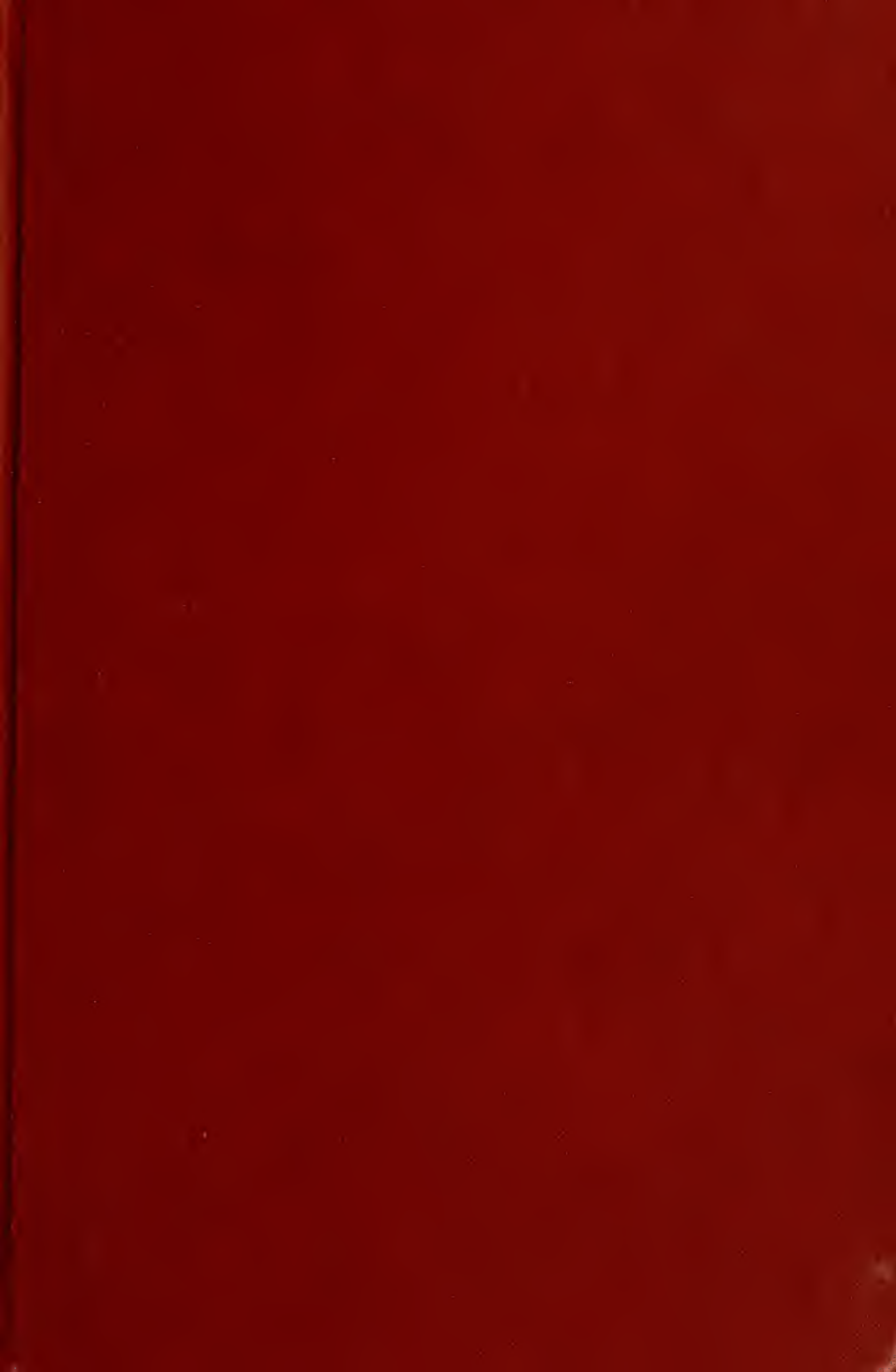

BACTERIAL PHOTOSYNTHESIS

Contribution No. 112 of The Charles F. Kettering Research Laboratory

BACTERIAL PHOTOSYNTHESIS

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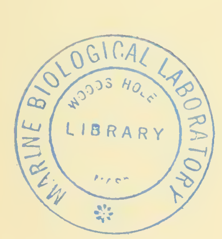

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INTRODUCTION

This book contains the papers presented at a small, invitational Symposium on Bacterial Photosynthesis held in Yellow Springs, Ohio on March 18-20, 1963. The Charles F. Kettering Research Laboratory was very pleased to serve as host for this conference. Research progress on bacterial systems is moving rapidly and a review of the present state of knowledge seemed appropriate. The organizing committee therefore invited some fifty-five overseas and American investigators to meet and exchange information at a small, informal meeting held under the Foundation's auspices. The staff of the Charles F. Kettering Research Laboratory was stimulated immensely by the conference; we hope these papers will serve as a point of departure for additional photosynthetic investigations.

E. W. Kettering

Left to right: H. Gest, H. Gaffron, C. B. van Niel, R. Hill, L. P. Vernon, A. San Pietro.

To KEES

In recognition of his pioneering research on bacterial photosynthesis, this volume is dedicated to Dr. C. B. van Niel, Herzstein Professor of Biology, Stanford University.

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PREFACE

The past ten years have witnessed a rapidly increasing tempo of research on bacterial photosynthesis. This is perhaps attributable, in part, to the fact that modern developments in microbiology and biochemistry have demonstrated the potential advantages of using bacteria as the source of experimental systems for investigation of numerous basic biological phenomena. The relatively large, and possibly unique, range of metabolic capacities shown by the photosynthetic bacteria has added to their appeal for such studies. There is little doubt, however, that the main stimulus for closer scrutiny of these organisms stems from the desire to understand the "comparative biochemistry" of photosynthesis in greater depth. Although considerable evidence has accumulated showing close similarities between green plant and bacterial photosynthesis, investigators have long been intrigued with the reasons for, and possible implications of, the differences observed between the two processes. Indeed, when a fundamental research advance is made with either type of photosynthetic system, pertinent reexamination of the other soon follows. This pattern of cross-checking, which has become more prominent in recent years, has unquestionably facilitated progress in elucidation of the mysteries of photosynthesis.

In the past, however, symposia on this important topic have been concerned primarily with green plant systems and only secondarily with photosynthetic bacteria. This realization and the conviction that an up-to-date review of the problem would stimulate further progress led to organization of the present symposium, devoted exclusively to the bacterial process. Inevitably, we were faced with the usual dilemmas posed by the attempt to arrange a meeting at which all investigators actively working in the field would be present and able to exchange ideas and viewpoints freely under informal circumstances. It is our hope that any shortcomings in this respect will be ameliorated by our efforts to make the proceedings of the symposium available to the scientific community at the earliest possible time.

A number of animated controversies developed during the course of the meeting and this we interpret as one of the signs of its success. Groups of participants directly interested in the debated questions met informally, as time permitted, with the aim of resolving basic issues. Our deepest gratitude goes to Dr. Martin Kamen who undertook the formidable task of presenting their conclusions to the symposium audience during his summarizing remarks.

It is a pleasure to acknowledge the valuable services of the following conveners: Dr. W. Arnold, Dr. C. S. French, Dr. R. Y. Stanier, and Dr. C. B. van Niel. Special notes of gratitude are due to Dr. R. K. Clayton and Miss Jane Finney for their editorial assistance and to Mr. Justin C. Crawford for his capable efforts in making the necessary arrangements. The editors are also indebted to those who contributed to the appendices, which contain frequently required experimental data and bibliographies of areas which could not be adequately covered due to lack of time.

We are particularly grateful to Antioch College for providing an auditorium and other attractive facilities for the symposium, and to the Charles F. Kettering Foundation for financially supporting the symposium.

