as a generic designation for the bacteria, as the name *Rhodosphaera* was given by Engler in 1881 to a genus in the family *Anacariaceae* among the flowering plants" (137, p. 449). Hence in the second and all subsequent editions of Bergey's Manual of Determinative Bacteriology it was replaced by *Rhodorhagus*.

Unquestionably the general tendency to accept Molisch's system so completely is due to the fact that so few investigators have afterwards studied this group of purple bacteria at first hand. Furthermore, the descriptions are clear, in spite of their brevity, and their value is tremendously enhanced by the excellent photomicrographs representing the seven genera. Even a superficial acquaintance with the various members of the group often suffices to identify a culture as *Rhodocystis*, *Rhodonostoc*, *Rhodobacillus*, or *Rhodospirillum*. The recent descriptions, published by Czurda and Maresch (20), of some representative strains appear, in comparison, far from satisfactory. Their paper would have benefited greatly from the inclusion of illustrations.

Because five of the seven genera are represented by a single species, while in *Rhodococcus* only two, and in *Rhodospirillum* three species were recognized by Molisch, the determination of a genus is practically equivalent to the complete identification of a given strain of purple bacteria on the basis of his system. Also in this respect very little of importance has been added by later workers. Two additional species of *Rhodospirillum* have been proposed by Hama (50, 155) on entirely insufficient grounds. Three attempts have been made at amplifying Molisch's descriptions. The first deals with *Spirillum* (*Rhodospirillum*) *rubrum* which Molisch recognized as a purple bacterium without describing it, probably because he felt that Esmarch's characterization was satisfactory. Of this bacterium Vahle (134) published a detailed and accurate description. Both the others pertain to *Rhodobacillus palustris*. Plowe *et al.* (156) made some observations on an organism isolated from the intestines of a Cerambycid beetle which they considered identical with the above-mentioned species. Claiming that Molisch's characterization was inadequate, they published a brief description of their culture which shows unmistakably that it cannot have been *Rhodobacillus palustris*, and makes it highly probable that it was not even a purple bacterium. Schneider's account (22) of *Rhodobacillus palustris* is rendered worthless by the fact that he did not use pure cultures.

However useful Molisch's system may have been, it cannot be denied that it presents a few features which to-day distinctly limit its validity. As has previously been stated, every one of the large numbers of cultures which I have examined during the past 12 years has appeared motile. In each case a polar arrangement of the flagella has also been demonstrated. Since there are good reasons for believing that all the previously observed nonsulfur purple bacteria are now represented in my collection, this finding invalidates the description of the genus *Rhodococcus* (*Rhodorhagus*), and eliminates altogether the genus *Rhodobacterium*. Furthermore, it is virtually impossible to decide what type of organism is to be designated as *Rhodovibrio*. This genus was described by Molisch on the basis of observations with impure cultures. Now it is perfectly true that one may find purple bacteria which agree with the characteristics of *Rhodovibrio*. But it is equally certain that under different conditions the same strains are morphologically indistinguishable from *Rhodobacillus*. This is, perhaps, most strikingly illustrated by the fact that Molisch himself has presented evidence to the same effect. A painstaking comparison of the photomicrographs of *Rhodobacillus palustris* from a gelatin plate culture, and of *Rhodovibrio parvus* (4, Plate 1, Fig. 1, and Plate 2, Fig. 10) fails to reveal any perceptible difference between the two organisms. On several occasions I have isolated what appeared to be indubitable *Rhodovibrio* strains, but they have invariably shown a range of morphological variation which includes the most typical habitus of *Rhodobacillus palustris* (4, Plate 1, Fig. 2). *Vice versa*, I have observed with all strains of the latter organism cultures which should be designated as characteristic *Rhodovibrio* cultures.

Not having been in a position to follow the development of *Rhodovibrio parvus* for lack of a pure culture, Molisch apparently considered the morphology of what he observed in some crude cultures as sufficiently distinctive and constant to warrant the creation of this genus. On the basis of our present information it seems to me necessary to conclude that *Rhodobacillus* and *Rhodovibrio* do not actually constitute distinguishable genera.

The genus *Rhodocystis* is morphologically characterized by the formation of cell masses embedded in a common slime capsule, while *Rhodonostoc* consists of single strands surrounded by mucus. There are in my collection 25 strains of purple bacteria which, on the basis of a number of morphological and physiological properties, I consider as closely related. Some of these show the typical *Rhodocystis* clumps, and produce cultures in which single individuals or strands are extremely rare. Yet other strains of this group, when grown in liquid media, give rise to a mucilaginous sediment that can easily be redispersed. In this case one finds few clumps; the cultures consist chiefly of capsulated chains of cells or even of single individuals. Again, the genus *Rhodocystis* was created on the basis of observations on crude cultures; only *Rhodonostoc* was apparently studied in cultures derived from single colonies, as witnessed by Molisch's description of a stab culture. Although from my own experience I am fully convinced that there undoubtedly exist organisms corresponding to these genera, and which are different in several respects, Molisch's method of differentiation must be deemed much too simple.

Yet another difficulty concerning the genus *Rhodonostoc* lies in its close similarity to *Rhodococcus*. This appears conclusively from an examination of the hundreds of photomicrographs of cultures which can be recognized as belonging to either one of these genera. Depending upon the culture conditions one and the same strain may, also in this case, suggest its identity with *Rhodonostoc* or *Rhodococcus*. The close resemblance can also be inferred from Molisch's own descriptions, although his illustrations show examples of cultures which can be easily differentiated on a morphological basis. In the description of *Rhodonostoc capsulatum* Molisch calls special attention to the brown color of this organism in stab cultures, and concludes: "Der braune Stich dieser Bakterie ist recht charakteristisch." (4, p. 23). But also the representatives of the genera *Rhodocystis* and *Rhodococcus* are brown instead of red in stab cultures. This must have escaped Molisch's notice because it is only under anaerobic conditions that the brown color of *Rhodococcus* is pronounced, and he evidently did not prepare stab cultures of *Rhodococcus*.

On account of these facts it seems very doubtful whether the three genera, each with one or two species, should be maintained. They might, at least for the present, be more logically combined. There appears to be some justification for preserving the genus *Rhodocystis;* the group of cultures of this type is characterized by a number of properties which set them apart from the other brown and purple bacteria. Since, however, the most important diagnostic feature of *Rhodocystis, viz.*, the occurrence of cell masses in a common slime capsule, is not shared by all the strains of this group, the name would be misleading. Also, the rather exclusive color of Molisch's *Rhodocystis gelatinosa*, referred to as "pale peach-colored," is characteristic only for some of this group of strains, and even for these not in all media. In yeast extract, for example, all strains are brown, though of various shades.

The *Rhodocystis* strains are the most outspokenly rod-shaped of the various brown bacteria. But even *Rhodococcus* develops in certain media as distinct rods. The three genera therefore possess in various degrees the morphological characters of the *Pseudomonadaceae*.

In view of the pseudomonas nature of the red organisms previously designated as *Rhodobacterium*, *Rhodobacillus*, and *Rhodovibrio*, Kluyver and van Niel have proposed the new generic name *Rhodomonas* for the species of this group (153). Czurda and Maresch have, however, pointed out (20) that this had previously been used by Karsten for a genus of cryptomonads. For this reason they replaced it by *Rhodopseudomonas*.

On similar grounds Kluyver and van Niel (153) tentatively proposed a genus *Phaeomonas* to include the different brown representatives of the non-sulfur purple bacteria. This was later accepted by Czurda and Maresch (20). As the type species for the genus the former authors designated *Phaeomonas* (*Streptococcus*) *varians* (Ewart). Unfortunately this

organism has not been characterized by its original author (19) in such a manner as to permit of its unequivocal identification. A careful appraisal of Ewart's description in the light of our present knowledge makes it even doubtful whether his *Streptococcus varians* is, indeed, a member of the purple bacteria group. Hence the genus *Phaeomonas* assumes a doubtful status. It appears, therefore, advisable to use a new generic name for which a more appropriate type species can be designated, or to dispense with the genus altogether.

The former procedure would make it possible to separate the "brown" and the "red" non-sulfur purple bacteria into groups of generic rank. But such a separation is not as easy as it might seem because a number of these brown bacteria develop with a red pigment under aerobic conditions. Even if in the diagnosis it were specified that the brown color is typical only for anaerobic cultures difficulties would still arise. I have frequently observed distinctly red cultures of certain of these strains under strictly anaerobic conditions, especially in malonate media. Furthermore, the characteristically red strains of the *Rhodobacillus palustris* type appear brown in certain media. It is clear, then, that at the present time a satisfactory differentiation would have to be based upon a completely arbitrary, and not even convenient, evaluation of color differences. A better understanding of the nature of the pigments themselves, and of their distribution in the various types may, of course, change the situation.

In view of these considerations, and also of the common morphological features of both the red and brown groups of these purple bacteria and the very small number of distinguishable species, it seems more appropriate to abandon such an attempt until more extensive data are available to suggest the desirability of recognizing more than one major group. For the time being it is consequently proposed to classify the rod-shaped and spherical to ellipsoidal non-sulfur purple bacteria as a single genus, *Rhodopseudomonas* Czurda and Maresch, with an amended diagnosis. The type species is *Rhodopseudomonas* (*Rhodobacillus*) *palustris* (Molisch).

The last genus of Molisch's system, *Rhodospirillum*, is morphologically so distinctive that little needs to be said about it. The existence of brown photosynthetic spirilla has previously (153) led to the proposal of the generic name *Phaeospirillum* for these bacteria. Since the four strains of brown spirilla which I have so far been able to study appear, apart from the color difference and a more pronounced tendency towards anaerobiosis, in all respects very similar to the red ones, such a generic segregation seems premature also in this case. At present I can see no objection to classifying all the photosynthetic spirilla as members of the single genus *Rhodospirillum* Molisch, with the type species *Rhodospirillum rubrum* (Esmarch) Molisch.

By thus reducing the number of genera of Molisch's family *Athiorhodaceae* to only two, the difficulties inherent in his system disappear. Still, this limitation to two genera is not entirely satisfactory. The fact is that the differences between e.g., *Rhodobacillus palustris* and *Rhodospirillum rubrum* do not appear larger nor more significant than between the former and *Rhodococcus capsulatus*. However, as has already been pointed out, the typically spherical *Rhodococcus*, itself occasionally occurring as rods, is linked to the characteristically rod-shaped purple bacteria by *Rhodonostoc* in such a manner that as yet it is too difficult to draw even an arbitrary line of demarkation which would result in a more acceptable system.

As definitions for the two genera the following are proposed.

Genus *Rhodopseudomonas* Czurda and Maresch. Emend.

Spherical and rod-shaped bacteria, motile by means of polar flagella. Non-sporeforming, gram negative. Contain bacteriochlorophyll which enables them to carry out a photosynthetic metabolism. The latter is dependent upon the presence of extraneous oxidizable substances, and proceeds without the evolution of molecular oxygen. Though some members are capable of oxidizing inorganic substrates, none is strictly autotrophic. Produce accessory pigments causing the cultures, especially in the light, to appear in various shades of brownish yellow to deep red.

The type species is *Rhodopseudomonas palustris* (Molisch). The genus includes the members of Molisch's genera *Rhodobacterium*, *Rhodobacillus*, *Rhodovibrio*, *Rhodocystis*, *Rhodonostoc*, and *Rhodococcus*, as well as the genera *Rhodosphaera* Buchanan, *Rhodorhagus* Bergey *et al.*, *Rhodomonas* Kluyver and van Niel, *Phaeomonas* Kluyver and van Niel, and *Rhodopseudomonas* Czurda and Maresch.

Genus *Rhodospirillum* Molisch.

Spiral-shaped bacteria, motile by means of polar flagella. Non-sporeforming, Gram negative. Contain bacteriochlorophyll, and are potentially photosynthetic in the presence of extraneous oxidizable substances. Molecular oxygen is not produced. Unable to live in strictly mineral media. Cultures in the light are brown to red, due to the formation of accessory pigments.

The type species is *Rhodospirillum rubrum* (Esmarch) Molisch. The genus includes *Phaeospirillum* Kluyver and van Niel.

It will be noted that no mention is made of the absence of sulfur globules in the cells; the separation from the sulfur purple bacteria rests upon the ability of the latter to develop strictly autotrophically. It is conceivable that some *Thiospirillum* (*Thiorhodospirillum*) species, none of which has so far been isolated or even studied with respect to their metabolism, may turn out to require organic substances. If this were the case, such species would be included in the genus *Rhodospirillum* as here outlined. I see no objection to this consequence.

A few words must yet be said about the brown bacteria reported by Utermöhl (18). Describing a remarkable combination (*Pelochromatium roseum* Lauterborn?) of small brown bacteria surrounding a larger organism, the author stated:

"Der Hauptgrund für meinen Zweifel, ob die vorliegende Form wirklich zu den Purpurbakterien gehört . . . . ist die Farbe, die durch ihr mattes braunrot auffällig von den mehr rosa- bis violettroten Farbtönen der übrigen Purpurbakterien absticht . . . ." (18, p. 606). "Aus Mangel an Zeit habe ich keine weiteren, mikrochemischen Untersuchungen vorgenommen, aus denen die Art der Farbstoffe näher zu ersehen wäre. Doch dürfte aus meinen Beobachtungen hervorgehen, dass es sich bei den Hüllbakterien um eine neue, den Purpur- und Chlorobakterien vergleichbare Reiche farbstoffführender Bakterien handelt, die ihrer braunen Färbung wegen wohl als Phaeobakterien bezeichnet werden können" (18, p. 607) "Das Binnenbakterium möchte ich als *Endosoma palleum*, die Hüllbakterien nach dem Entdecker dieses Zellverbandes als *Lauterborniola minima* bezeichnen" (18, p. 609).

The description is extremely cursory and so incomplete that it is not even possible to decide whether *Lauterborniola* has a definite connection with the brown members of the photosynthetic bacteria. Utermöhl qualified his first publication as a preliminary com-

munication; the only later reference I have been able to find occurs in an extensive paper by the same author on the phytoplancton of the lakes of Eastern Holstein (157). Here, however, the section on Phaeobacteria occupies only a single paragraph, with the remark:

"Über diese Formen habe ich bereits an anderer Stelle ausführlich berichtet (reference to (18)), so dass ich mich hier sehr kurz fassen kann."

Until further studies will have clarified the nature of "*Lauterborniola*" it is, therefore, necessary to omit it from the various genera now included in *Rhodopseudomonas*.