The Editors

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LIST OF ABBREVIATIONS

A(OD)	absorbance (optical density)
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BChl	bacteriochlorophyll
Chl	chlorophyll
pCMB	p-chloromercuribenzoate
Cyt	cytochrome
DCMU	3-(3-4-dichlorophenyl)-1,1-dimethylurea
DPIP, $DPIPH_2$	2,6-dichlorophenolindophenol and its reduced form
EDTA	ethylenediaminetetraacetic acid
ev	electron-volt
ESR (EPR)	electron spin resonance
FAD, $FADH_2$	flavin adenine dinucleotide and its reduced form
FMN, $FMNH_2$	flavin mononucleotide and its reduced form
IDP	inosine diphosphate
ITP	inosine triphosphate
mv	millivolt
NAD, NADH (DPN, DPNH)	nicotinamide adenine dinucleotide and its reduced form
NADP, NADPH (TPN, TPNH)	nicotinamide adenine dinucleotide phosphate and its reduced form
PCMB	*p*-chloromercuribenzoate
Pi	orthophosphate
PMS (MPM)	phenazine methosulfate (methyl phenazonium methosulfate)
PPNR	photosynthetic pyridine nucleotide reductase
PQ	plastoquinone
RHP	*Rhodospirillum rubrum* heme protein (cytochromoid C)
Tris	tris(hydroxymethyl)aminomethane
UQ (CoQ)	ubiquinone

H. Gaffron addressing the opening session of the symposium.

OPENING ADDRESS

Opening Address

VAN NIEL'S THEORY: THIRTY YEARS AFTER

HANS GAFFRON
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Tallahassee, Florida

Ladies and Gentlemen:

Considering that we are, as I thought we would be, a gathering of experts, there is really not much excuse for an opening address. The purpose of such an address is probably to remind the participants of the few major problems which the meeting is about, in order that we do not lose sight of them when we begin to discuss the countless details and ramifications into which a topic like Bacterial Photosynthesis must of necessity be subdivided. On the other hand we have had lately symposia and meetings contributing to the problem of photosynthesis at the rate of one or two per year. It is unlikely, therefore, that any one of us could have lost sight of the major problem.

Even with modern teamwork, progress in terms of essential new discoveries is not so fast that a proposed meeting here and there could not be skipped. But the fact that we are all here shows that the idea of the organizing committee to hold this particular symposium must nevertheless have struck today's guests as an attractive proposition.

Two reasons can be pointed out immediately. Though we have been acquainted with the Kettering Foundation as a place of research in our field since the days of Inman, Albers and Knorr, Rothemund and later of Clendenning and Eyster, the Laboratory has lately undergone a rebuilding and an expansion which has moved it into the front line of modern research on the photochemistry in living cells.

One attraction must have been the desire to visit the Kettering Laboratory, and the second was the idea to single out the phototrophic bacteria for special consideration. This plan has automatically brought together not only the young keen minds for whom history begins after 1945, but also those of us who, in a much more leisurely way than is the fashion today, began once upon a time to investigate those reactions which still provide so much material for lively discussions.

A look at the elegantly done Symposium program has sharpened our anticipation of the coming intellectual pleasures. Mr. Kettering and Dr. Vernon deserve thanks indeed for having called us together.

And then there was the prospect that we might have among us our good colleague, the eminent and wise scholar Cornelis Benardus van Niel of Pacific Grove, whom his friends and pupils call Kees. Actually

I did not believe he would show up—too many of our meetings during past years had to be held without him. But to my surprise, and to everybody's pleasure, he arrived yesterday evening.

Our program promises us the description of quite a number of new observations, experimental techniques and contributions for or against certain hypotheses aimed at explaining the particular kind of metabolism that sets the purple bacteria apart from the green plant.

Hardly any one of us who were around twenty years ago would have believed that van Niel's idea of a photolysis of water as the core of the photosynthesis problem could still elicit a vivid discussion today. For the green plants it had been proven as correct by Hill's reaction. And as a reasonable interpretation also for the anaerobic photometabolism of purple bacteria there was the indirect evidence of the adaptable hydrogenase-containing algae.

Purple bacteria furnished van Niel the key to the first generally convincing picture of the photosynthetic process in terms of modern metabolic ideas. And purple bacteria are now believed to provide clear evidence that a photolysis of water—water as an intermediate hydrogen donor—should not be accepted as part of the hypothetical picture for bacterial photosynthesis. That is, van Niel's generalization of 1935 is disallowed.

It is about this question mainly that I would like to speak to you. Usually after thirty years a theory ought to have been transformed into fact or replaced by a better one. With van Niel's theory it so happened that after ten years there were no doubts left that the oxygen of photosynthesis originates from water. This we have accepted as fact.

I propose to show that, like any good scientific theory which managed to live in these hectic times for thirty years, van Niel's extended version is still useful. A truly good theory never dies—it only becomes more refined. This may make it more difficult to explain and to teach—but it does not render the simpler version wrong.

It is often repeated that one new fact which does not fit destroys a hypothesis. This is not true. As long as this new observation does not give birth to a better theory—and better is by definition the more encompassing view—it should be noted but treated as if with a little more thought and patience it may soon find its place within the existing order.

We have accepted the proposition that light will split, oxidize, dehydrogenate, or photolyze water in green plants, because on the face of so much evidence we cannot explain from where else the oxygen could originate. On the other hand, purple bacteria do not evolve oxygen. Why should we assume that water is involved in the photochemical process, even as an intermediate and incomplete process, when there is as yet no incontrovertible evidence that the assumption is warranted? How sound a viewpoint—and what a dull one. As I pointed out recently somewhere else, the mechanism to release oxygen from water with

eight quanta is too remarkable and complex a mechanism not to have a long evolutionary history. And there are too many parallels in the behavior of photosynthetic bacteria and plants not to be intrigued by what I am willing to call the more interesting and therefore more rewarding hypothetical proposition. And perhaps I am biased because it took me once so long to recognize its elegance.

Certainly thirty years ago I simply could not see why I should accept van Niel's proposition that organic substances serve purple bacteria exclusively as hydrogen donors (just like H_2S, sulfur or hydrogen) for the reduction of carbon dioxide to carbohydrate, and thence to bacterial substance.

My own results with purple bacteria did not show this at all. Quite independently (never having heard of van Niel) I had started about 1929 on investigations on purple bacteria after Warburg had mentioned to me that Stalfeld had told him of these strange organisms. As a chemist I had never looked at a microbe before and knew only Warburg's great discovery—the alga *Chlorella*. Soon I discovered that the reddish microbes behaved quite differently from green plants. They refused to do photosynthesis but evidently ate organic acids in the light without further ado, either with a stoichiometrically determined amount of carbon dioxide, or, if available, also with hydrogen. The product of the photometabolism was partly a substance ($C_4H_6O_2$) (which Hans Fisher later depolymerized into crotonic acid) and for the greater part just more bacteria. Later, when working with *Chromatium*, the purple sulfur bacteria, I found that they produced lots of H_2S in the dark, particularly when previously illuminated in presence of butyrate. So I concluded that the light reactions with sulfur were reversible and that this was the mechanism by which they were able to attack organic substances. Many of you will remember that van Niel challenged this vigorously. Years later Henley in my laboratory confirmed the fermentative sulfide formation from internally stored sulfur but not from sulfate. My observation of a particular accelerating effect of added sulfate was indeed, as van Niel had shown, a nonspecific salt effect.

In 1935 van Niel extended his special theory so that it included also the metabolism of the heterotrophic purple bacteria. In this paper he quotes Gaffron's statement that photosynthesis of the purple bacteria involves the cooperation of a larger number of molecules and that several intermediate reactions occur before the first stable reaction products appear. Van Niel then wrote, "This statement seems to contain an argument against a unified concept of photosynthesis in green plants and purple bacteria." Because I could not see eye to eye with a Dr. Roelofsen, working in Kluyver's laboratory, van Niel had spent a year in Holland devising a good number of experiments to prove convincingly that sulfur bacteria can use organic substances directly as hydrogen donors, just like the Athiorhodaceae. He then came to Berlin to see me, we set up one or two experiments, they were absolutely

convincing, and I conceded defeat quickly so that we could go sight-seeing. Soon a long publication appeared in which van Niel explained every one of my experiments according to his views.

My experiments were perfectly reproducible. But except for some evidence that the photochemistry with aliphatic acids was much more complex than the simple overall equation of green plant photosynthesis allowed for, I had no rational hypothesis at all. It was only a few years later, after Hill's chloroplast reactions and mainly on account of my own photoreduction experiments, that I understood fully the power of van Niel's concept. Because I firmly believe that the mere description of new phenomena is the lesser half of any scientific task and that facts, unless they can be used to support or to revise current theoretical opinion, remain just memorable curiosities until someone provides the theoretical connection with existing knowledge, I would like to point out the following. Considering that van Niel's was the first comprehensive and fruitful theory of photosynthesis which had been proposed until that time, he had to make the attempt to keep the fundamental principle intact.

It has been very agreeable, satisfying, and flattering, of course, that recently Stanier and Doudoroff did prove that the reactions in purple bacteria I had written about really exist. And I can only recommend warmly the technique of staying alive long enough to see such vindications happen. But does this invalidate van Niel's general concept of photosynthesis as it applies to the metabolism of purple bacteria? I do not think so for a moment.

Here I would like to digress with a remark on the importance of schools and the influence of masters. When I first met van Niel in 1935 I was absolutely under the spell of Warburg. He was twenty years older, in the prime of his productivity with a dozen fundamental discoveries already to his credit and many more ahead of him. He was then an implacable foe of Wieland's dehydrogenation theory. He believed (as most of you are well aware he still does) in the direct photochemical decomposition of carbon dioxide, and this was sufficient to put a block into my brain. The Wieland-Kluyver-van Niel way of looking at the same factual material I rejected for reasons only a psychologist may be able to explain to us in the future. I tell this story because the same strange power is, as you know, still at work today. And it means that conscientious teachers should, after having convinced their pupils of their own viewpoint, challenge them to find faults with the ruling theory of the laboratory and help them even in this exercise.

Another digression is on the lucky choice of material to work with. If Willstätter and Stoll had studied minced spinach extracts instead of minced sunflower leaves they might have found what Hill found twenty years later. The fabulous success of Warburg's choice of *Chlorella* we need only to mention in passing. If I had started with *Chromatium* instead of with Athiorhodaceae the confusing conflict with van Niel might

not have arisen. On the other hand, as I pointed out, the wrong theory exerts perhaps an even stronger inhibitory influence.

And then there is costly apparatus—it too can hinder progress when you believe that because it was acquired on a special government grant it has to be used for its money's worth. It made a great impression on me when a new-fangled very expensive light source (I believe specially built by Siemens) had arrived at Warburg's laboratory. The great man and his assistants spent an afternoon trying to make it work. When it turned out that the thing was a disappointment, Warburg coolly said, "Negelein—store it in the attic," and never looked at it again.

To return to the photolysis of water. What I have been able to understand about our experiments on adapted algae during the past twenty years I managed on the basis of the idea of internal back reaction involving water. That is, internal oxido-reductions involving hydrogen donors have to be assumed.

As you know, during recent years the question concerning the nature of the primary process has been progressively restricted to a smaller and smaller field of enquiry (Compare Fig. 1). The entire carbon assimilation mechanism has been cleared away as a typical example of orthodox biochemistry upon which plants and photosynthetic bacteria have no exclusive property right. And a look at our program will tell you that the discussion has narrowed to the question of how much phosphorylation, TPN reduction, and oxygen evolution have to do with the light energy conversion process. Thus the difference between bacteria and plants brings us to the question: What happens in the pigment complex of bacteriochlorophyll which distinguishes the end result from that we find in the green plants?

Let us enumerate what purple bacteria and plants have in common. 1) A chlorophyll *a* type pigment. The bacterial form contains two more hydrogens and handles light quanta at a discount of ten kilocalories per mole quanta as compared with the green chlorophyll, because the singlet absorption band lies around λ 890 mμ. 2) Different percentages of one chlorophyll *a* are distributed among several binding sites, as attested to by the various humps in the main absorption bands seen in living cells. 3) Plants and bacteria contain carotenes and quinones, not quite identical chemically but very likely serving the same functions. 4) Not only the aerobic plants as shown by Hill, but also the obligate anaerobic purple bacteria have, as Kamen and his co-workers discovered, cytochromes—not one, but at least two, and with oxidation-reduction potentials that are 0.2 volts apart. This is true for plants as well as bacteria, and we ought to remember this when we come to discuss the role of cytochromes. 5) Photophosphorylation. The observation of a light-induced phosphate turnover in intact purple and green cells preceded by several years the demonstration that a respiratory type of phosphorylation occurs in cell-free preparations. Light and water replace, as van Niel would say, the role which a hydrogen donor

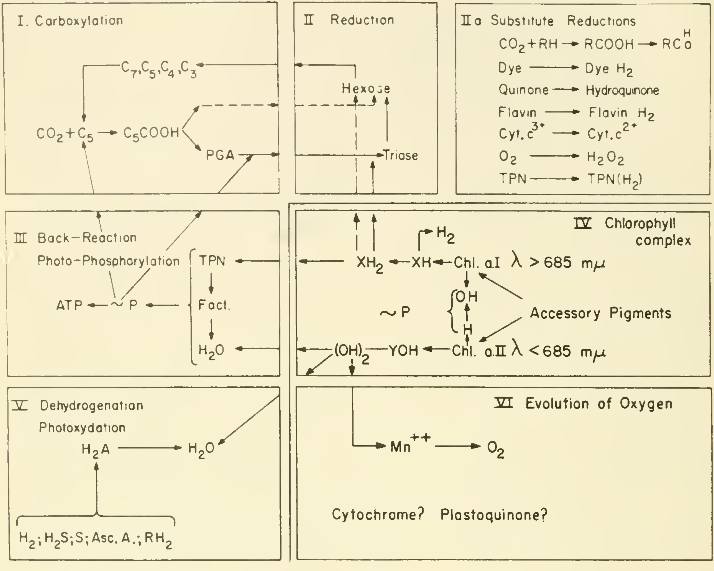


Fig. 1. Sum of the recognized partial enzymatic systems which together constitute the mechanics of photosynthesis. All except the chlorophyll complex are able to function without light.

and oxygen play in the corresponding ordinary dark metabolism. Thus a cell-free system can be set up which converts light energy into typical phosphate energy: ATP. The experiment works with green or purple chromatophores—there is no difference. 6) Identical or very similar enzymes serve as reducing agents for the carbon dioxide reduction system and for the initial carboxylation. 7) Photosynthetic units exist in both types of organisms, according to Arnold's experiments. This implies at least two differently bound molecules of the same pigment class in bacteria just as well as in plants: the photon-collecting and the photon-converting molecules of which the latter, according to Franck, must have its main absorption at the long wavelength end of the complex spectrum shown by living chlorophyll. 8) Transfer of energy from light-absorbing accessory pigments happens in both classes of organisms. To these eight points others equally important may soon be added. In short, there is so much in common that the problem of utilization of water as hydrogen donor might better

be approached by calling attention to the differences. Which, and how important, are they?

Is the color difference important? Probably not, because the green bacteria use quanta of very nearly the same energy level as the green plants, yet do not evolve oxygen.