III. Detailed classification and description of the non-sulfur purple and brown bacteria: the species

> "For in all analysis it is the business of the analyser to get at the ultimate unities; when he has reached the ultimate unities it is also his business to respect them: further division will show acuteness, but it will not show judgment."
>
> Hillaire Belloc "On the simplicity of words". (160, p. 23)

In the previous section it has been pointed out that the morphological characteristics on which Molisch based six of his seven genera of *Athiorhodaceae* do not suffice for a satisfactory differentiation of the organisms. It is true that a thorough familiarity with the various representatives, such as comes with a prolonged study of the group, will enable one to recognize special types which correspond more or less closely to some of Molisch's species. Nevertheless, the information now available concerning the morphology and physiology of a large number of strains should permit of a description of such types in a more immediately useful manner.

Observations on the morphological properties of well over 150 strains, cultured in a large variety of media and under different conditions, have shown that none of the organisms can be adequately characterized by describing its size and shape in a single culture. In the course of time permanent slides, mostly nigrosin mounts, have been prepared of all cultures that seemed of interest from the point of view of keeping a record of these studies. Dr. Arthur L. Cohen has made close to 2000 photomicrographs of these slides, which have made it possible to compare accurately the behavior of individual isolates. The following descriptions are based partly on this material.

Furthermore, the interpretation of the basic physiological properties of this group has led to a study of the growth of each one of the strains in the presence of 40 different, simple substrates. The technique used for these experiments has already been described (cf. p. 33-36). The results have revealed that there exist a number of close correlations between the morphological types and the utilizability of a variety of compounds. Since a large number of strains of each type was available for these studies, the range of physiological variation within each group can also be indicated with a fair prospect of being more or less representative in this respect.

A survey of these morphological and physiological data indicates that six groups of non-sulfur purple bacteria can readily be distinguished. Four of these belong to the genus *Rhodopseudomonas* as previously defined; the remaining two comprise the spiral-shaped organisms, united in the genus *Rhodospirillum*.

On the whole, the various strains which constitute one such group form a rather

homogeneous assemblage. Its members have a number of properties in common which permit of their allocation by means of a variety of morphological, physiological, and biochemical criteria. It seems, therefore, fully justifiable and expedient to consider each such group as a species.

I realize that it would be possible to stress individual strain differences so as to provide for a larger number of species. However, such a procedure is not at present advisable inasmuch as the significance of the differences cannot yet be evaluated in association with other aspects of the organisms. Especially in the absence of extensive observations on the constancy and variability of most of the physiological and biochemical characteristics of any one strain it seems more logical to adopt a policy in which the species are limited to groups with a number of correlated properties. I am wholeheartedly in agreement with the conclusion, reached by Parr and Robbins (161): "It is obvious that our results favor the view that classification should be conservative. New species should only be recognized on the basis of thoroughly well-worked-out cultural differences having real significance, and, if possible, correlated with other characters."

If, later, the need for a further differentiation arises, it is obvious that the reasons prompting it can be more readily incorporated in the manner of subdivision as long as details of uncertain value have not previously been used for the classification of these organisms.

The four groups representing the genus *Rhodopseudomonas* correspond, as far as can be ascertained, rather closely with the species *Rhodobacillus palustris*, *Rhodocystis gelatinosa*, *Rhodonostoc capsulatus* and *Rhodococcus capsulatus*, all of them described by Molisch. They will be designated as *Rhodopseudomonas palustris*, *Rhodopseudomonas gelatinosa*, *Rhodopseudomonas capsulatus*, and *Rhodopseudomonas spheroides*. Molisch's species *Rhodococcus minor*, *Rhodovibrio parvus*, and *Rhodobacterium capsulatum* seem to be, at least to-day, unrecognizable entities.

The fifth group, comprising the *Rhodospirillum* strains, presents a similar problem. Also here two subgroups can readily be segregated. They are composed of the red and the brown photosynthetic spirilla respectively. Since, in the strains I have studied, the pigmentation is correlated with other distinct morphological differences, the recognition of at least two separate species is indicated. But in addition, the various isolates of the red spirilla show unmistakable evidence of further mutual differences. There is, consequently, some reason for increasing the number of species. Here again, with but one exception of a morphologically outstanding type, I have not actually been able to decide, on the basis of the available data, how a further splitting up of the remaining group of red spirilla should be carried out. This group has, therefore, been considered provisionally as a single species, and future investigations will have to show the way in which a more satisfactory treatment of this group is to be achieved in case a need for a continued subdivision will be felt.

In the following description of the species use has been made of those morphological and physiological characteristics which appear to me as significant, both from the point of view of identification and differentiation. A number of commonly recommended descriptive characters have been omitted because they are either in no way characteristic for any one

species, or because they have appeared so variable and dependent upon environmental conditions that no useful purpose is served in mentioning them. This latter case applies, for example, to the shape and size of colonies. Also, certain of the routine procedures for the study of pure cultures of bacteria have not been included in the present investigation since they did not *a priori* offer much promise that information of a basic nature could thereby be obtained. It is granted that, by so doing, the descriptions are "incomplete." But the same may be said of the published accounts of those bacterial species whose only characterization is based upon the use of standardized techniques. It is not uncommon to find that such descriptions convey a rather misleading impression concerning the general nature of the organisms and their outstanding physiological functions, even though some arbitrary tests may serve the useful purpose of permitting a rapid differential diagnosis. An outstanding example of this has been cited by Otto Rahn (158) when he pointed out that, merely on the outcome of the routine tests, *Hydrogenomonas pantotropha* would become indistinguishable from *Phytomonas vascularum*. This may simply mean that a more complete investigation of *Hydrogenomonas pantotropha* with respect to its possible plant pathogenicity, and of *Phytomonas vascularum* as a potential hydrogen oxidizing bacterium would actually establish their identity. In view of the recent claims (Elrod and Braun, 159) that *Phytomonas polycolor* would be the same organism as *Pseudomonas aeruginosa* this would hardly be surprising. It only serves to bring out the fact that the ordinarily practiced description of a bacterium is not necessarily the most satisfactory, nor can it lay claim to any degree of completeness. It is to be anticipated that each particular group of microorganisms can best be studied by some special methodology which would be of little use elsewhere.

These introductory remarks may suffice to justify the approach here used for the following descriptions of the species of non-sulfur purple bacteria. Since the generic diagnoses given in the preceding section cover certain common features, these will not again be mentioned.

Rhodopseudomonas palustris

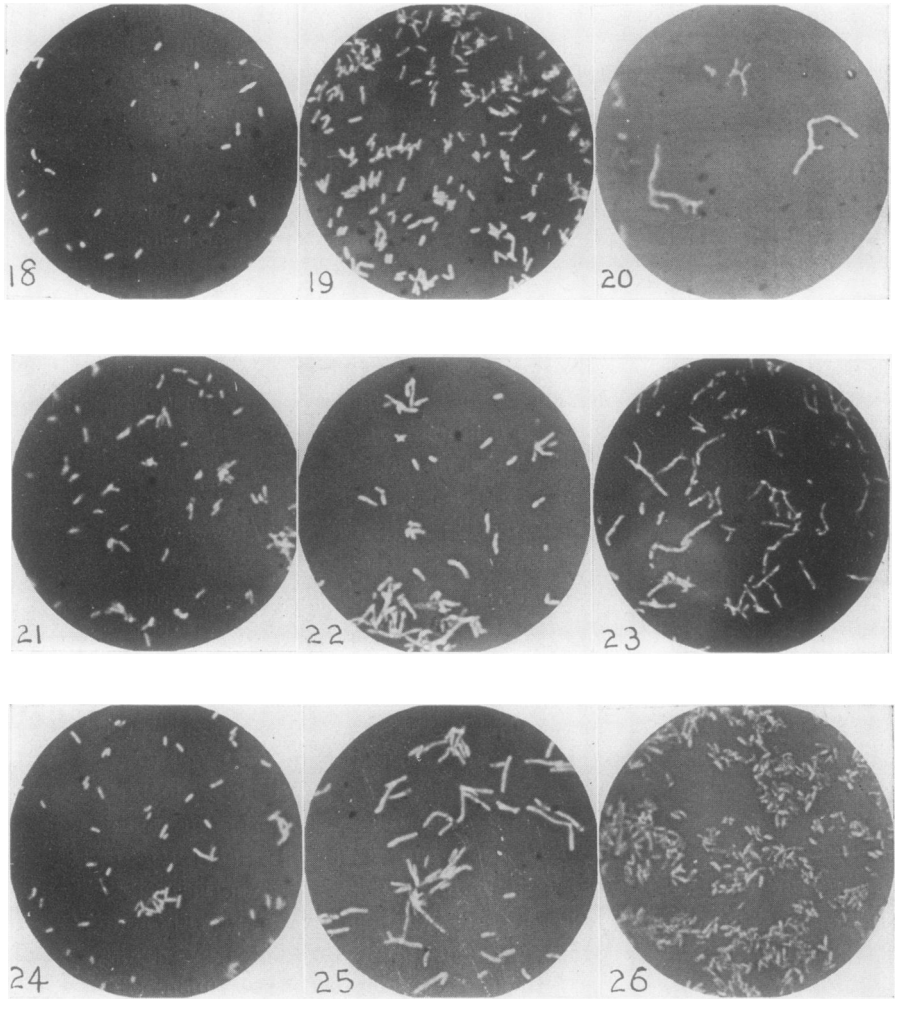
Synon. *Rhodobacillus palustris* Molisch.
Rhodovibrio parvus Molisch.
Rhodomonas palustris Kluyver and van Niel.

This species is represented by 48 individual strains, obtained at various times by a number of different methods. Included are isolations from surface water and mud samples in Holland, California, and Cuba. The Cuban mud samples were made available through the kindness of Dr. J. Heath under whose supervision they were collected, and who forwarded them to me. They have been invaluable in permitting me to test the general validity of the specific enrichment culture methods with material from a far removed locality. Three strains were isolated from impure cultures, kindly furnished by Drs. E. Schneider, Breslau, and H. Gaffron, then at Berlin-Dahlem.

Morphology. (figs. 1–3; 18–26) The bacteria are usually distinctly rod-shaped, although in certain media, and especially in young cultures, very short, vibrio-like organisms may predominate. The size is extraordinarily variable, even for one and the same strain, and is strongly influenced by the age of the culture and by the composition of the medium. The most consistently short cells have been observed in young yeast extract cultures, especially when incubated anaerobically in the light (figs. 1, 26), or in media in which development is generally slow and scanty, such as in the basal medium with malonate (figs. 18, 21, 24). They then measure about 0.6–0.8 x 1.2–2μ, and often appear slightly curved. More frequently the cells are considerably longer, and may attain a length of 10 μ.

Highly characteristic is the pronounced tendency to the formation of rather

irregularly shaped, bent and crooked, long rods, occasionally swollen at one or both extremities, and frequently suggesting branching. These forms can always be found in older cultures, where they occur as clusters, strikingly reminiscent of



FIGS. 18–26. *Rhodopseudomonas palustris*, grown anaerobically in basal medium with various substrates; × 800.

18–20. Strain No. 52, with:
- 18 ..... 0.2% Na-malonate
- 19 ..... 0.2% $Na_2S_2O_3$
- 20 ..... 0.1% Na-*n*-caproate

21–23. Strain No. 66, with:
- 21 ..... 0.2% Na-malonate
- 22 ..... 0.2% Na-crotonate
- 23 ..... 0.2% Na-propionate

24–26. Strain No. 82, with:
- 24 ..... 0.2% Na-malonate
- 25 ..... 0.05% Na-*n*-caprylate
- 26 ..... 1% yeast autolysate

Corynebacterium and *Mycobacterium* species. Such cells are, as a rule, immotile. This behavior is the most readily distinguishing morphological feature of the species (figs. 3, 20, 23, 25).

Growth in liquid media is never mucoid; the sediment which is deposited as the cultures grow older appears homogeneous and smooth, and can readily be redispersed.

Color. The pigment production leads to cultures varying from a light pink to a dark brownish red. This again depends upon the medium; where development is slight, as in malonate, thiosulfate, and usually, glycerol, the lighter shades predominate, while in media containing fatty acids the cultures become more nearly brown.

Most strains produce a water-soluble pigment which tinges the supernatant liquid of older cultures a clear red.

Physiology. The development in yeast extract is not markedly influenced by the reaction of the medium over a range from pH 6 to 8.5. With fatty acids as the main substrate the combined effect of low pH and relatively high fatty acid concentration (0.1–0.2%) may prevent growth. The temperature optimum is generally rather high, good development being possible at 37°C. In this respect there exist, however, strain differences, somewhat lower temperature optima being exhibited by isolates which have been maintained in pure culture for a long period of time.

Characteristic odors are not observable, save that old cultures may develop a faint ionone-like fragrance.

Most strains are able to grow on the surface of agar plates; a few are considerably more sensitive to oxygen and develop only in stabs, in which the upper region may remain free of growth. This behavior is capable of being changed, apparently through adaptation; some strains which behaved like true anaerobes when first isolated are now uninhibited by atmospheric oxygen tension.

None of the strains liquefies gelatin, as shown by gelatin yeast extract stabs kept under observation for as long as 60 days.

Biochemical characteristics. Outstanding among the biochemical characteristics, as revealed by the comparative studies in the basal medium with 40 simple organic compounds (See Chapter 5, Section II), are the ability to grow in thiosulfate, and the rapid and profuse development in glutarate and ethanol media. All strains have failed to grow in solutions in which mannitol, sorbitol, glucose, or mannose were supplied as hydrogen donors; if the media contain an additional, utilizable hydrogen donor development is normal, so that the carbohydrates do not appear to be inhibitory.

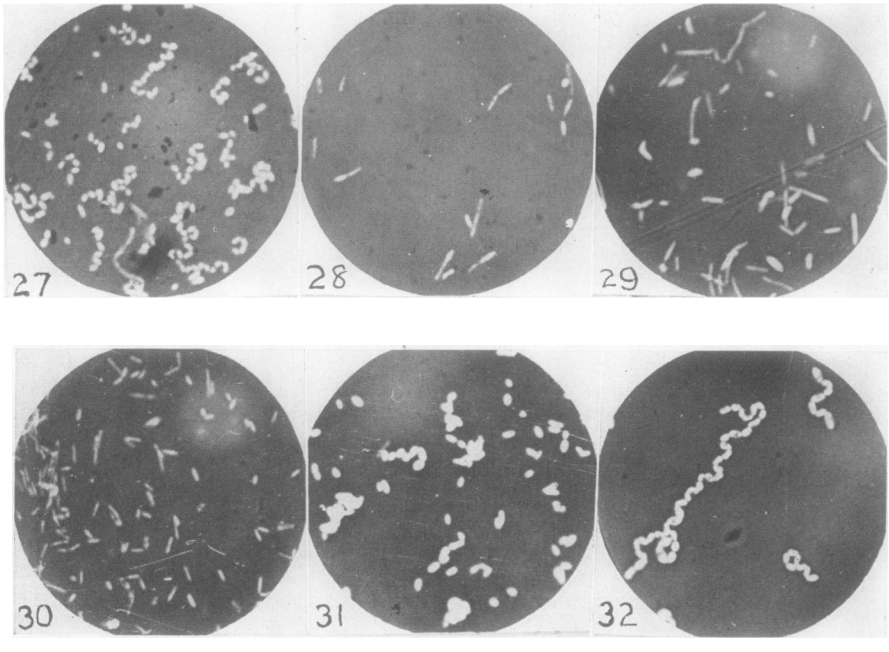
Though not all strains have been tested as to their ability to oxidize molecular hydrogen, those which have been so examined have given positive results.