Are the enzymes important that permit the purple and green bacteria to use inorganic or organic hydrogen donors for their photosynthesis? Again I believe the answer is no, because we have now a dozen or more typical oxygen-releasing algae which can skip the oxygen part and do a bacterial photoreduction with hydrogen just like obligate anaerobes. Furthermore, Pringsheim and Wiessner discovered a green flagellate that, at best, shows only a marginal capacity for normal photosynthesis. Like a purple bacterium, it cannot grow at all in the light when presented only with carbon dioxide and water. It cannot even grow like *Rhodospirillum* with acetate and oxygen in the dark. It grows exclusively with acetate in the light.

In short, the more we look around the more we find gradual transitions between the photochemical capacities of a typical purple bacterium and that of the most specialized oxygen-evolving green plants.

The only clear-cut difference I know, in respect to the ability to release oxygen, is the need for manganese first shown by Kessler in our laboratory, and confirmed for the Hill reaction by Eyster in this laboratory. Wiessner later found that purple bacteria thrive on a low manganese diet which forces the algae to turn heterotroph for dear life. Then there is a difference in the quinones which might be important. Finally the absence of an Emerson effect in green algae after adaptation to hydrogen, a condition that parallels again, as I have just learned, the behavior of purple bacteria. In these van Niel and Blinks could find no Emerson effect.

The spectral investigations of Duysens, Kok, Rabinowitch; the gas exchange measurements by French, Myers, Govindjee; and whoever may by now have acquired the proper monochromators have established that in photosynthesis two or more pigments which are activated by distinctly different wavelengths have to cooperate. The products of the two separate photochemical processes are chemical substances, not physical states, because, according to Myers and French, they live for seconds. And they both must be present in order that oxygen can appear, and without oxygen evolution there is no reduction under anaerobic conditions.

The two-pigment problem has now merged with the two-quanta-per-hydrogen problem, first clearly enunciated by Franck and Herzfeld many years ago. Hill, Kok, Duysens and Witt, all independently, gave reasons why one part of the tandem pigment should be assigned to the reduction, the other to the oxidation side. A year ago Dr. Franck paid us a visit. Dr. Clayton happened to be there too. Naturally we discussed the Emerson effect, about which Franck had published some very in-

teresting theoretical propositions. Franck suggested that the long wavelength pigment was likely to be oxidized more easily than the rest of the chlorophyll.

If this were truly so, it would follow that our algae might be able to produce oxygen in the dark red under anaerobic conditions. Bishop and I made the experiment and it turned out that an adaptation to photoreduction was necessary. Anaerobic treatment was not enough to make the dark red radiation efficient. I reported this at the Paris meeting last summer. Of course this confirms the assumptions of Kok, et al.

If it is possible to set the entire carbon dioxide reduction machinery in motion, as we have done with that part of the twin-pigment system which for itself alone cannot produce oxygen, then one is tempted to relegate the photolysis of water entirely to the other twin. Instead of making things easy, the now popular schemes of Kok, Duysens, etc., introduce, however, two problems at once—or, rather, make them especially conspicuous.

According to well-known measurements of quantum yields, photoreduction on the one hand turns out to be singularly inefficient and uses at least twice or three times as many quanta as it ought to, while the evolution of oxygen per se might become, in these schemes, a four-quanta process.

Instead of continuing to speculate how near or far from the double primary process van Niel's photolysis may be found, we may look at some of the available experiments.

Arnon, in the Proceedings of the National Academy of Sciences, has in a charming way enumerated the stages of the strip tease which have followed each other for a century to get at the true first stable chemical products of the primary process. The accent is on stable, as I would like to emphasize. First there was starch. Then numerous intermediates known from the respiratory sugar metabolism. Then specifically PGA, then finally ATP. And here the strip comes dangerously close to the end, because the photochemically reduced enzyme PPNR of San Pietro, now rebaptized ferredoxin, is stable only in the absence of oxygen. Thus the first stable primary product appears to be reduced TPN. This is also the beginning of the back flow of electrons (hydrogen) which leads to phosphorylation, the only chemical energy source which, according to a new generalized concept, is necessary under anaerobic conditions to give us the metabolism of purple bacteria or adapted algae.

Twenty years ago Gaffron and Rubin observed a photochemical evolution of hydrogen from adapted algae when they were put in nitrogen or helium, without carbon dioxide and preferably in the presence of dinitrophenol. Some time later, Kamen, Breghoff and Gest discovered a photohydrogen evolution which they considered as being much more significant than the reaction in green algae because it was so much

bigger. A while ago Bishop in our laboratory noted that a mutant of *Scenedesmus*, which cannot evolve oxygen but handles photoreduction very well, never produced hydrogen under conditions when we would expect it to do so. In normal *Scenedesmus* evolution of hydrogen was found to be sensitive to typical oxygen evolution inhibition. Putting the old and the new experiments together, it seemed clear why light-induced evolution of hydrogen is poor in algae and good in bacteria.

The purple bacteria are set to use organic material, and if there is a separation of [H] and [OH] the latter can easily be used to oxidize the organic substrate. This is supported by Gest's recent studies where hydrogen and carbon dioxide appear simultaneously and in proper proportion. The green plants, on the other hand, have either put up a permeability barrier against, or lost the enzymes for, the same organic hydrogen donors. This apparently has been one condition for the efficient evolution of free oxygen. What happens in the green plants is that there will be back reactions whenever the reducing intermediates of the light reaction, such as reduced ferredoxin, are not utilized. Some of these back reactions may be the ones that produce cyclic phosphorylation.

Assume now that we activate the hydrogenase in a normal green plant, replace the hydrogen by nitrogen, remove the carbon dioxide, poison the phosphorylating back reactions, and illuminate. What do we get? A complete and direct photolysis of water by light. Hydrogen and oxygen are evolved in about the right proportions and in impressive amounts.

If the oxygen evolution is stopped specifically by any of the three possible methods—poison, manganese deficiency, or mutation—there is no hydrogen evolution. No hydrogen without oxygen. It follows in our opinion that this is the reaction nearest to the photochemical process which gives you stable, usable products (Fig. 2).

Thus we have reached the end of the line. This is what light is able to produce. Once there is nascent hydrogen and nascent oxygen, everything that follows falls under the category of enzymatic dark reactions. Dr. Bishop will present these results at the Atlantic City Federation meeting.

Let us come back to the two-pigment problem and van Niel's theory. The basic two-pigment skeleton of a general photosynthetic system thus looks like the scheme of Fig. 3. It is of utmost importance, of course, to fill in the arrows correctly with the proper enzymes, metals, proton and electron transfer agents. Only then shall we know for sure how the photolysis of water proceeds with two quanta.

The water splitting is the result of the whole sequence and there may be no one particular place to point to where we can say: here photolysis happens. There are too many spots (at any one-electron transfer metal catalysis, for instance) where we have to formulate the reaction with the aid of water to balance the charges.

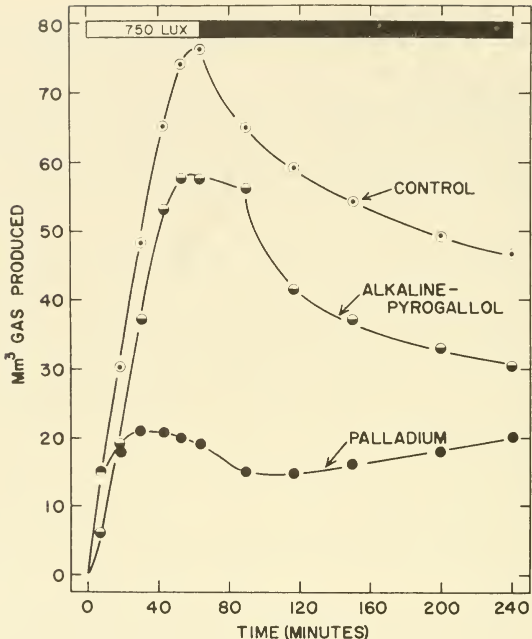


Fig. 2. Photolysis of water in *Scenedesmus* as a simple manometric experiment. Hydrogen and oxygen are produced in about the ratio 2:1 if photosynthesis, photoreduction and phosphorylation are suppressed. Adapted algae are illuminated in absence of CO_2, under nitrogen and in presence of an inhibitor of phosphorylation. The mixed character of the gas evolved becomes apparent by the way it reacts after the light has been turned off.

Unless a water molecule is shown to be firmly bound as part of a molecule in such a way that it is not exchangeable, metabolic reactions in aqueous media will not distinguish one water molecule from another. When we speak of the photolysis of water, we do not mean that the emerging hydrogen and oxygen molecules stem from one and the same molecule as earlier hypotheses assumed. Nobody ever suggested this for electrolysis, for instance. Movements of electrons and holes, or charge transfer, are part of the mechanisms of reversible oxido reductions which in turn are the wheels of the apparatus which brings

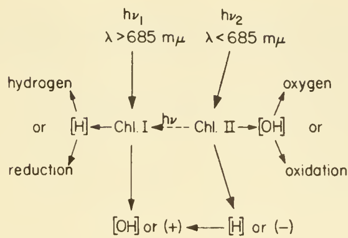


Fig. 3. The basic mechanism of photosynthesis which in a two pigment system produces overall what amounts to a photolysis of water.

about the final result, the photolysis of water. This final result is not the appearance of positive holes on the one side, or a corresponding stream of electrons ejected on the other, but the appearance of the elements hydrogen and oxygen, or in their place such permanent chemical changes as may be brought about metabolically by either hydrogen or oxygen.

To achieve present day photosynthesis, it was necessary in the course of natural evolution to prevent as far as possible all types of intermediary re-oxidations. Free oxygen had to be eliminated as a waste product. And also the release of free hydrogen had to be prevented in order that it could be used instead in intermediate forms for synthetic reactions.

Shall we believe now that the purple bacteria just have only one half of the system and that nowhere water enters into their photochemical mechanism? This would mean, if we think of evolution, that the plants arose by doubling the arrangement of the purple bacteria. If so, this would leave us with the dilemma of the eight quanta in photoreduction mentioned above. Duysens, in desperation, believes the quantum number to be an accident. Let me point out that the obligate anaerobic phototrophic bacteria, as well as *Chlamydobotrys* of Pringsheim and Wiessner, have to grow while the light is shining. No fermentation supports growth in the dark. In the green plants we keep respiration and growth on a separate energy balance sheet, while with the bacteria our energy measurements include everything. This may equalize the energy requirements of plants and bacteria in an accidental way.

But a look at the regularity of Larsen's results in his beautiful measurements of the quantum requirements in green and red bacteria makes the chance hypothesis appear rather weak. Regardless of the substrate, H_2, H_2S or thiosulfate, and the correspondingly different

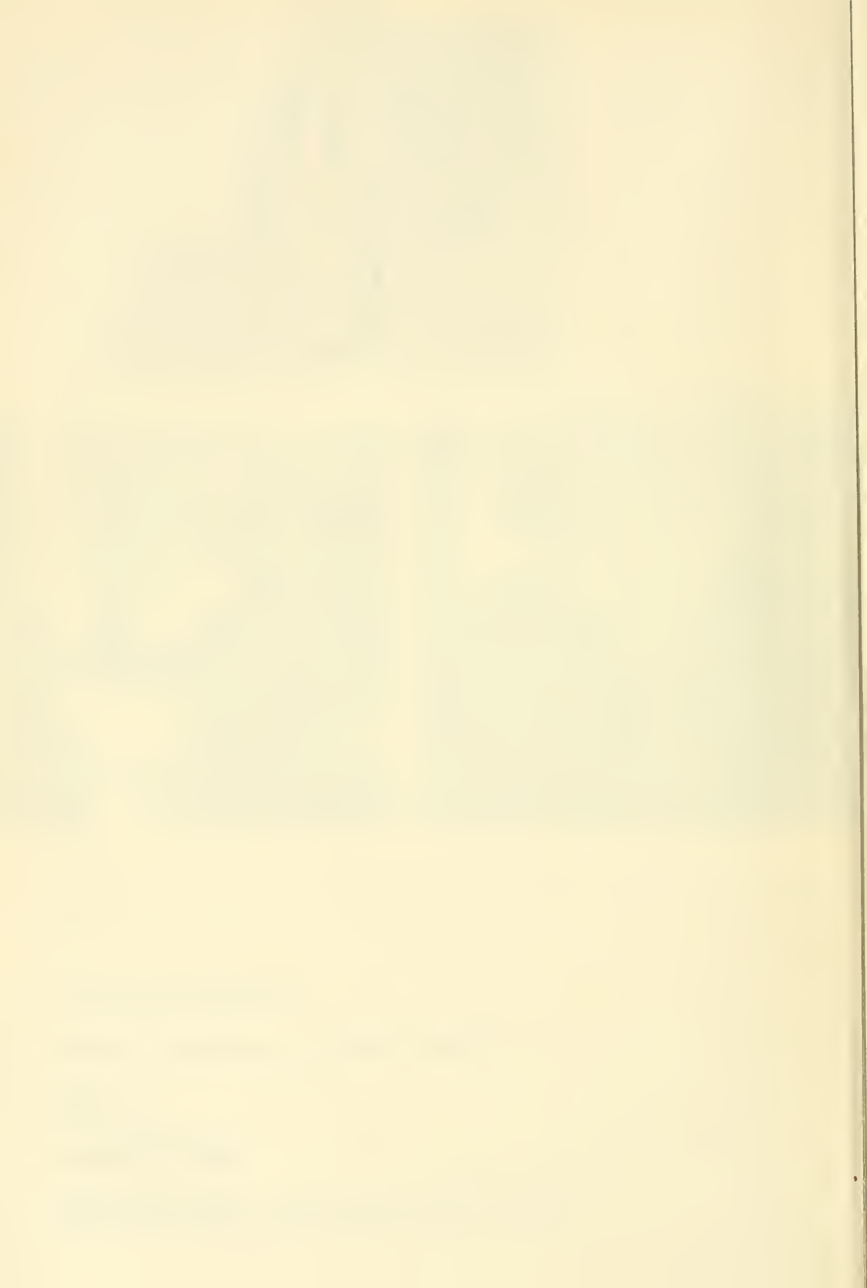
efficiency in energy utilization, the quantum number remained about nine, and constant. If purple bacteria and adapted algae use only one half of the mechanism shown in Fig. 3 which is required for full photosynthesis—i.e., for the complete photolysis of water—then there is no obvious spot for the noncyclic phosphorylation which everybody likes now to place at the junction of the two-pigment systems. The bacteria are forced to put extra energy into cyclic phosphorylation. This too might explain an equal quantum number per carbon dioxide reduced.

If we accept at all the idea that green plant photosynthesis evolved from the simpler system still to be found in purple bacteria, it is not so unreasonable to believe that the twin-pigment arrangement is already present in the latter. Only the differences in potential between the left and right halves of the tandem arrangement did not yet shift far enough apart to allow for a spontaneous dismutation of [OH] into free oxygen with the aid of newly added enzymes such as the manganese-containing one I mentioned earlier. Complicated as this all sounds, I need not remind you that in reality it will eventually turn out to be more intricate still. There are the various sets of accessory pigments, for instance, which seem to be attached either to the bright red or to the dark red absorbing chlorophyll. Why under these circumstances van Niel's simple concept of an intermediary photolysis of water should have aroused so much opposition, I cannot see. If a theory explains a series of rather diverse observations in a consistent manner, and makes sense from the point of view of increased metabolic potentialities, I prefer it to a disjointed set of explanations of which each one does not reach further than a narrowly circumscribed set of experimental conditions. I am quite confident that despite the great number of new phenomena we are going to discuss during the coming session, there will not be one observation which clearly demands that we abandon van Niel's hypothesis of 1935. In other words, at the end of this week it will be just as much alive as during the years gone by.