Of the six amino acids used in this investigation (glycine, alanine, leucine, asparagine, aspartic and glutamic acids) only leucine has invariably given positive cultures; only four strains have grown feebly with alanine, six with glutamic acid, three with asparagine, and two with aspartic acid.

Distinguishing features of this species are its morphological resemblance to

Mycobacterium species, its ability to grow with thiosulfate as oxidizable material, and its inability to attack mannitol, sorbitol, and the carbohydrates.

Enrichment cultures. In accordance with the general biochemical characteristics of the species, it is easily obtained by enrichment cultures, using the basal medium with ethanol, glutarate, or thiosulfate. The last-mentioned solution will, of course, give rise to the simultaneous development of purple sulfur bacteria. For this reason it is simplest to start cultures with alcohol or glutarate. If other non-sulfur purple bacteria are present in such abundance that the isolation of *Rhodopseudomonas palustris* presents difficulties—which, in my experience, has never been the case—subcultures in thiosulfate medium will eliminate the other species almost entirely.



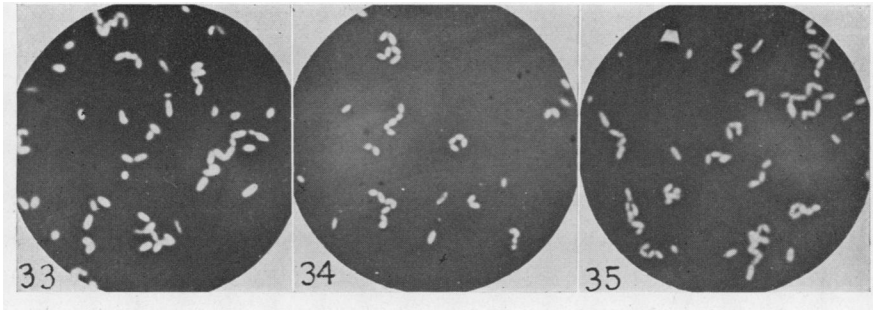
FIGS. 27–32. *Rhodopseudomonas capsulatus*, grown anaerobically in basal medium with various substrates; × 800.
27. Strain No. 49 with Na-*i*-caproate
28. Strain No. 102 with Na-lactate
29. Strain No. 26 with Na-propionate
30. Strain No. 26 with Na-acetate
31. Strain No. 42 with glucose
32. Strain No. 44 with fructose

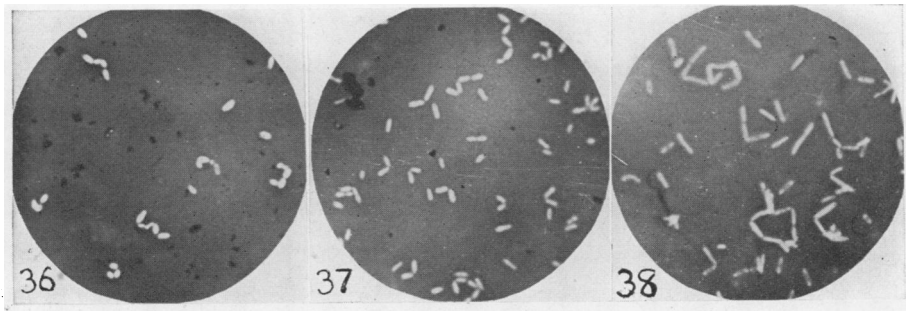
Rhodopseudomonas capsulatus
Synon. *Rhodonostoc capsulatus* Molisch.

Only 16 strains of this species are present in my collection. They have been isolated from enrichment cultures with various organic acids as substrates, and using mud samples from different parts of California, and from Cuba. The 16 representatives show a remarkably uniform behavior.

Morphology (figs. 4–6; 27–38). Depending upon the pH of the medium, the organisms occur as motile, nearly spherical cells, or as distinct rods. The former

are found in media with a pH below 7; they often form chains, and may thus present some resemblance to streptococci (figs. 27, 32, 33, 34). The rod-shaped bacteria are characteristic for media at a pH above 7, and the higher the pH, the longer are also the rods (figs. 28–30). The individual cells usually measure slightly less than 1 μ in width, though in an alkaline environment (pH 8 or above) the rods are attenuated, and often not more than 0.5 μ wide (fig. 30), while in the presence of glucose or fructose slightly swollen individuals can be observed (1.2 μ; figs. 31, 32). The length of the organisms ranges from 1 to 6 μ; in neutral





FIGS. 33–38. *Rhodopseudomonas capsulatus*, grown anaerobically in basal medium with various substrates; × 800.
33. Strain No. 44 with Na-*i*-butyrate
34. Strain No. 44 with Na-*i*-valerate
35. Strain No. 44 with Na-lactate
36. Strain No. 102 with Na-malonate
37. Strain No. 34 with Na-propionate
38. Strain No. 27 with Na-*i*-butyrate

to slightly alkaline solutions the most frequent size is 2 to 2.5 μ. At pH 8 or above an irregular, filamentous and quite abnormal growth occurs.

Special mention should be made of the zigzaggy arrangement of the cells in chains; the angular aspect of the latter is so outstanding a feature that on this alone an identification can be based (figs. 33–38). Cultures in media at pH 8 or above are slimy.

Color. Practically all anaerobic cultures appear brown, the shade ranging from a light, yellowish-brown to a deep mahogany brown. Only in malonate media, where development is not abundant, do the organisms produce a reddish-

brown growth. With none of the strains has the formation of a water-soluble red pigment been observed.

In the presence of oxygen, both in aerobic liquid cultures and on agar plates, additional pigments are produced; the growth under these conditions is distinctly red.

Physiology. Also this organism can develop in yeast extract media over a pH range from at least 6 to 8.5, although at the higher pH the growth is morphologically abnormal. The available strains show a decidedly lower temperature optimum than those of *Rhodopseudomonas palustris*; none develops at a temperature above 30°C.

Most cultures are practically odorless; occasionally a very faint peach-like flavor has been detected.

Growth is not inhibited by the presence of oxygen.

Gelatin liquefaction has never been observed.

Biochemical characteristics. Of the 40 substrates tested, the fatty acids and many of the substituted acids are good carbon sources. All strains grow rapidly and abundantly in propionate media. Glutaric acid leads, at best, to very meager cultures; tartrate, citrate, and gluconate are not utilized. Also ethanol and glycerol, as well as mannitol and sorbitol, fail to produce growth. Glucose and fructose, on the other hand, appear to be satisfactory substrates, while mannose is not attacked. Further diagnostically valuable properties are the ability of all strains to grow in alanine and glutamic acid media, while they fail to do so with leucine.

Thiosulfate is not oxidized; a few strains have been found capable of oxidizing molecular hydrogen, the others have not yet been tested in this respect.

Distinguishing properties. While the most useful morphological criteria are the brown color of anaerobic cultures and the cell-shape of the organisms, especially the appearance of the chains, *Rhodopseudomonas capsulatus* can also be readily distinguished from other species by its ability to grow with propionate, glucose, fructose, alanine, and glutamic acid, and by the absence of development with the above-mentioned alcohols, and with mannose, leucine, and thiosulfate.

Enrichment cultures. The organism can usually be obtained from enrichment cultures with any one of a number of organic acids, particularly propionic, lactic, and succinic acids. Frequently this species also predominates in peptone or yeast extract media. Since the latter are, however, always badly contaminated with a large number of non-photosynthetic bacteria, isolation may be greatly facilitated by one or more transfers to a propionate medium with around 0.2% propionate. By streaking from successful bottle cultures on yeast or peptone agar plates and incubation under aerobic conditions in the dark, the elimination of some other purple bacteria can be accomplished.

The utilizability of glucose and fructose cannot, of course, be made the basis of a satisfactory enrichment method. There are too many non-photosynthetic bacteria which can develop anaerobically with these sugars. Under their influence the substrate is rapidly transformed into a number of decomposition products before the *Rhodopseudomonas gelatinosa* cells have become numerically preponderant. Meanwhile, the initial medium is changed so as to be no longer specific for any one member of the non-sulfur purple bacteria.

Rhodopseudomonas spheroides

Synon. *Rhodococcus capsulatus* Molisch
Rhodococcus minor Molisch
Rhodosphaera capsulata Buchanan
Rhodorhagus capsulatus Bergey *et al.*
Rhodosphaera minor Buchanan
Rhodorhagus minor Bergey *et al.*

A total of 19 strains with a number of common characteristics has been singled out as representatives of this species. They have been obtained in Delft, Holland, and in California from a variety of enrichment cultures, using different media and inocula.

Morphology (figs. 7, 8; 39–54). The bacteria of this group are the most nearly spherical of any of the non-sulfur purple bacteria. In young cultures actively motile, they lose their motility with age, especially if the medium becomes alkaline. The size of the individual cells is extremely variable; without capsule they measure from 0.7 to as much as 4 μ in diameter (figs. 39–50). In media which are or become very alkaline, irregular, swollen and distorted rods are produced, having the appearance of involution forms (figs. 49; 51–54); sugar-containing solutions frequently give rise to egg-shaped, ovoid cells, the dimensions of which generally vary from 2 x 2.5 to 2.5 x 3.5 μ (figs. 45, 50). Although such cells look much more "normal" than the before-mentioned "involution forms" it should be pointed out that in cultures of one and the same strain numerous intermediate shapes have been found between slender, rod-shaped organisms, the ovoid cells, and large, bizarre forms, 2.5μ wide, and often attaining a length of more than 10 μ (figs. 49–54).

The individuals regularly occur singly, only rarely does one encounter short chains.

Considering the variable size of these bacteria it becomes clear that the recognition of species within this group cannot be based solely on cell dimensions, as practiced by Molisch for the separation of *Rhodococcus capsulatus* and *Rhodococcus minor*. Since furthermore the physiological and biochemical characteristics of the strains are quite homogeneous, I have deemed it desirable to recognize for the present only a single species.

Color. As in the case of *Rhodopseudomonas capsulatus*, the anaerobic cultures of *R. spheroides* are brown in color, varying in shade from a light dirty greenish to dark brown. With but four exceptions all strains are capable of producing a water-soluble red pigment with the characteristic absorption maxima of 535 and 565 mμ. When grown in the presence of oxygen the organisms are distinctly red.

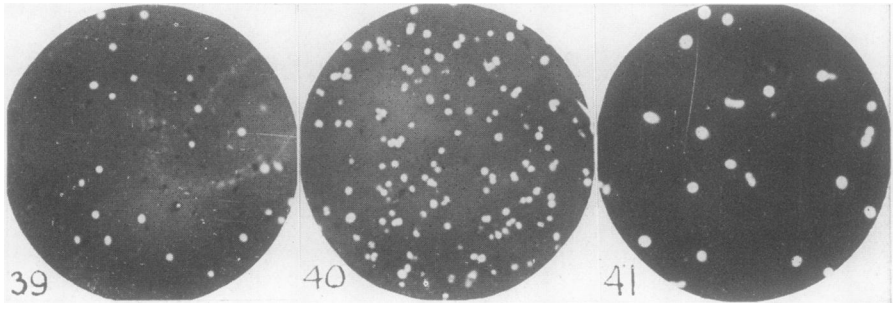
Physiology. Again the pH range over which development is possible is wide, and extends from at least pH 6 to 8.5. The temperature optimum is relatively low, and lies below 30°C.

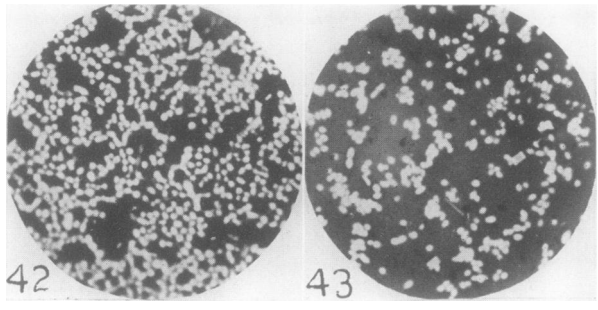
All cultures exhibit an unpleasant, putrid odor; those in which the pH has risen to 7 or above are stringy, due to abundant mucus production.

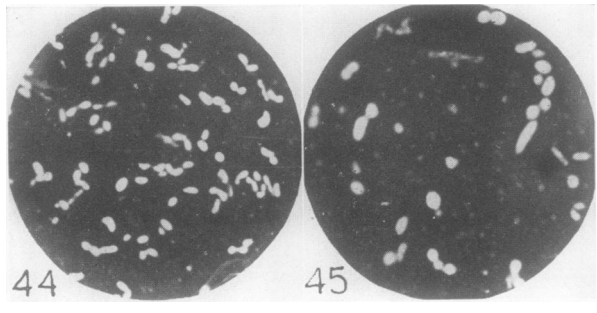
None of the strains liquefies gelatin.