Top: C. S. French; *right:* C. Black, R. A. Lazzarini, R. Bartsch;
left: N. Good, G. Hind.

I

COMPONENTS, STRUCTURE, and FUNCTION of the BACTERIAL PHOTOCHEMICAL APPARATUS



CAROTENOIDS OF PHOTOSYNTHETIC BACTERIA—DISTRIBUTION, STRUCTURE AND BIOSYNTHESIS

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A characteristic feature of all photosynthetic bacteria is their content of yellow to violet carotenoid pigments, which contribute to the spectacular colours occasionally exhibited by the bacterial cell.

Elegant studies of such bacteria, performed by R. Y. Stanier, L. N. M. Duysens and others, have demonstrated the function of the carotenoids as protectors against photo-oxidation and as auxiliary absorbers of radiant energy for photosynthesis and phototaxis. Other functions have been claimed, but not rigidly proven. Pertinent reviews in this field are available (see, for example, 1,2,3).

In most of the photosynthetic bacteria the biosynthesis of carotenoids proceeds in such a manner that considerable amounts of intermediates can be isolated in addition to end products. The carotenoids of these organisms therefore represent a unique array of compounds which are biochemically and structurally very closely related. In addition, conditions can be created under which otherwise inaccessible intermediates accumulate, and their interconversions can be studied. Mainly for the above reasons investigations on photosynthetic bacteria have contributed much to the solution of problems connected with the biosynthesis of this class of natural products.

The number of known carotenoid pigments has increased significantly as a result of study of the pigment complex of such bacteria. Despite the fact that the carotenoids of photosynthetic bacteria are chemically closely related, sufficient variation does occur to make them useful for the characterization of these bacteria.

The present paper will be limited to recent progress in our knowledge of the distribution, chemical structure, and biosynthesis of coloured carotenoids of photosynthetic bacteria—a topic which reflects the special interest of the author. I hope the limitation is justified by the rapid and perhaps unexpected expansion of this field during the last few years. Much of the work to be discussed has not yet been published, although it is in press.

The carotenoids of photosynthetic bacteria are, with a few exceptions, aliphatic. They often carry tertiary hydroxyl and methoxyl groups located in the 1,1'-positions, and sometimes contain conjugated keto-groups. The chromophore, which consists of a variable number of conjugated double bonds, causing the yellow to pink-blue colour characteristic of these pigments, is often located rather unsymmetrically in the molecule. In addition, isolated double bonds may be present in agreement with the formal composition of the carotenoids by combination of eight isoprene units followed by dehydrogenations. As an example is shown the assumed structure (4) of OH-spheroidenone (Formula I).

(I)

Table 1 gives a summary of the characteristic structural features of carotenoids in the photosynthetic units of various groups of organisms. The carotenoid pigments of the photosynthetic bacteria are distinguished from those of algae and higher plants by their aliphatic and frequently unsymmetric nature and the presence in the molecule of tertiary hydroxyl or methoxyl groups. The photosynthetic tissue of the algae and higher plants generally contain bicyclic carotenoids of the α- and β-carotene type, often substituted in the 3-positions with secondary hydroxyl groups; epoxy-carotenoids are here quite abundant.

From photosynthetic bacteria there have been isolated to date 32 different carotenoids (including colourless forms). To the majority of these pigments fairly reliable chemical structures have been ascribed. In Table 2 is presented the distribution pattern of coloured carotenoids in 16 species of photosynthetic bacteria belonging to six different genera and four families. Aliphatic, hydroxylated and methoxylated carotenoids of what we shall call the normal spirilloxanthin series (involving the seven carotenoids participating in the transformation of lycopene to spirilloxanthin in *R. rubrum* (6,7), are abundant in the genus *Rhodospirillum*. This series occurs also in some species of *Rhodopseudomonas*, *Chromatium*, *Thiospirillum*, and in *Rhodomicrobium vannielii*. Keto-carotenoids of the spheroidenone type are restricted to three species of *Rhodopseudomonas*, whereas another type of keto-carotenoids is present in some Thiorhodaceae spp. Cyclic carotenoids have not so far been found in pure cultures of any of the Athiorhodaceae or Thiorhodaceae. Derivatives of the monocyclic γ-carotene are, so far as is known, restricted to the green bacteria, and β-carotene has been found only in *Rhodomicrobium vannielii*.

TABLE 1.

Characteristic structural features of the coloured carotenoids in the photosynthetic units of various groups of organisms

Type of organism		Aliphatic	Tertiary hydroxyl groups in 1-positions	Tertiary methoxyl groups	Mono-cyclic	Bi-cyclic	Secondary hydroxyl groups	Epoxydic oxygen
Photosynthetic bacteria	Purple bacteria	+	+	+	–*	–	–*	–
	Green bacteria	+	+	+?	+	–	–	–
	Rhodomicrobium vannielii	+	+	+	–	+	–	–
Algae and higher plants		–	–	–	–**	+	+	+

* with one exception
** with the exceptions stated in ref. 17

TABLE 2.

The distribution pattern of coloured carotenoids in photosynthetic bacteria

Family	Genus	Species	References	Aliphatic carotenoids: Neurosporene group		Lycopene group				P481 group			Spirilloxanthin group		Spheroidene group			Keto-carotenoids: Spheroidenone group							Cyclic carotenoids			
				Neurosporene	Chloroxanthin	Lycopene	Rhodopin (OH-Lycopene)	di-OH-Lycopene	Pigment 3	3,4-Dehydro-rhodopin	Anhydro-rhodovibrin (P481)	Rhodovibrin (OH-P481)	Monodemeth. spirilloxanthin	Spirilloxanthin	Demethylated spheroidene	Spheroidene (Y)	OH-Spheroidene (OH-Y)	Spheroidenone	OH-Spheroidenone	2-Keto-rhodovibrin	2-Keto-spirilloxanthin	Warmingone (Pigment 2)	Pigment 1	Okenone	γ-Carotene	OH-γ-Carotene	β-Carotene	Total number of coloured carotenoids
Athiorhodaceae	*Rhodospirillum*	*R. molischianum*	5			+	+																					2
		R. photometricum	5			+	+	?			+	+																5
		R. rubrum	6	+	⟨?⟩	+	+	+			+	+	+	+														9
	Rhodopseudomonas	*Rps. palustris*	5,7	+	⟨?⟩	+	+	+		+	+	+	+	+		⟨+⟩	⟨+⟩											12
		Rps. gelatinosa	4,8	+	+						+	+	+	+		+	+	+	+	+	+							12
		Rps. spheroides	5,9,10,11	+	[+]	?										+	+	+	+		+							8
		Rps. capsulata	5	+		+										+	+	+	+									6
		Rps. sp.	*	+		+																						2
Thiorhodaceae	*Chromatium*	*Chr.* sp. (strain D)	12,13	?	⟨?⟩	+	(+)	(+)			+	+	+	+														9
		Chr. vinosum	14			+	+	+		+	+		+	+														7
		Chr. okenii	15	⟨+⟩		⟨+⟩																		+				3
		Chr. warmingii	16		?	+	+		+													+	+					6
	Thiospirillum	*T. jenense*	14			+	+																					2
Chlorobacteriaceae	*Chlorobium*	*Chl. limicola*	17																						+	+		2
		Chl. thiosulfatoph.	17																						+	+		2
Hyphomicrobiaceae	*Rhodomicrobium*	*Rhm. vannielii*	18,19,**			+	+	+			+	+	+	+													+	8
	Total	16		8	6	13	9	6	1	2	7	6	6	6	0	4	4	3	3	1	2	1	1	1	2	2	1	

[ ] in mutants.

⟨ ⟩ in DPA-cultures only.

? presumably present, but wrongly identified.