Biochemical characteristics. Cultures of this group in the basal medium with various substrates show, as a rule, much less copious development than those of

the previous two species. Because better results are obtained by increasing the amount of yeast extract in the medium it seems likely that the growth-factor







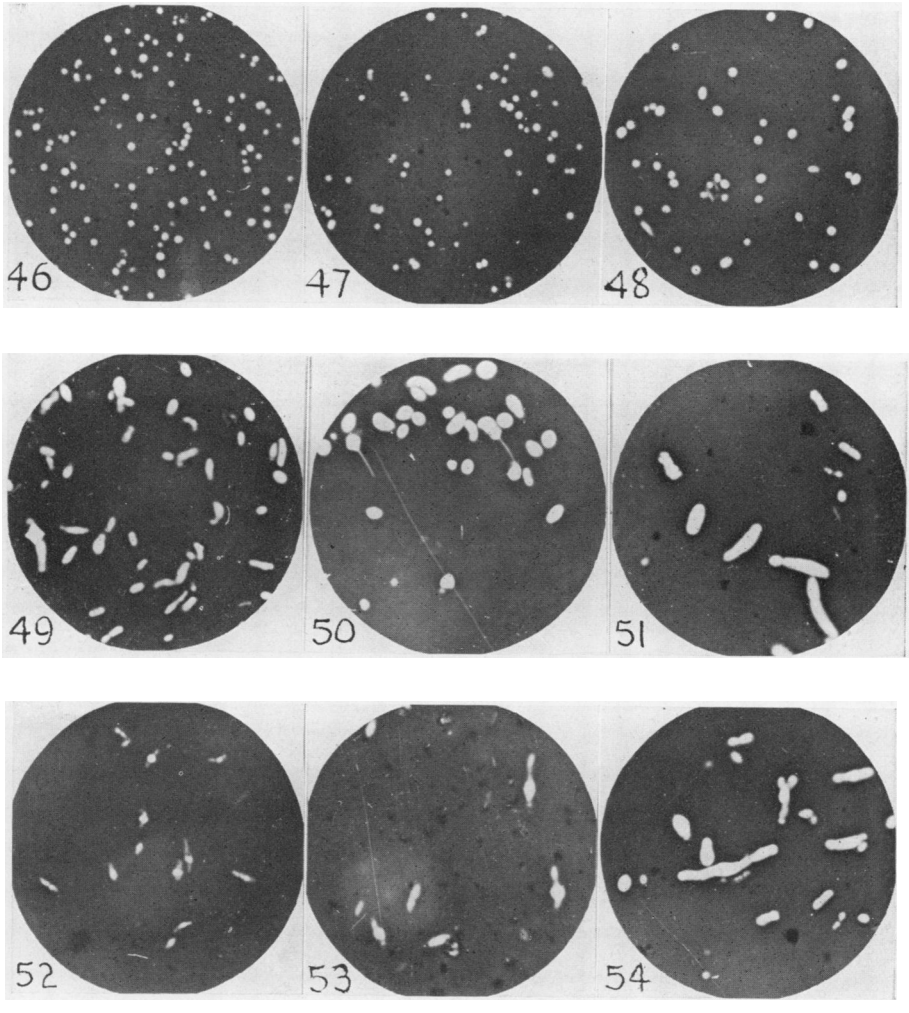
FIGS. 39–45. *Rhodopseudomonas spheroides*, grown anaerobically in basal medium with various substrates; × 800.

39. Strain No. 106 with yeast autolysate
40. Strain No. 106 with Na-aspartate
41. Strain No. 106 with Na-malate
42. Strain No. 33 with yeast autolysate
43. Strain No. 33 with Na-crotonate
44. Strain No. 33 with sorbitol
45. Strain No. 33 with fructose

requirements of *Rhodopseudomonas spheroides* are greater than those of *R. palustris* and of *R. capsulatus*.

Also the strains are much more adversely affected by fatty acids; growth in

media with the normally used concentrations of propionic, caproic, or pelargonic acid has never been obtained.



FIGS. 46–54. *Rhodopseudomonas spheroides*, grown anaerobically in basal medium with various substrates; × 800.

46. Strain No. 80 with Na-succinate
47. Strain No. 80 with ethanol
48. Strain No. 80 with Na-*n*-valerate
49. Strain No. 80 with Na-*n*-caproate
50. Strain No. 80 with glucose
51. Strain No. 80 with Na-*n*-butyrate
52. Strain No. 48 with yeast autolysate, pH 8.5
53. Strain No. 28 with " " pH 9
54. Strain No. 29 with Na-malonate

All of the members of this group have developed in media with tartrate, gluconate, ethanol, glycerol, mannitol, sorbitol, glucose, fructose, and mannose.

Growth in glucose media is accompanied by acid production. The pH of such media may decrease to 4.0 before development stops. The acidic decomposition of glucose occurs both in the presence and in the absence of air, and in light as well as in darkness. In illuminated cultures the acid tends to disappear later on. None of the amino acids used has given consistent results; only two strains have shown growth in alanine, 5 in asparagine, 7 in aspartic and glutamic acids, and 9 in leucine. Correlations have not been observed, however. One of the strains growing slightly with alanine also produced a meager culture with leucine, while the other developed in the presence of aspartic acid.

Growth on the basis of thiosulfate oxidation has not been observed; and the three strains which have been investigated with respect to their ability to oxidize hydrogen have given negative results.

Distinguishing characteristics are, in addition to the typical cell-shape, and the color of the cultures, especially the growth in tartrate and gluconate, in ethanol, glycerol, mannitol, sorbitol, glucose, fructose, mannose, and the failure to grow with thiosulfate.

Enrichment cultures. Since, for the reasons given above (p. 94), glycerol, mannitol, sorbitol, glucose and mannose are not suited as substrates for the direct enrichment of this species from mud or water samples, the best method is based upon a combination of a number of its characteristics. Sometimes it is possible to isolate pure cultures from enrichment cultures with methanol, ethanol, and other alcohols, with fatty acids, or with tartrate. Excellent results are in general obtained by transferring crude cultures in alcohol media to tartrate, by which procedure *Rhodopseudomonas palustris*, the more abundant species in the former medium, can gradually be eliminated while at the same time insuring an inoculum in which purple bacteria are present in far greater numbers than colorless organisms which can be enriched with tartrate in the absence of air (27). Streaks on yeast agar plates, incubated aerobically in the dark, readily lead to pure cultures.

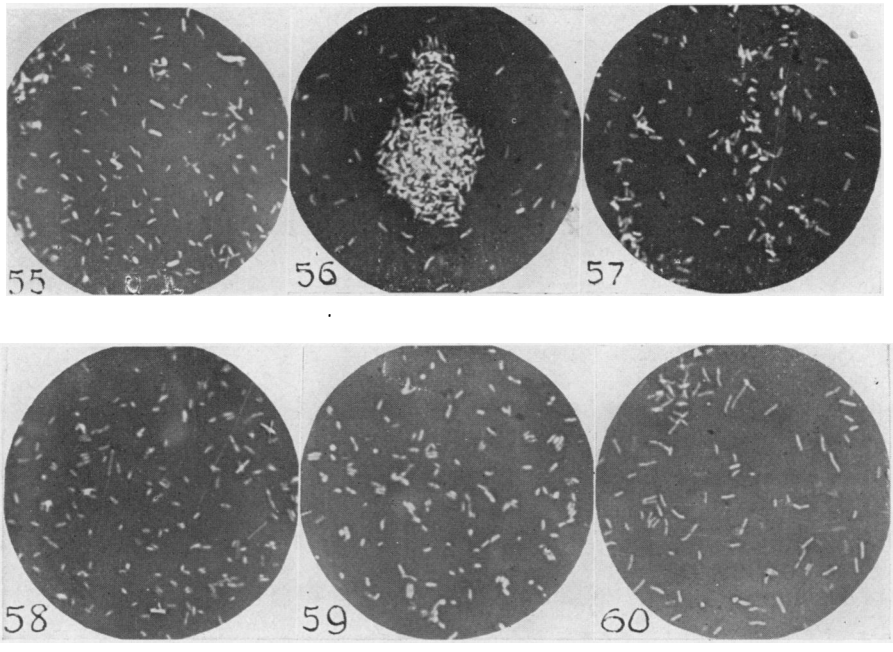
Rhodopseudomonas gelatinosa
Synon. *Rhodocystis gelatinosa* Molisch

The description of this species is based upon the study of a group of 24 strains with a number of common characteristics. All of them have been isolated in California, from a variety of mud samples, including some from Cuba.

Morphology (figs. 55–66). In young cultures the cells appear as short and small rods, approximately 0.5 μ wide, and 1 to 2 μ long (figs. 55–60). They are actively motile, but this is often difficult to ascertain because of the very extensive mucus production which causes the individuals to clump together (See p. 15). This smallest of the *Rhodopseudomonas* species displays, like the others, a striking morphological variability. Most characteristic are the cell shapes found in old cultures; they are much longer, at times swollen, and appear as irregularly curved rods, up to 15 μ long and in places 1 μ wide (figs. 64–66). In this stage the cells bear some superficial resemblance to those of old *R. palustris* cultures, but the typical mycobacterium-like clusters of the latter are here replaced by more irregularly curved conglomerates.

Since the cultures are extraordinarily slimy, individual bacteria are only observable when a thorough homogenizing procedure, such as shaking with glass beads, is practiced. Frequently even slides made from such material still reveal a certain degree of orientation of the elements (fig. 56).

Color. Of diagnostic significance, which cannot yet be associated with any specific pigments, is the color of anaerobic cultures in most liquid media. It has already been likened to the color of a culture of sulfur purple bacteria in which the cells are stuffed with sulfur; Molisch used the designation "peach color." It is quite pale, distinctly pinkish, and delicate. Only in yeast extract media, where growth is considerably heavier than in the basal medium with one of the



FIGS. 55–60. *Rhodopseudomonas gelatinosa.* Magnified × 800.
55–57. Four-day-old yeast extract cultures of strains No. 37, 104, and 115 respectively; homogenized with glass beads, incompletely so in Fig. 56.
58–60. Five-day-old homogenized cultures of strain No. 62, in basal medium with Na-formate, acetate, and succinate respectively.

various organic substrates, does the appearance differ from the above; in such cultures the slimy cell masses appear a dirty, faded brown.

The production of a soluble red pigment has been observed with six of the strains.

Physiology. In yeast extract, growth has been obtained when the initial pH of the medium ranged from 6 to 8.5. The temperature relations of none of the strains are accurately known.

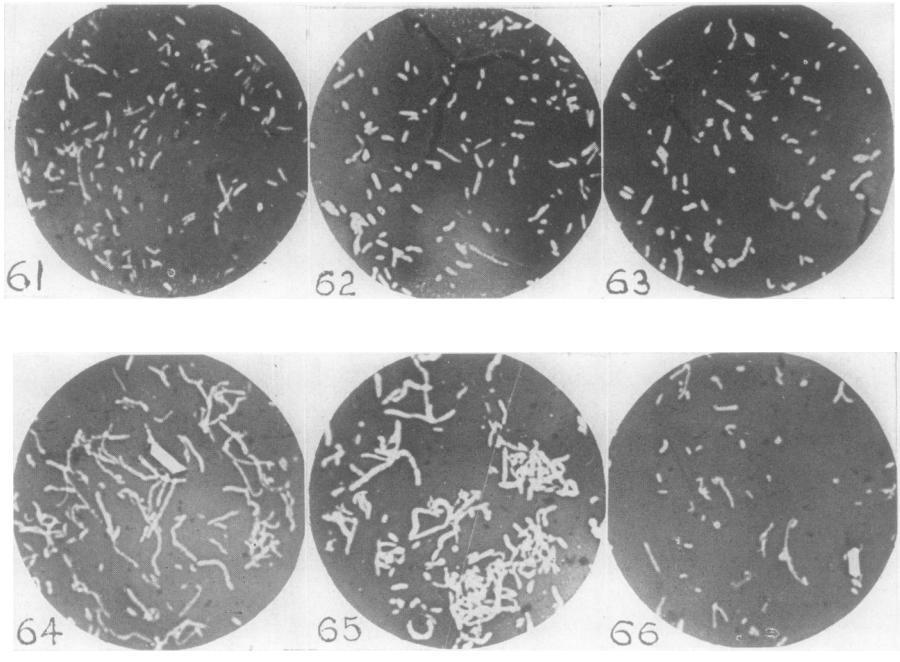
The cultures possess an acrid odor, particularly when, as in yeast extract, growth has been appreciable.

On aerobic yeast agar plates many of the strains fail to develop, but none can

be considered a strict anaerobe. All will grow normally when inoculated into a relatively shallow layer of yeast extract, incubated under aerobic conditions.

The most outstanding physiological property of this group of cultures is that they all liquefy gelatin.

Biochemical characteristics. As in the case of *R. spheroides* growth is adversely effected by many of the higher fatty acids; transfers in propionate media do not develop. Apart from the complex substrates like yeast extract or peptone, the best single substrates appear to be ethanol, glucose, fructose, and mannose, and the amino acids alanine, asparagine, aspartic and glutamic acids. The



FIGS. 61–66. *Rhodopseudomonas gelatinosa;* × 800.
61–63. Seven-day-old homogenized cultures of strain No. 67, in basal medium with Na-acetate, *n*-butyrate, and lactate respectively.
64–66. Thirty-day-old homogenized cultures of strain No. 38, in basal medium with Na-lactate, ethanol, and asparagine respectively.

representatives of this group show considerable growth also in citrate. On the other hand, glycerol, mannitol, sorbitol and tartrate do not allow development. Leucine gives rise to poor cultures with about half of the strains; the rest do not grow.

No action on thiosulfate has been detected; whether any of the strains can oxidize molecular hydrogen has not yet been investigated.

Distinguishing characteristics. The combination of morphology, particularly the small size of the individual cells and the stringiness of the cultures, the unusual color of the cell masses, the ability to liquefy gelatin and to utilize amino acids and citrate serve to mark this group off sharply. Significant negative characteristics are the failure to grow in propionate, tartrate, and glycerol.

Enrichment cultures. The most certain way for isolating representatives of this species consists of making enrichment cultures in ethanol, and after some subcultures in the same medium, which serve to reduce the number of colorless organisms introduced with the inoculum, transferring these to citrate, or to a medium with one of the four amino acids which support good growth.

Since streak cultures on aerobic plates often give negative results, pour plates with yeast gelatin, and subculturing from liquefying colonies of purple bacteria, rapidly and certainly lead to the desired result.

Table 12 may serve to summarize the most important characteristics of the various species of the genus *Rhodopseudomonas* here described. The key for the identification of the species of non-sulfur purple bacteria, to be found in the next section, is in part based upon this table.

Rhodospirillum rubrum (Esmarch) Molisch
Synon. *Spirillum rubrum* Esmarch
Rhodospirillum photometricum Molisch
Rhodospirillum giganteum Molisch
Rhodospirillum longum Hama
Rhodospirillum gracile Hama

Twenty strains of non-sulfur purple spirilla have been included in the present study. Sixteen of these are red, the others brown in various culture media. The former are provisionally considered as a single species, *Rhodospirillum rubrum*. One strain was originally obtained from the National Type Culture Collection, Lister Institute, London, and presumably represents a subculture of the original Esmarch strain. All others are new isolations, performed in Delft, Holland, and in Pacific Grove, California. The source material for two of these was an impure culture of *Rhodobacillus palustris*, kindly furnished by Dr. E. Schneider, Breslau, Germany. The remaining seventeen strains were isolated from enrichment cultures started with different mud and water samples.

The comparative study of the sixteen red strains has revealed the existence of differences, especially in morphological respect. Some of these differences appear rather marked, even to the extent of suggesting the advisability of a segregation of distinct species (figs. 11–16; 67–96). But the variable morphology of each individual strain (figs. 11–16; 67–75) makes it exceedingly difficult to propose a rational subdivision because the customarily used and easily ascertainable morphological criteria for characterizing spirilla overlap so considerably as to obliterate their usefulness as specific characters. Furthermore, a number of strains, when originally isolated, presented certain features which were apparently quite unlike those of previously studied red spirilla. In some cases this must be ascribed to special culture conditions realized during the isolation procedure; in others it appears clearly the result of gradual modifications in the behavior of particular strains. Noticeable color differences which depend upon the composition of the culture medium must be assigned to the former category; in the latter belong such phenomena as the earlier mentioned changes in the degree of tolerance for oxygen. In the course of time such initially observed differences have, therefore, tended to disappear more or less completely. Thus it became increasingly clear that the distinguishing features did not satisfy the

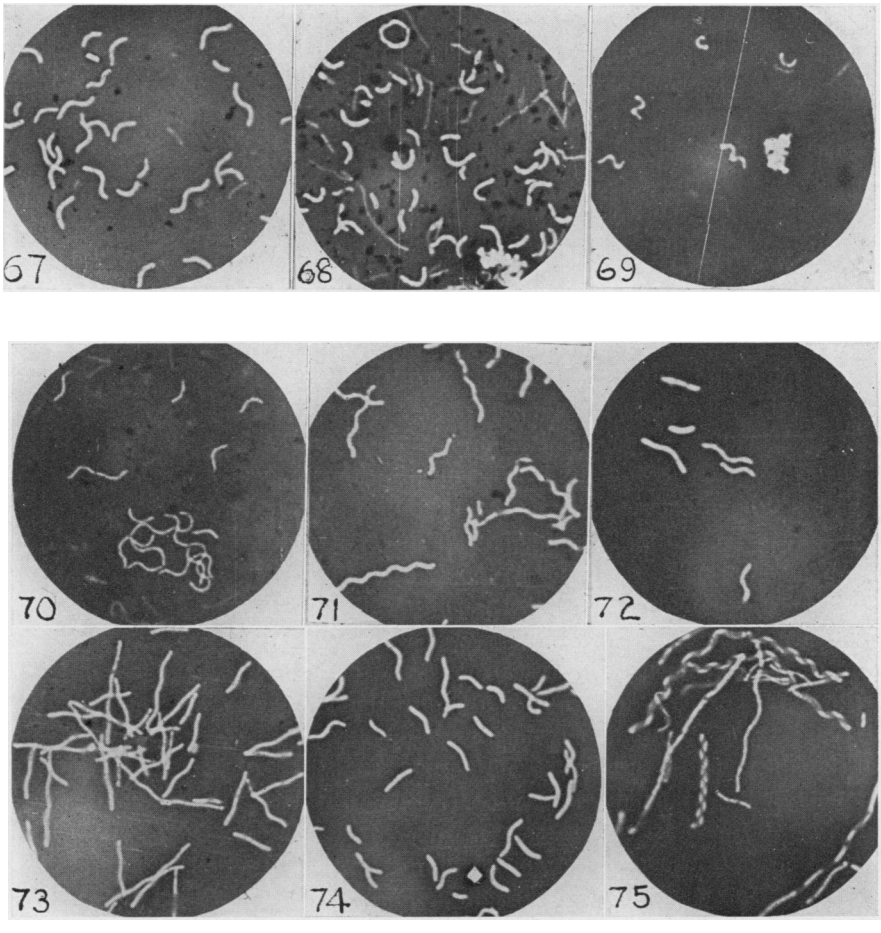
TABLE 12

Summary of characteristics of the Rhodopseudomonas species

SPECIES NAME	MORPHOLOGY			COLOR		SLIME PRODUCTION	GELATIN LIQUEFACTION	GROWTH IN BASAL MEDIUM WITH																	
	Young cultures		Special Features	Anaerobic	Aerobic			Propionate	Glutarate	Tartrate	Citrate	Ethanol	Glycerol	Mannitol	Sorbitol	Glucose	Mannose	Alanine	Leucine	Asparagine	Aspartic acid	Glutamic acid	Thiosulfate
	Shape	Size in μ																					
R. palustris	Rods	0.7–0.8 ×1.2–2	Long rods bent, branched ("Mycob.") in older cultures	Red to brown-red	Red to brown-red	−	−	+	+!	−	−	+!	+	−	−	−	−		+				+
R. capsulatus	Cocci at pH <7; rods at pH > 7	± 1 × 2–2.5	Chains of cells in zigzag arrangement	Brown	Red	Above pH 8	−	+!	−	−	−	−	−	−	−	+	−	+	−	+	−	±	−
R. spheroides	Cocci	0.7 to 4.0	No chains; swollen involutions forms	Brown	Red	Above pH 7	−	−	+	+	−	+	+	+	+	+	+	−	±	∓	∓	±	−
R. gelatinosa	Rods	0.5 × 1–2	Clumped; curved involution forms, thin and long	Pale peach color	−	Very marked	+	−	±	−	+	+	−	−	−	+	+	+	±	+!	+	+	−

± indicates that some strains develop, others fail to grow.
∓ indicates that a few strains develop, most fail to grow.
! indicates exceptionally copious development.

requirements of adequate differential characteristics. On the other hand, it has occasionally happened that a strain which had been in the collection for several years was observed to yield, upon plating, colonies of more than one type, especially as far as the morphology of the individual cells was concerned. Observations of this kind have been followed by re-isolation of each one of the



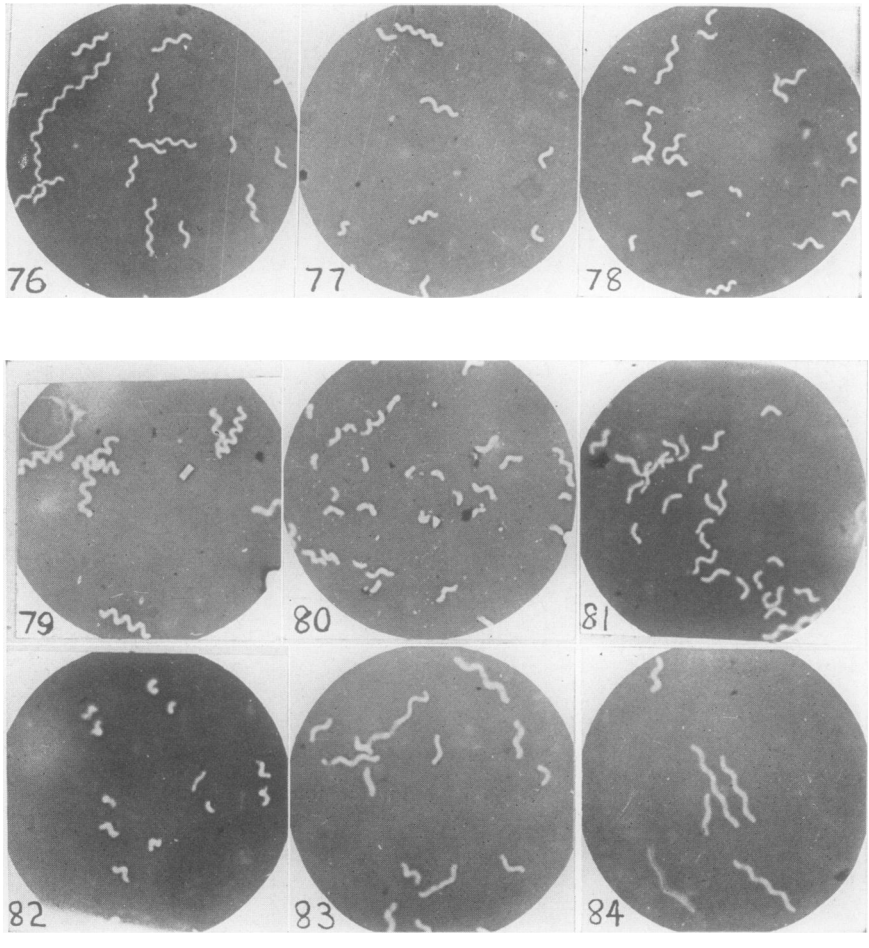
FIGS. 67–75. *Rhodospirillum rubrum*, Strain No. 2. Magnified 800 ×.
67–69. Five-day-old, anaerobic cultures in yeast extract at pH 7.0, 7.5, and 8.0 respectively.
70–75. Seven-day-old, anaerobic cultures in basal medium with Na-*n*-butyrate, succinate, glutarate, malate, ethanol, and asparagine respectively.

distinctive types, and by a careful comparison of the new isolates. The results have been uniform in all cases; the various sub-strains did not exhibit consistent differences either among themselves or with other members of the group.

In view of these facts it seems at present impossible to propose a satisfactory subdivision of the sixteen red strains. It is, of course, possible that future investigations may lead to the discovery of appropriate means for a more refined

classification. As in previous instances, it would appear that attempts of this kind will be less hampered if at this time the common characteristics only are considered.

As a specific name for the group of red spirilla *Rhodospirillum rubrum* (Esmarch) Molisch will be retained. It is hard to see on what basis Molisch proposed two



FIGS. 76–84. *Rhodospirillum rubrum*, Strain No. 4. × 800.
76–78. Three-day-old, anaerobic cultures in yeast extract at pH 6.0, 7.0, and 8.0 respectively.
79–84. Eight-day-old anaerobic cultures in basal medium with Na-*i*-butyrate, *n*-caproate, succinate, glutarate, fumarate, and malate respectively.

new species, *Rhodospirillum photometricum* and *Rhodospirillum giganteum*, in addition to Esmarch's organism which he included in the genus as *Rhodospirillum rubrum* (Esmarch) Molisch (4, p. 24–25). In Esmarch's account the width of this first spirillum to be obtained in pure culture is fixed as "etwa doppelt so stark wie die der Choleraspirillen" (36, p. 227), which would be around 1 μ. The

length is emphatically stated as extremely variable; from a gelatin culture cells with 1 to 2 or 3 turns were observed, while in broth at 37°C the individuals were much longer, many measuring 30 to 40, and even up to 50 complete turns. Migula gives more precise data, and lists the width as 1 to 1.2 μ, the length quite indefinite, often reaching 100 μ (129, p. 1027).

From Molisch's statement concerning Esmarch's organism: "Wie ich mich überzeugte, enthält diese Bakterie Bakteriopurpurin und Bakteriochlorin . . ." (4, p. 25), one must obviously conclude that he examined an authentic strain. Yet, Molisch neither furnishes morphological data concerning this species, nor does he make clear in what particular respect it differs from *Rhodospirillum photometricum* and *Rhodospirillum giganteum*, of which only the former was studied in pure culture. It is described as 5 to 13 μ long and 1.4 μ thick; the second species, "Sehr nahe verwandt und übereinstimmend," was measured as 9 to 70 μ long by about 1.2 μ thick.

Since pure culture studies have shown that also the width may vary considerably in one and the same strain, I see no reason for maintaining Molisch's species side by side with *Rhodospirillum rubrum*. The same argument applies to Hama's *Rhodospirillum longum* and *Rhodospirillum gracile*, both described from crude cultures only (50,155) and differentiated from previously described species on the basis of cell sizes. The latter are published as 1–1.2 μ wide by 7–250 μ, generally 7–30 μ long, and 1 μ wide by 5–16 μ long respectively.

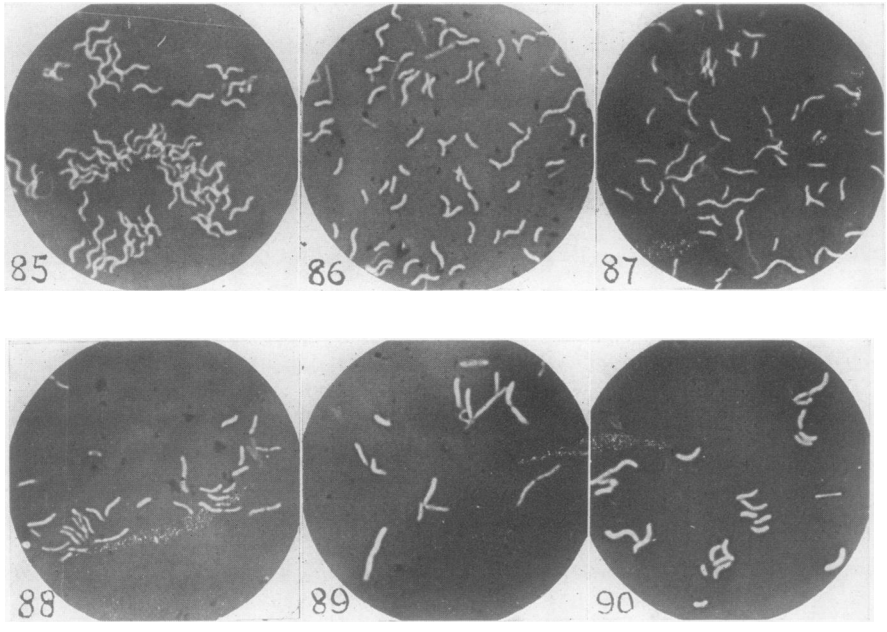
Morphology. The individual cells are characteristically spiral-shaped. However, the size of the elements is variable within rather wide limits. Depending upon environmental conditions the organisms may be from 0.5 to 1.5 μ wide; in one and the same strain, even in a single culture—with progressive development the composition of the medium may undergo considerable changes!—the dimensions may vary this much. Also the length is far from constant; small spirilla, representing one-half of a complete turn, are usually about 2 to 3 μ long, but elements of 25 to 50 μ in length can frequently be encountered. Nor is the length and width of the turns a fixed morphological character. The following data, pertaining to cultures of one strain and in yeast extract media only, may serve as an illustration.

CELLS		SPIRALS	
Width	Length	Width of turn	Length of turn
0.5–1.2μ	2–50μ	1–4μ	1.5–7μ

In different media the variations appear even more pronounced. At the same time, a more or less consistent relation between the shape and size of the cells and the composition of the medium here becomes apparent. Among the most striking examples of this pattern is the microscopic aspect of cultures in the basal medium with alanine and with malate. In the former, the majority of the cells is found in the form of half-circles to complete rings (Figs. 91–93); in the latter all strains exhibit a tendency towards the formation of elements which are much flattened out in appearance (figs. 73; 88–90).