* Aasmundrud, O. and Eimhjellen, K. E. Unpublished observations.

** Ryvarden, L. and Liaaen Jensen, S. Unpublished observations.

Lycopene is the carotenoid most widely distributed—it is found in 13 of the 16 species. The composition of the carotenoid mixture from *Rhodopseudomonas gelatinosa* is the most complex, consisting of 12 coloured components in cells grown under semi-aerobic conditions.

In turning to a discussion of the biosynthesis of these pigments I would like to refer to recent reviews (1,20,21), and to take as the starting point the structural scheme in Fig. 1, suggested for carotenoid biosynthesis in purple bacteria two years ago by Stanier's group (22, 23,7). This pathway offered a simple and logical picture of the carotenoid biosynthesis in non-sulphur purple bacteria. The scheme was based on a number of studies, *viz.*, the carotenoid composition of mutants of *Rhodopseudomonas spheroides* with deranged carotenoid synthesis (24), the diphenylamine-effect and carotenoid transformations

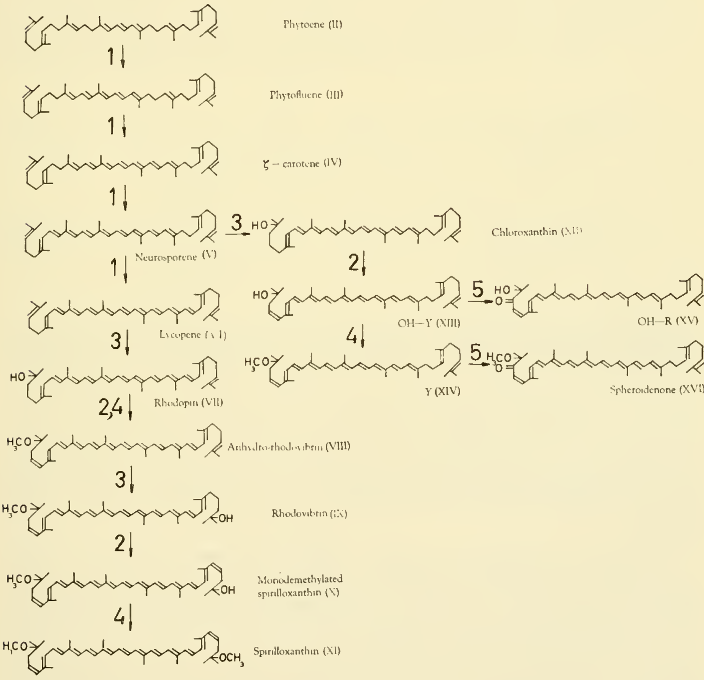

Fig. 1. Structural scheme for the pathway of carotenoid biosynthesis in purple bacteria (23,7).

TABLE 3.

The categories of step-reactions operative in the biosynthesis of coloured carotenoids in photosynthetic bacteria

Type no.	Reaction type	Structural features
1	Dehydrogenation	→
2	Dehydrogenation	OH → OH
3	Hydration	→ OH
4	Methylation	OH → OCH_3
5	Oxidation (aerobic)	OCH_3 → OCH_3 O
6	Hydroxylation	→ OH
7	Oxidation (anaerobic)	OH → O
8	Cyclization	? →

in washed cell suspensions of *R. rubrum* (6), the effect of oxygen on anaerobic cultures of *Rps. spheroides* (25,26), and finally the structural determinations of the carotenoids involved (27,7). The biochemical reactions participating in these transformations were grouped into five different categories (23,7), namely, two types of dehydrogenations, a hydration reaction consisting of addition of water to an isopropylidene double bond, methylation of a tertiary hydroxyl group, and finally a type of oxidation involving introduction of a conjugated keto-group. These reaction types are represented by numbers 1-5, depicted in Table 3.

Tentative chemical structures, (XIII), (XV) and (XVII a or b), in agreement with the limited data available for these trace compounds, were assigned to the three carotenoids designated by Goodwin as hydroxy-Y, hydroxy-R and P512 (5). The carotenoid P512, now referred to as P518 (11,4), exhibits absorption maxima at longer wavelengths than any other known carotenoid. This carotenoid has now been isolated from *Rps. spheroides* and *Rps. gelatinosa*, and its structure appears very likely to be that of a 2-keto-spirilloxanthin (XVIII) (11).

(XVII) a) R=H R'= ; b) R=CH_3 R'= OH

P518 (2-keto-spirilloxanthin) (XVIII)

The carotenoids OH-Y and OH-R have furthermore been shown to be the major carotenoids of anaerobic and illuminated aerobic cultures respectively, of *Rps. gelatinosa*, and can, according to recent investigations (8,4), presumably be depicted as (XIX) and (I). These two compounds both contain a tertiary methoxyl and a tertiary hydroxyl group and can formally be considered as having arisen from pigment Y (XIV), more recently named spheroidene (28), and spheroidenone (pigment R) (XVI) by a type 3 reaction (23), that is, addition of H_2O to an isopropylidene end-group. Hence OH-spheroidene (XIV) and OH-spheroidenone (I) should be successors instead of precursors of spheroidene (XIV) and spheroidenone (XVI).

Demethylated spheroidene ≠OH-Y (XIII)

≠OH-R (XV)

Spheroidene (Y) (XIV)

Spheroidenone (R) (XVI)

OH-Spheroidene (OH-Y) (XIX)

OH-Spheroidenone (OH-R) (I)

This assumption is supported by recent studies on the carotenoid biosynthesis in *Rps. gelatinosa* carried out in collaboration with Eimhjellen (29). The carotenoids of this organism had previously been studied by Goodwin (5). A reinvestigation under more defined conditions gave a rather different picture.

As seen from Table 4, cells grown photosynthetically under anaerobic conditions in light contain spheroidene, OH-spheroidene and spirilloxanthin. These carotenoids are not present in cultures grown in the light with suitable aeration. Instead, the corresponding keto-carotenoids appear in approximately the same relative amounts. The carotenoid complex of semi-aerobic cultures includes all these carotenoids. Neurosporene is always present at a low concentration in young cultures.

TABLE 4.

Carotenoid composition of Rhodopseudomonas gelatinosa *grown in the light and harvested in the late exponential stage of growth*

Carotenoid	Anaerobic cultures	Semi-aerobic cultures	Aerated cultures
Neurosporene	+	+	+
Spheroidene (Y)	+ (15)*	+	÷
OH-Spheroidene (OH-Y)	+ (65)	+	÷
Spirilloxanthin	+ (20)	+	÷
Spheroidenone (R)	÷	+	+ (15)
OH-Spheroidenone (OH-R)	÷	+	+ (65)
P518 (2-keto-spirilloxanthin)	÷	+	+ (20)

* These values are the approximate per cent of total carotenoid.

Further kinetic studies of the carotenoid content of washed cell suspensions incubated in buffer solution in the light revealed that transformations occurred within the carotenoids present, but no net carotenoid synthesis was observed. We consider the results of such experiments to be support for the interconversions depicted in Fig. 2: under anaerobic conditions from neurosporene and spheroidene to OH-spheroidene and spirilloxanthin, and in aerated cultures from neurosporene and spheroidenone to OH-spheroidenone and P518.

The transformations of spheroidene to spirilloxanthin under anaerobic conditions thus represent an alternative pathway to spirilloxanthin. Hitherto, the lycopene pathway (as in *R. rubrum*) has been considered exclusive.

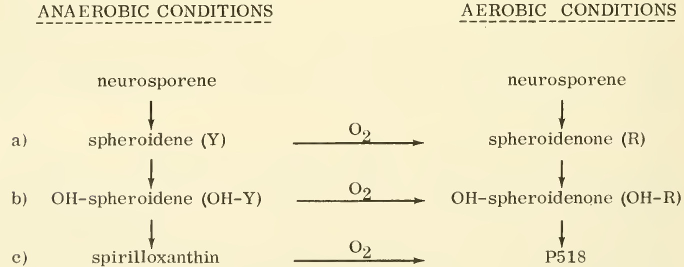

Fig. 2. Carotenoid interconversions in washed cell suspensions of *Rhodopseudomonas gelatinosa*.