Among the special involution forms, often consisting of straightened spirals, may be mentioned the irregularly swollen cells characteristic of media with higher fatty acids in initial concentration of over 0.05%. Such organisms stain irregularly, contain fatty inclusions, and are sometimes clearly branched (figs. 94–96).

None of the strains produces mucus. In calcium-deficient media the growth is flocculent, as if agglutinated. It may then form a loose to very compact sediment, or adhere to the sides of the culture vessels. The majority of the individual cells are immotile in such cultures, and cell masses hard to break up. With an adequate supply of calcium the growth in liquid media is suspended and



FIGS. 85–90. *Rhodospirillum rubrum*. Magnified × 800.
85–87. Anaerobic cultures in yeast extract at pH 8.0, of strains No. 7, 9, and 10 respectively.
88–90. Anaerobic malate cultures of strains No. 6, 11, and 1 respectively.

consists of separate, motile individuals. The sediment developing in old cultures of this kind can readily be re-dispersed. While anaerobic cultures display an even distribution of the bacteria as long as growth proceeds, a distinct layering becomes apparent upon admission of oxygen. The behavior of individual strains then is that of more or less pronounced micro-aerophils.

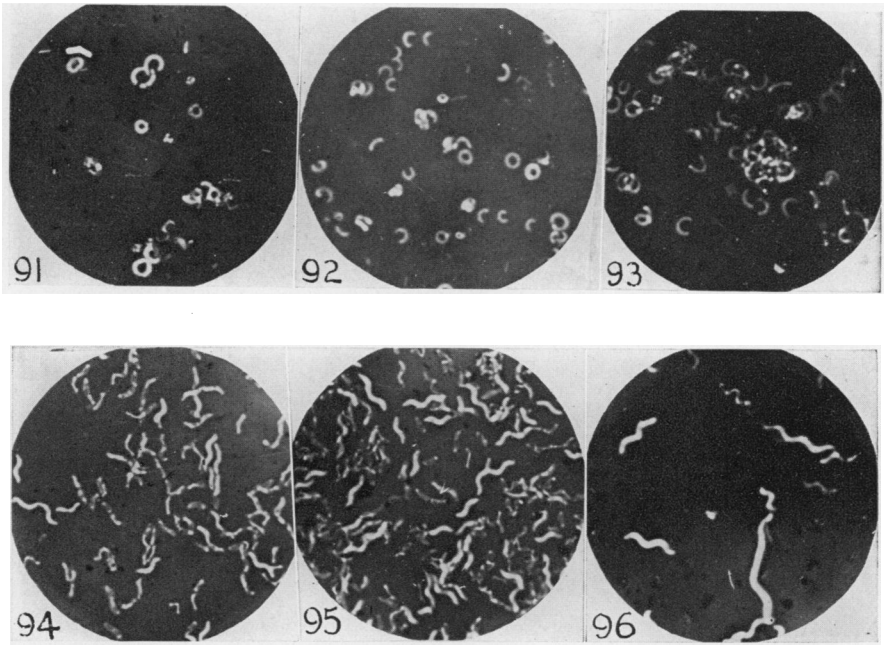
Color. Ordinarily deep and dark red, without any brownish tinge. In ethanol media it is of a decidedly different shade, and characteristically pink.

Pigment production is markedly inhibited by the presence of oxygen and the absence of light, the former apparently having an even greater effect than the latter. Thus slants, incubated in the dark, present a pale, greyish surface growth with a very faint reddish hue, while often showing deep-red cell masses

in the region between the glass wall and the agar surface where conditions are still sufficiently aerobic to induce development.

All strains show a marked absorption band with a maximum at about 550 mμ in the intact organisms. None produces a diffusible, water-soluble pigment.

Physiology. Growth in complex media, such as yeast extract, occurs over the entire range tested, *i.e.* from pH 6.0 to 8.5, although it is somewhat retarded in the most acid medium. As with other non-sulfur purple bacteria, the combination of a high fatty acid concentration and a neutral to acid reaction may prevent growth.



FIGS. 91–96. *Rhodospirillum rubrum.* × 800.
91–93. Anaerobic cultures in basal medium with alanine of strains No. 10, 13, and 15 respectively.
94–96. "Involution forms."
94. Strain No. 9 in acetate, 38 days
95. Strain No. 10 in *n*-butyrate, 26 days
96. Strain No. 11 in glucose, 42 days.

The temperature optimum lies between 30 and 37°C for all strains, and varies slightly with different isolates.

Cultures of *Rhodospirillum rubrum* have a distinctive odor which is, however, difficult to define. It is slightly putrid, and somewhat yeast-like, characteristically different from that of other species of purple bacteria.

Gelatin is not liquefied by any of the strains.

Biochemical characteristics. Growth is generally good with all fatty acids tested, except formate and propionate, in the latter case due to too high a concentration in the experimental media (0.2%). No appreciable development

occurs with tartrate, gluconate, or citrate. Ethanol is a favorable substrate, whereas the carbohydrates and their corresponding polyalcohols are not utilized. Of the amino acids tested alanine, asparagine, aspartic and glutamic acids are satisfactory; glycine and leucine give rise, at best, to slight development.

Not a single strain has been found capable of using thiosulfate. The few isolates which have so far been examined for their ability to oxidize molecular hydrogen have given positive results.

Distinguishing properties. The most important characteristics of the species are the spiral shape combined with the ability to produce a red pigment with a definite absorption maximum at 550 mμ in the intact cells. Diagnostically useful also are the good growth in ethanol, alanine, asparagine, aspartic and glutamic acid media, and the unsuitability of carbohydrates and thiosulfate as substrates.

Enrichment cultures. Most enrichment cultures for non-sulfur purple bacteria contain a larger or smaller proportion of *Rhodospirillum rubrum* cells. Agar shakes from such crude cultures therefore seldom fail to reveal the dark red colonies of this species. More specific enrichment cultures consist of such with various simple alcohols, especially ethanol and *n*-amyl alcohol, or with alanine. By combining its ability to grow in media with both types of substrates it is possible to achieve regularly enrichment cultures in which the majority of organisms consist of *Rhodospirillum rubrum*. In order to forestall an excessive development of *Rhodopseudomonas palustris* it is best to start with alanine media, followed after one or two transfers by cultures in ethanol. The growth of *Rhodopseudomonas capsulatus* is thereby eliminated, while *Rhodopseudomonas gelatinosa* can readily be outgrown by *Rhodospirillum rubrum*, especially if the concentration of yeast autolysate in the enrichment medium does not exceed 0.2%.

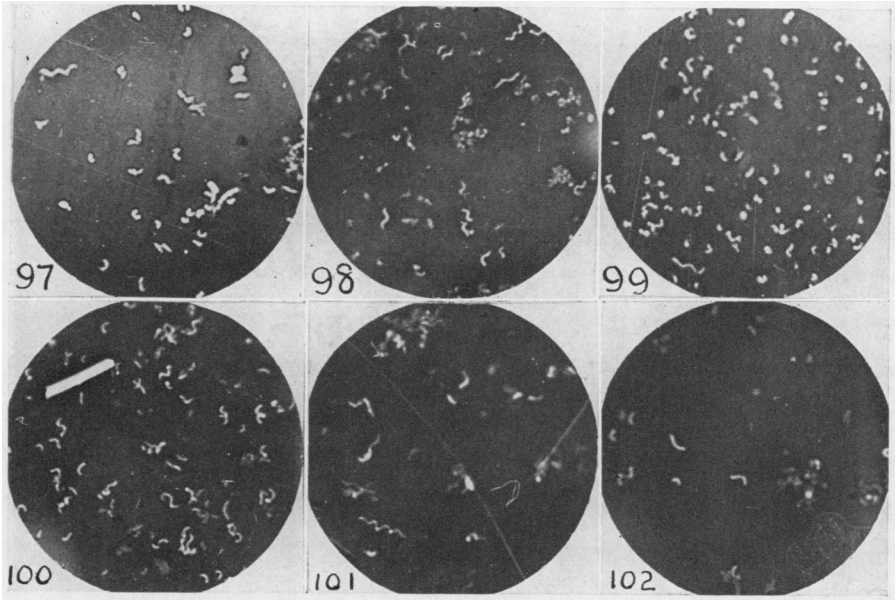
Rhodospirillum fulvum

Clearly differentiated from the red *Rhodospirillum rubrum* strains are the brown photosynthetic spirilla. They are here assembled as a single species, *Rhodospirillum fulvum*, readily distinguishable on the basis of a number of morphological and physiological characteristics. Such organisms have not before been described in sufficient detail to make them recognizable, although Molisch and Buder, referring to *Rhodospirillum spec.*, have undoubtedly recorded observations made with representatives of this type. (See pp. 66–68). For this reason a new name has been used here, emphasizing one of the obvious differences from *Rhodospirillum rubrum*.

Unfortunately the four strains of brown spirilla to which I have had access have not been investigated as extensively as the other cultures of non-sulfur purple bacteria. From the beginning they have appeared to be by far the most sensitive members of the group, and could not be cultured except under rigorously anaerobic conditions. In spite of frequent transfers, three strains had perished before the detailed comparative physiological and biochemical studies were started, so that relatively few observations on the morphology under different environmental conditions have been recorded. The one surviving strain with which most of these studies have been carried out has died since.

The following description rests, therefore, mainly on the behavior of a single isolate. Even though the more striking general characteristics have been ascertained for all four strains, this seriously limits its value.

Morphology (figs. 97–102). Characteristic for the species is the very small size of the individual cells. According to the available data, they are not over 0.5 μ thick, and rarely longer than 2.5 μ. A single complete turn of about 1 by 1.5 μ represents the most common shape. In media with a higher fatty acid as a substrate the spirals appear steeper than in succinate, fumarate, and malate cultures. But the difference is not great and, in view of the minute size of the cells, difficult to appreciate.



FIGS. 97–102. *Rhodospirillum fulvum*. × 800.
Anaerobic cultures of strain No. 123 in basal medium with Na-*n*-butyrate, *n*-caproate, pelargonate, fumarate, malate, and succinate respectively.

Swollen individuals with a resemblance to vibrios can be encountered in cultures which do not appear too healthy. They have been considered as involution forms.

Formation of mucus or clumping in liquid cultures has not been observed.

Color. Quite as distinctive as the size is the color of this species. Individual colonies and stab cultures appear reddish-brown; the shade of certain liquid cultures can be described more accurately as brownish-orange. In agreement herewith is the fact that the absorption maximum at 550 mμ, so characteristic for the red spirilla, is absent in cultures of *Rhodospirillum fulvum*. The production of water-soluble, diffusible pigments has not been observed.

Physiology. No detailed information concerning the effect of the reaction of the medium and of temperature on the development of the brown spirilla is

available; all strains have shown development at 30°C. None has caused gelatin liquefaction.

The behavior towards oxygen indicates that the organism is a strict anaerobe; development in media exposed to air has invariably failed to occur. Even in deep yeast agar columns, rapidly cooled and heavily inoculated by a stab immediately after sterilization, growth is very uncertain if the medium is left without a seal. If a culture is obtained, the upper part, extending over at least 5 cm., remains blank, and subcultures generally fail after more than 10 days.

In agreement with this behavior is the strongly negative aerotaxis, spectacularly evident in liquid media. After lifting the stopper of a uniformly turbid bottle culture, the bacteria disappear rapidly from the upper layers, and in a short period of time the liquid becomes entirely clear with the organisms aggregated at the bottom. Here they may remain actively motile for more than 24 hours provided the culture is not shaken.

Several attempts at adaptation to oxygen have been made, but all with negative results.

Biochemical characteristics. Fatty acids and the four-carbon dicarboxylic acids are uniformly good substrates, while glutaric acid has proved unsuitable for the one strain studied. Abundant development of this isolate has also been observed with ethanol and, curiously, with glucose as a substrate. Other carbohydrates and the corresponding polyalcohols have given negative results. Aspartic acid is the only amino acid which has served as a satisfactory substrate. Thiosulfate is not utilized. No data are available with respect to the oxidation of molecular hydrogen.

Not too much value should be attached to these findings since they are limited to the behavior of a single strain, and, in some cases (glucose, for example) to a single experiment.

Distinguishing properties. The small size of the cells and the color serve as sufficiently characteristic criteria to distinguish this species from the red spirilla. The strictly anaerobic nature and the failure to grow with glutarate and various amino acids except aspartate may be used as supplementary specific properties for this organism.

Enrichment cultures. The present information is entirely inadequate to serve as a guide for selecting specific enrichment media. Successful isolations have been achieved with caprylate and pelargonate media. Whether this is generally satisfactory must, however, await the accumulation of more extensive observations.

IV. Keys for the identification of the species of the non-sulfur purple and brown bacteria

The recognition of only two genera and six species among the non-sulfur purple bacteria makes it a simple matter to devise keys for their determination. The most satisfactory procedure consists of an initial segregation of the two genera, *Rhodopseudomonas* and *Rhodospirillum*, on the basis of cell shape. A rapid determination of the species can then be based upon morphological, physiological or biochemical characters.

Dichotomous keys can be adequately constructed for any one of these various properties. Depending upon the nature of the characters used, one species can usually be singled out immediately. The following keys are examples of some possible arrangements.

Key for the determination of genera:

1. Cells rod-shaped or spherical, not spiral-shaped..... Genus *Rhodopseudomonas*
2. Cells spiral-shaped..... Genus *Rhodospirillum*

Key for the determination of Rhosopseudomonas species based upon morphological characters

1. a. Cells clearly rod-shaped in all media..... 2
   b. Cells more or less spherical in media at pH below 7..... 3
2. a. Cells short, somewhat curved, to long branched rods, size 0.7–0.8 by 1.2–2 μ, do not form mucus, liquid cultures evenly turbid. Color red to dark brown-red..... *Rhodopseudomonas palustris*
   b. Cells slender rods, 0.5 by 1.2 μ, usually clumped together in extensive slime masses. Cultures pale brown to peach-colored..... *Rhodopseudomonas gelatinosa*
3. a. In media at pH above 7 clearly rod-shaped, 1 by 1–2.5 μ. Chains of cells frequent in zigzag arrangement..... *Rhodopseudomonas capsulatus*
   b. In media at pH above 7 cells still predominantly spherical, 0.7–4 μ in diameter. Mostly single, no chain formation..... *Rhodopseudomonas spheroides*

Key for the determination of Rhodopseudomonas species, principally based upon physiological properties.

1. a. Gelatin liquefied..... *Rhodopseudomonas gelatinosa*
   b. Gelatin not liquefied..... 2
2. a. Do not produce mucus in media at pH above 8. Color the same under aerobic and anaerobic conditions..... *Rhodopseudomonas palustris*
   b. Produce mucus in media at pH above 8. Color brown under anaerobic, red under aerobic conditions..... 3
3. a. Develop readily in media with 0.2% propionate as the main substrate. Mucus production marked at pH above 8..... *Rhodopseudomonas capsulatus*
   b. Do not develop in media with 0.2% propionate as the main substrate. Slime formation evident at pH above 7.0..... *Rhodopseudomonas spheroides*

Key for determination of Rhodopseudomonas species mainly based upon biochemical properties.

1. a. Thiosulfate used as main oxidation substrate..... *Rhodopseudomonas palustris*
   b. Thiosulfate not used..... 2
2. a. Propionate (0.2%) used..... *Rhodopseudomonas capsulatus*
   b. Propionate not used..... 3
3. a. Mannitol and sorbose utilized..... *Rhodopseudomonas spheroides*
   b. Mannitol and sorbose not utilized..... *Rhodopseudomonas gelatinosa*

Key for the determination of Rhodospirillum species.

1. Cultures red; cells over 0.5 μ, usually about 1–1.2 μ wide..... *Rhodospirillum rubrum*
2. Cultures brown to orange; cells 0.5 μ or less in width..... *Rhodospirillum fulvum*

Acknowledgements. It is a pleasure to express, also at this place, my gratitude to those who have contributed towards the completion of this monograph. More especially, thanks are due to Dr. Arthur L. Cohen, to whom I am deeply indebted for his preparation of the huge documentary material in the form of some 2000 individual photomicrographs; to Dr. William Arnold for stimulating advice and help with some of the experiments; to Miss Pearl Murray for her faithful and competent care of the pure culture collection to which the numerous strains of non-sulfur purple bacteria have added a not inconsiderable burden;

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REFERENCES

1. GAFFRON, H. 1933 Über den Stoffwechsel der schwefelfreien Purpurbakterien. Biochem. Z., **260**, 1–17.
2. VAN NIEL, C. B. 1929 Photosynthesis of bacteria. In: Contributions to Marine Biology, Stanford Univ. Press, 161–169.
3. VAN NIEL, C. B. 1931 On the morphology and physiology of the purple and green sulphur bacteria. Arch. Mikrobiol., **3**, 1–112.
4. MOLISCH, H. 1907 Die Purpurbakterien nach neuen Untersuchungen. G. Fischer, Jena.
5. GAFFRON, H. 1935 Über den Stoffwechsel der Purpurbakterien. II. Biochem. Z., **275**, 301–319.
6. FOSTER, J. W. 1940 The rôle of organic substrates in photosynthesis of purple bacteria. J. Gen. Physiol., **24**, 123–134.
7. MULLER, F. M. 1933 On the metabolism of the purple sulphur bacteria in organic media. Arch. Mikrobiol., **4**, 131–166.
8. VAN NIEL, C. B. 1941 The bacterial photosyntheses and their importance for the general problems of photosynthesis. In: Advances in Enzymology, **1**, 263–328.
9. BUDER, J. 1919 Zur Biologie des Bakteriopurpurins und der Purpurbakterien. Jahrb. wiss. Botan., **58**, 525–628.
10. WINOGRADSKY, S. 1887 Über Schwefelbakterien. Botan. Ztg., **45**, 489 ff, (Nos. 31–37).
11. WINOGRADSKY, S. 1888 Beiträge zur Morphologie und Physiologie der Bakterien. Heft 1; Schwefelbakterien. Arthur Felix, Leipzig.
12. PRINGSHEIM, E. G. 1932 Neues über Purpurbakterien. Naturwissenschaften, **20**, 479–483.
13. NADSON, G. A. 1903 Observations sur les bactéries pourprées. Bull. Jard. Imp. Bot. St. Pétersb., **3**, 99–109.
14. GAFFRON, H. 1934 Über die Kohlensäure-Assimilation der roten Schwefelbakterien. I. Biochem. Z., **269**, 447–453.
15. GAFFRON, H. 1935 Über die Kohlensäureassimilation der roten Schwefelbakterien. II. Biochem. Z., **279**, 1–33.
16. VAN NIEL, C. B. 1936 On the metabolism of the *Thiorhodaceae*. Arch. Mikrobiol., **7**, 323–358.
17. WINOGRADSKY, S. 1933 Review of (7) in Bull. inst. Pasteur, **31**, 972–974.
18. UTERMÖHL, H. 1924 Phaeobakterien. Biol. Zentr., **43**, 605–610.
19. EWART, A. J. 1897 On the evolution of oxygen from colored bacteria. J. Linnean Soc. (Bot.), **33**, 123–155.
20. CZURDA, V., UND MARESCH, E. 1937 Beiträge zur Kenntnis der Athiorhodobakterien-Gesellschaften. Arch. Mikrobiol., **8**, 99–124.
21. LJUBIMENKO, V. 1921 Recherches sur les pigments des bactéries pourpres. J. Soc. Botan. Russie, **6**, 107–119.
22. SCHNEIDER, E. 1930 Beiträge zur Physiologie der Farbstoffe der Purpurbakterien. 1. Mitteilung: Die Reinkultur des *Rhodobacillus palustris* Molisch und die Gewinnung seiner Pigmente. Beitr. Biol. Pflanz., **18**, 81–115.
23. SELIBER, G. 1928 La culture des bactéries pourprées à la lumière électrique. Bull. inst. Lesshaft, **14**, 55–57.
24. SCHRAMMECK, J. 1935 Untersuchungen über die Phototaxis der Purpurbakterien. Beitr. Biol. Pflanz., **22**, 314–380.
25. BAARS, J. K. 1930 Over Sulfaatreductie door bacterien. Dissert., Delft.

26. BRAAK, H. R. 1928 Onderzoekingen over de vergisting van glycerine. Dissert., Groningen.
27. BARKER, H. A. 1936 On the fermentation of some dibasic C_4-acids by *Aerobacter aerogenes*. Proc. Koninkl. Akad. Wetenschappen Amsterdam, **39**, 674–683.
28. WERKMAN, C. H., AND WOOD, H. G. 1942 On the metabolism of bacteria. Botan. Rev., **8**, 1–68.
29. SLADE, H. D., AND WERKMAN, C. H. 1941 The anaerobic dissimilation of citric acid by cell suspensions of *Streptococcus paracitrovorus*. J. Bact., **41**, 675–684.
30. ORLA-JENSEN, S. 1919 The lactic acid bacteria. Kgl. Danske Videnskb. Selskb. Skrifter, Naturvidenskab math. Afdel., **8**, Raekke V, 2.
31. ENGELMANN, TH. W. 1888 Die Purpurbacterien und ihre Beziehungen zum Licht. Botan. Ztg., **46**, 661 ff.; Reprint pp. 1–50.
32. FRENCH, C. S. 1937 The rate of CO_2 assimilation by purple bacteria at various wave lengths of light. J. Gen. Physiol., **21**, 71–87.
33. FRENCH, C. S. 1940 The pigment-protein compound in photosynthetic bacteria. II. The absorption curves of photosynthin from several species of bacteria. J. Gen. Physiol., **23**, 483–494.
34. BAVENDAMM, W. 1924 Die farblosen und roten Schwefelbakterien. G. Fisher, Jena.
35. FORTI, A. 1933 Il "Lago di Sangre" a Pergusa in Sicilia e la prima piaga d'Egitto. Il Naturalista Siciliano, Anno 28, Nuova Serie, vol 8°, 1932-XI, 63–86.
36. ESMARCH, E. 1887 Ueber die Reincultur eines Spirillum. Centr. Bakt., **1**, 225–230.
37. KLUYVER, A. J. 1929 Over slijmvorming in melk. Nederland. Tijdschr. Hyg., Microbiol. Serol., **3**, 301–307.
38. GRAY, P. H. H. 1941 A solution for staining differentially the spores and vegetative cells of micro-organisms. Can. J. Research, **19**, 95–98.
39. LEWIS, I. M. 1937 Cell inclusions and the life cycle of Azotobacter. J. Bact., **34**, 191–204.
40. LEWIS, I. M. 1938 Cell inclusions and the life cycle of Rhizobia. J. Bact., **35**, 573–586.
41. LEWIS, I. M. 1940 The genus *Spirillum* Ehbg. with special reference to cell inclusions and the chromidial theory. J. Bact., **40**, 271–284.
42. LEWIS, I. M. 1941 The cytology of bacteria. Bact. Rev., **5**, 181–230.
43. HARTMAN, T. L. 1940 The use of Sudan Black B as a bacterial fat stain. Stain Tech., **15**, 23–28.
44. KLUYVER, A. J., AND VAN NIEL, C. B. 1936 Prospects for a natural system of classification of bacteria. Centr. Bakt., II. Abt., **94**, 369–403.
45. FRENCH, C. S. 1940 The pigment-protein compound in photosynthetic bacteria. I. The extraction and properties of Photosynthin. J. Gen. Physiol., **23**, 469–481.
46. Committee on Bacteriologic Technic. Manual of methods for pure culture study of bacteria. Soc. Am. Bacteriologists.
47. BEIJERINCK, M. W. 1892 La biologie d'une bactérie pigmentaire. Arch. néerland. sci., Haarlem, **25**, 227–280. Also in: Verzam. Werken, Delft, 1921, Vol. II, p. 327–358.
48. GIESBERGER, G. 1936 Beiträge zur Kenntnis der Gattung *Spirillum* Ehbg. Dissert. Utrecht.
49. MYERS, J. 1940 Studies on the Spirilleae. J. Bact., **40**, 705–720.
50. HAMA, Y. 1933 Studien über eine neue Rhodospirillumart aus Yumoto bei Nikko. J. Sci., Hiroshima Univ., Ser. B, Div. 2, **1**, 135–156.
51. BEIJERINCK, M. W. 1902 Photobacteria as a reactive in the investigation of the chlorophyllfunction. Proc. Koninkl. Akad. Wetenschappen., Amsterdam, **4**, 5–9.
52. VAN NIEL, C. B. 1935 Photosynthesis of bacteria. Cold Spring Harbor Symposia, **3**, 138–150.
53. VAN NIEL, C. B. 1937 Les photosynthèses bactériennes. Bull. assoc. diplomés microbiol. faculté phar. Nancy, Nr. **13**, 3–18.
54. ROELOFSEN, P. A. 1935 On photosynthesis of the Thiorhodaceae. Dissert. Utrecht.

55. CORYELL, C. D. 1940 The proposed terms "exergonic" and "endergonic" for thermodynamics. Science, **92**, 380.
56. PREVOT, A.-ROMAIN 1933 Études de systématique bactérienne. I. Lois générales. II. Cocci anaérobies. Ann. sci. nat., Botan., 10e Série, **15**, 23–261.
57. NAKAMURA, H. 1937 Über die Photosynthese bei der schwefelfreien Purpurbakterie, *Rhodobazillus palustris*. Beiträge zur Stoffwechselphysiologie der Purpurbakterien, I. Acta Phytochim., **9**, 189–229.
58. NAKAMURA, H. 1937 Über die Kohlensäureassimilation von *Rhodospirillum gigan-teum*. Beiträge zur Stoffwechselphysiologie der Purpurbakterien, II. Acta Phytochim., **9**, 231–234.
59. VAN NIEL, C. B. 1943 Biochemical problems of the chemo-autotrophic bacteria. Physiol. Rev., **23**, 338–354.
60. LWOFF, A. 1936 Études sur les fonctions perdues. Ann. fermentations, **2**, 419–428.
61. LWOFF, A. 1938 Les facteurs de croissance pour les microorganismes. Ann. inst. Pasteur, **61**, 580–617.
62. VAN NIEL, C. B. AND SMITH, J. H. C. 1935 Studies on the pigments of the purple bacteria. Arch. Mikrobiol., **6**, 219–229.
63. KLUYVER, A. J. AND CUSTERS, M. TH. J. 1940 The suitability of disaccharides as respiration and assimilation substrates for yeasts which do not ferment these sugars. Antonie van Leeuwenhoek, **6**, 121–162.
64. STANIER, R. Y. 1942 Are there obligate cellulose-decomposing bacteria? Soil Sci., **53**, 479–480.
65. SELIBER, G. 1924 La décomposition des grasses par les bactéries pourprées. Bull. inst. Leshaft, **9**, 229–234.
66. PIGULEWSKI, G. UND CHARIK, M. 1928 Zersetzung des Olivenöls unter dem Einfluss der vitalen Tätigkeit einiger Mikroorganismen. Biochem. Z., **200**, 201–210.
67. HEILBRUNN, L. V. 1940 Protoplasm and colloids. In: The Cell and Protoplasm, Am. Assoc. Advancement Sci. Publication No. 14, 188–198.
68. ROELOFSEN, P. A. 1934 On the metabolism of the purple sulphur bacteria. Proc. Koninkl. Akad. Wetenschappen, Amsterdam, **37**, 660–669.
69. FRENCH, C. S. 1937 The quantum yield of hydrogen and carbon dioxide assimilation in purple bacteria. J. Gen. Physiol., **20**, 711–735.
70. WESSLER, S., AND FRENCH, C. S. 1939 The photosynthetic quotient H_2/CO_2 for *Streptococcus varians*. J. Cellular Comp. Physiol., **13**, 327–334.
71. NAKAMURA, H. 1937 Über das Vorkommen der Hydrogenlyase in *Rhodobacillus palustris* und über ihre Rolle im Mechanismus der bakteriellen Photosynthese. Acta Phytochim., **10**, 211–218.
72. NAKAMURA, H. 1938 Über die Rolle der Hydrogenase im Stoffwechsel von *Rhodo-bacillus palustris*. Acta Phytochim., **10**, 259–270.
73. YAMAGATA, S., UND NAKAMURA, H. 1938 Über die Hydrogenase, nebst einer Bemerkung über den Mechanismus der bakteriellen Knallgasreaktion. Acta Phytochim., **10**, 297–311.
74. DEN DOOREN DE JONG, L. E. 1926 Bijdrage tot de kennis van het mineralisatieproces. Dissert. Delft; Nijgh & van Ditmar, Rotterdam.
75. DEN DOOREN DE JONG, L. E. 1927 Über protaminophage Bakterien. Centr. Bakt., II. Abt., **71**, 193–232.
76. JANKE, A. 1928 Über den dissimilatorischen Abbau niederer Alkylamine durch Bakterien. Centr. Bakt., II. Abt., **74**, 25–26.
77. BARKER, H. A. 1936 The oxidative metabolism of the colorless Alga, *Prototheca zopffii*. J. Cellular Comp. Physiol., **8**, 231–250.
78. CLIFTON, C. E. 1937 On the possibility of preventing assimilation in respiring cells. Enzymologia, **4**, 246–253.
79. CLIFTON, C. E. AND LOGAN, W. A. 1939 On the relation between assimilation and respiration in suspensions and in cultures of Escherichia coli. J. Bact., **37**, 523–540.

80. WINZLER, R. J., AND BAUMBERGER, J. P. 1938 The degradation of energy in the metabolism of yeast cells. J. Cellular Comp. Physiol., **12**, 183–211.
81. WINZLER, R. J. 1940 The oxidation of acetate by baker's yeast. J. Cellular Comp. Physiol., **15**, 343–354.
82. DOUDOROFF, M. 1940 The oxidative assimilation of sugars and related substances by *Pseudomonas saccharophila*. Enzymologia, **9**, 59–72.
83. VAN NIEL, C. B. 1940 The biochemistry of microorganisms. In: The Cell and Protoplasm. Am. Assoc. Advancement Sci., Publication No. 14, p. 106–119.
84. BARKER, H. A. 1936 On the biochemistry of the methane fermentation. Arch. Mikrobiol., **7**, 404–419.
85. BARKER, H. A., RUBEN, S., AND KAMEN, M. D. 1940 The reduction of radioactive carbon dioxide by methane-producing bacteria. Proc. Natl. Acad. Sci., U.S., **26**, 426–430.
86. BARKER, H. A. 1940 Studies upon the methane fermentation. Antonie van Leeuwenhoek, **6**, 201–220.
87. BARKER, H. A. 1941 Studies on the methane fermentation. V. Biochemical activities of *Methanobacterium omelianskii*. J. Biol. Chem., **137**, 153–167.
88. GLADSTONE, G. P., FILDES, P., AND RICHARDSON, G. M. 1935 Carbon dioxide as an essential factor in the growth of bacteria. Brit. J. Exptl. Path., **16**, 335–348.
89. PIRIE, N. W. 1937 The meaninglessness of the terms life and living. In: Perspectives of Biochemistry, Cambridge Univ. Press, p. 11–22.
90. ENGELMANN, TH. W. 1883 *Bacterium photometricum*. Ein Beitrag zur vergleichenden Physiologie des Licht- und Farbensinnes. Pflügers Arch. ges. Physiol., **30**, 95–124.
91. ENGELMANN, TH. W. 1888 Die Purpurbakterien und ihre Beziehungen zum Licht. Bot. Zeitg., **46**, 661 ff. Also in Arch. Néerland., **23**, 151–198.
92. EWART, A. J. 1897 Bacteria with assimilatory pigments, found in the tropics. Ann. Botany, **11**, 486–487.
93. NADSON, G. A. 1903 Observations sur les bactéries pourprées. Bull. Jard. Imp. Bot. St. Pétersb., **3**, 109–119.
94. ARCICHOVSKIJ, V. 1904 Zur Frage über das Bakteriopurpurin. Bull. Jard. Imp. Bot. St. Pétersb., **4**, 81–98.
95. LÉVY R., TEISSIER, G., ET WURMSER, R. 1925 Étude des pigments d'une bactériacée sulfureuse. Ann. physiol. physicochim. biol., **1**, 298–311.
96. VERMEULEN, D., WASSINK, E. C., AND REMAN, G. H. 1937 On the fluorescence of photosynthesizing cells. Enzymologia, **4**, 254–268.
97. FRANCK, J., AND GAFFRON, H. 1941 Photosynthesis, Facts and Interpretations. Advances in Enzymology, **1**, 199–262.
98. WOHL, K. 1941 On the mechanism of photosynthesis in purple bacteria and green plants. New Phytologist, **40**, 34–55.
99. KATZ, E., AND WASSINK, E. C. 1939 Infrared absorption spectra of chlorophyllous pigments in living cells and in extra-cellular states. Enzymologia, **7**, 97–112.
100. WASSINK, E. C., KATZ, E., AND DORRESTEIN, R. 1939 Infrared absorption spectra of various strains of purple bacteria. Enzymologia, **7**, 113–129.
101. FRENCH, C. S. 1938 The chromoproteins of photosynthetic purple bacteria. Science, **88**, 60–62.
102. MESTRE, H. 1930 The investigation of the pigments of the living photosynthetic cell. In: Contrib. to Marine Biology, Stanford Univ. Press, p. 170–187.
103. MESTRE, H. 1935 The absorption of radiation by leaves and algae. Cold Spring Harbor Symp., **3**, 191–209.
104. STOLL, A., UND WIEDEMANN, E. 1938 Chlorophyll. In: Fortschr. Chem. organ. Naturst., **1**, 159–254.
105. SMITH, E. L. 1940 Chlorophyll as the prosthetic group of a protein in the green leaf. Science, **91**, 199–200.

106. SMITH, E. L. 1941 The chlorophyll-protein compound of the green leaf. J. Gen. Physiol., **24**, 565–582.
107. SMITH, E. L. AND PICKLES, E. G. 1941 The effect of detergents on the chlorophyll-protein compound of spinach as studied in the ultracentrifuge. J. Gen. Physiol., **24**, 753–764.
108. SCHNEIDER, E. 1934 Über chlorophyllartige Farbstoffe bei den Purpurbakterien. Ber. deut. botan. Ges., **52**, 96–100.
109. SCHNEIDER, E. 1934 Über das Bakteriochlorophyll der Purpurbakterien. Z. physiol. Chem., **226**, 221–254.
110. FISCHER, H., RIEDMAIR, J., UND HASENKAMP, J. 1934 Über Oxo-porphyrine: Ein Beitrag zur Kenntnis der Feinstruktur von Chlorophyll *a*. Ann. Chem., **508**, 224–249.
111. FISCHER, H., UND HASENKAMP, J. 1935 Über die Konstitution des Farbstoffes der Purpurbakterien und über 9-Oxy-desoxo-Phäoporphyrin a_5. Ann. Chem., **515**, 148–164.
112. FISCHER, H., UND LAMBRECHT, R. 1937 Über Bacteriochlorophyll *a*. Z. physiol. Chem., **249**, I–III.
113. FISCHER, H., LAMBRECHT, R., UND MITTENZWEI, H. 1938 Über Bacterio-Chlorophyll. Z. physiol. Chem., **253**, 1–39.
114. FISCHER, H., LAUTSCH, W., UND LIN, K. H. 1938 Teilsynthesen von Dehydro-bacterio-phorbid und Dehydro-bacterio-chlorin. Ann. Chem., **534**, 1–22.
115. FISCHER, H., UND WENDEROTH, H. 1939 Zur Kenntnis von Chlorophyll. Ann. Chem., **537**, 170–177.
116. FISCHER, H., MITTENZWEI, H., UND HEVÉR, D. B. 1940 Überführung von Dehydro-bacterio-phäophorbid *a* in Chlorophyll *a*. Ann. Chem., **545**, 154–178.
117. VAN NIEL, C. B., AND ARNOLD, W. 1938 The quantitative estimation of bacterio-chlorophyll. Enzymologia, **5**, 244–250.
118. VAN NIEL, C. B. 1933 In: Year Book No. 32 of the Carnegie Institution of Washington, p. 184.
119. KARRER, P., UND SOLMSSEN, U. 1935 Die Carotinoide der Purpurbakterien I. Helv. Chim. Acta, **18**, 1306–1315.
120. KARRER, P., UND SOLMSSEN, U. 1936 Die Carotinoide der Purpurbakterien II. Über Rhodoviolascin. Helv. Chim. Acta, **19**, 3–5.
121. KARRER, P., UND SOLMSSEN, U. 1936 Carotinoide aus Purpurbakterien III. Helv. Chim. Acta, **19**, 1019–1024.
122. KARRER, P., SOLMSSEN, U., UND KOENIG, H. 1938 Carotinoide aus Purpurbakterien IV. Helv. Chim. Acta, **21**, 454–455.
123. KARRER, P., UND KOENIG, H. 1940 Carotinoide der Purpurbakterien V. Über Rhodoviolascin. Helv. Chim. Acta, **23**, 460–463.
124. SCHNEIDER, E. 1936 Über die Carotinoide der Purpurbakterien. Beiträge zur Physiologie der Farbstoffe der Purpurbakterien III. Rev. faculté sci. univ. Istanbul, **1**, 74–80.
125. FRENCH, C. S. 1940 Absorption spectra of the carotenoids in the red and brown forms of a photosynthetic bacterium. Botan. Gaz., **102**, 406–409.
126. MACKINNEY, G. 1940 Plant pigments. In: Ann. Rev. Biochem., **9**, 459–490.
127. MIGULA, W. 1895 Schizophyta. In: Engler und Prantl, Die natürlichen Pflanzenfamilien I, 1a, 1–44.
128. MIGULA, W. 1897 System der Bakterien, I. Bd., Allgemeiner Teil. G. Fischer, Jena.
129. MIGULA, W. 1900 System der Bakterien, II. Bd., Specielle Systematik der Bakterien. G. Fischer, Jena.
130. RICHTER, O. 1907 Die Bedeutung der Reinkultur. Bornträger, Berlin.
131. JENSEN, (S.) O. 1909 Die Hauptlinien des natürlichen Bakteriensystems. Centr. Bakt., II. Abt., **22**, 305–346.

132. BENECKE, W. 1912 Bau und Leben der Bakterien. Teubner, Leipzig.
133. KRUSE, W. 1910 Allgemeine Mikrobiologie. Vogel, Leipzig.
134. VAHLE, C. 1909 Vergleichende Untersuchungen über die Myxobakteriazeen und Bakteriazeen, sowie die Rhodobakteriazeen und Spirillazeen. Dissert. Marburg. Also in Centr. Bakt., 1910, II. Abt., **25**, 178–260.
135. BUCHANAN, R. E. 1917 Studies in the nomenclature and classification of the bacteria. II. The primary subdivisions of the *Schizomycetes*. J. Bact., **2**, 155–164.
136. BUCHANAN, R. E. 1918 IX. The subgroups and genera of the *Thiobacteriales*. J. Bact., **3**, 461–474.
137. BUCHANAN, R. E. 1925 General systematic bacteriology. Williams & Wilkins, Baltimore.
138. WINSLOW, C.-E. A., BROADHURST, J., BUCHANAN, R. E., KRUMWIEDE, C., ROGERS, L. A., AND SMITH, G. H. 1917 The families and genera of the bacteria. J. Bact., **2**, 505–566.
139. BREED, R. S., CONN, H. J., AND BAKER, J. C. 1918 Comments on the evolution and classification of bacteria. J. Bact., **3**, 445–459.
140. PRIBRAM, E. 1929 A contribution to the classification of microörganisms. J. Bact., **18**, 361–394.
141. NATHANSOHN, A. 1902 Über eine neue Gruppe von Schwefelbakterien und ihren Stoffwechsel. Mitt. Zool. Stat. Neapel, **15**, 655–680.
142. JACOBSEN, H. C. 1912 Die Oxydation von elementarem Schwefel durch Bakterien. Folia Microbiol., **1**, 487–496.
143. JACOBSEN, H. C. 1914 Die Oxydation von Schwefelwasserstoff durch Bakterien. Folia Microbiol., **3**, 155–162.
144. BEIJERINCK, M. W. 1904 Ueber die Bakterien, welche sich im Dunkeln mit Kohlensäure als Kohlenstoffquelle ernähren können. Centr. Bakt., II. Abt., **11**, 593–599. Also in: Verzam. Werken, IV, 242–248.
145. BEIJERINCK, M. W. 1920 Chemosynthesis at denitrification with sulfur as source of energy. Proc. Kon. Akad. Wetensch., Amsterdam, **22**, 899–908. Also in: Verzam. Werken, V, 281–288.
146. LIESKE, R. 1912 Untersuchungen über die Physiologie denitrifizierender Schwefelbakterien. Sitzungsber. Heidelb. Akad. Wiss., Abt. B., **1912**, 63–91.
147. WAKSMAN, S. A., AND JOFFE, J. S. 1922 Microörganisms concerned in the oxidation of sulfur in the soil. II. *Thiobacillus thiooxidans*, a new sulfur-oxidizing organism isolated from the soil. J. Bact., **7**, 239–256.
148. STARKEY, R. L. 1935 Isolation of some bacteria which oxidize thiosulfate. Soil Sci., **39**, 197–216.
149. MIYOSHI, M. 1897 Studien über die Schwefelrasenbildung und die Schwefelbacterien der Thermen von Yumoto bei Nikko. J. Coll. Sci., Imp. Univ., Tokyo, **10**, 143–173.
150. PRINGSHEIM, E. G. 1923 Zur Kritik der Bakteriensystematik. Lotos, Prag, **71**, 357–377.
151. BAVENDAMM, W. 1936 Die Physiologie der schwefelspeichernden und schwefelfreien Purpurbakterien. Ergeb. Biol., **13**, 1–53.
152. PRIBRAM, E. 1933 Klassifikation der Schizomyceten. Deuticke, Leipzig.
153. KLUYVER, A. J., AND VAN NIEL, C. B. 1936 Prospects for a natural system of classification of bacteria. Centr. Bakt., II. Abt., **94**, 369–403.
154. STANIER, R. Y., AND VAN NIEL, C. B. 1941 The main outlines of bacterial classification. J. Bact., **42**, 437–466.
155. HAMA, T. 1933 Nine species belonging to the order *Thiobacteriales* Buchanan, found in Hiroshima. J. Sci. Hiroshima Univ., Ser. B, Div. 2, **1**, 157–164.
156. PLOWE, J. Q., SAPERO, J. J., MOY, H. B., AND BECKING, L. B. 1926 A preliminary study of *Rhodobacillus palustris*, Molisch. Proc. Soc. Exptl. Biol. Med., **24**, 73–75.
157. UTERMÖHL, H. 1925 Limnologische Phytoplanktonstudien. Arch. Hydrobiol., Suppl. Bd. V, 1–527.

158. RAHN, O. 1929 Contributions to the classification of bacteria. Centr. Bakt., II. Abt., **78**, 1–21.
159. ELROD, R. P., AND BRAUN, A. C. 1941 A phytopathogenic bacterium fatal to laboratory animals. Science, **94**, 520–521.
160. BELLOC, H. 1910 On Anything. Dutton & Co., New York.
161. PARR, L. W., AND ROBBINS, M. L. 1942 The concept of stability and some of its implications. J. Bact., **43**, 661–684.
162. STERN, A., UND PRUCKNER, F. 1940 Lichtabsorption einiger Derivate des Bakteriochlorophylls. Z. physik. Chem., Abt. A, **185**, 141–151.