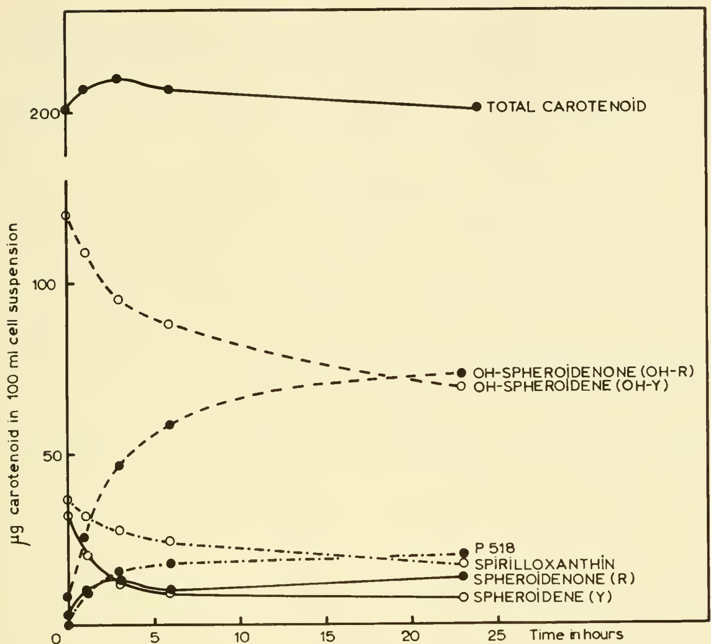

Fig. 3. The influence of air on the carotenoids in a resting cell suspension of anaerobically grown *Rhodopseudomonas gelatinosa*. Incubated at 30°C in light. From ref. (29).

Also the interconversions from neurosporene and spheroidenone to P518 observed in aerated cell suspensions represent an extension of the scheme earlier suggested by us (7,23) for carotenoid biosynthesis in Athiorhodaceae. Evidence has been obtained demonstrating that the last-mentioned transformations from spheroidenone can also take place under anaerobic conditions (29).

As indicated by the carotenoid composition (Table 4) of cells grown with various degrees of aeration, definite transformations occurred on the introduction of air to a washed cell suspension of anaerobically grown cells, incubated in buffer solution in the light. The results of such an experiment are presented in Fig. 3. It is seen that the total carotenoid content remains approximately constant. The decrease in the concentration of OH-spheroidene broadly equals the increase in

that of OH-spheroidenone. The same is true for the decrease and increase in the concentration of spirilloxanthin and P518, and also for the concentration changes of spheroidene and spheroidenone. The result can best be interpreted in terms of three pairs of transformations, a), b) and c) indicated by the horizontal arrows in Fig. 2, although the reactions mentioned above (vertical arrows) evidently took place also during this experiment, but to a lesser extent.

The path which we suggest (29) for carotenoid synthesis in *Rps. gelatinosa*, can therefore be structurally depicted as shown in Fig. 4.

The scheme is based on the results of the kinetic studies cited above and the presence of minor carotenoids in large scale anaerobic cultures, *e.g.*, chloroxanthin (XII), anhydrorhodovibrin (VIII), rhodovibrin (IX) and monodemethylated spirilloxanthin (X) (8). A new carotenoid with physical properties strongly indicative of a 2-keto-rhodovibrin (XVIIb) has recently been isolated in minute amounts (30). Demethylated spheroidene (XIII) and OH-P518 (XX) are hypothetical intermediates, and have not yet been isolated.

Since the qualitative distribution pattern of carotenoids in *Rps. spheroides* (5,9,10,11) and *Rps. capsulata* (5) is rather similar to that in *Rps. gelatinosa* (4,8), it can be tentatively assumed that the carot-

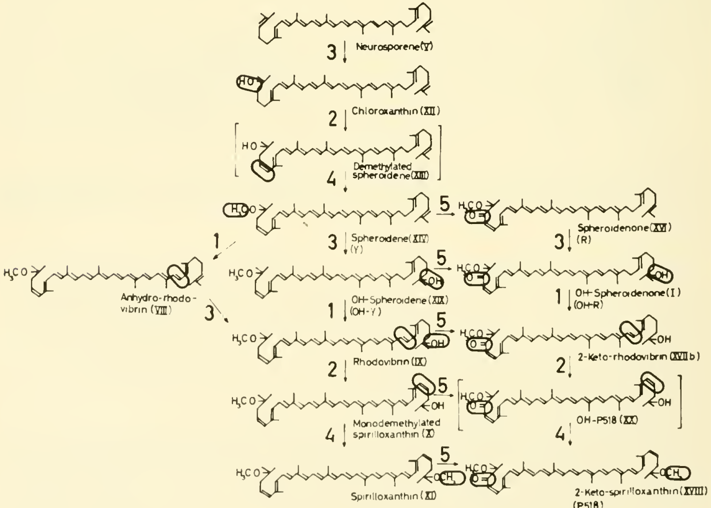

Fig. 4. Structural scheme for the pathway of carotenoid biosynthesis in *Rhodopseudomonas gelatinosa* (29).

enoid synthesis in these organisms proceeds along the same lines. *Rps. capsulata,* however, does not seem to carry the synthesis beyond the stage of OH-spheroidene and OH-spheroidenone. In Fig. 4 the enclosures indicate the reaction points at each step, and the reaction types, given in numbers, are still restricted to the five first categories presented in Table 3.

Turning now to the Thiorhodaceae carotenoids, it is evident that additional reaction types occur. Previous reports available on the carotenoid composition of *Chromatium* spp. (12,13) can probably be interpreted in favour of a distribution pattern similar to that in *R. rubrum* and *Rps. palustris, i. e.,* of the normal spirilloxanthin series. It should be stressed at this point that the presence of lycoxanthin and lycophyll has not been satisfactorily proven in any of the photosynthetic bacteria. The pigments isolated might well have been identical with rhodopin and its di-hydroxy-analogue (7). Studies with labelled carbon reported by Benedict, Fuller and Bergeron (13) seem to support the same reaction sequence for the carotenoid synthesis in *Chromatium* strain D as in *R. rubrum.*

The carotenoids of four new Thiorhodaceae species have recently been examined in collaboration with H. G. Schlegel's group in Gõttingen. *Chromatium vinosum* exhibits presumably the same carotenoid composition as does *Chromatium* strain D, whereas *Thiospirillum jenense* produces lycopene and rhodopin only, according to Schmidt (14). Interesting and new features have been encountered in *Chromatium warmingii,* which synthesizes, in addition to lycopene and rhodopin, three new carotenoids, *i. e.,* two new keto-carotenoids and one which presumably is a hydroxy derivative of rhodopin with a secondary hydroxyl group (16). The huge bacterium *Chromatium okenii* produces a major new keto-carotenoid, named okenone (15). The structure of this compound is not yet established; it represents presumably a new type of keto-carotenoid with a tertiary methoxyl group (15).

The introduction of the conjugated keto group in the keto-carotenoids of *Rps.* spp. is strictly oxygen dependent (25,26,29,31). This raises the question as to the mode of biosynthesis of keto-carotenoids in obligately anaerobic bacteria, like *Chromatium warmingii* and *Chromatium okenii* (32). The distribution pattern and the present, although incomplete, knowledge of the structures of the carotenoids in *Chromatium warmingii* might give a clue to this question. To the left in Fig. 5 is depicted the molecular environment at the reaction point on introduction of the keto-group of spheroidenone (XVI), OH-spheroidenone (I), or 2-keto-spirilloxanthin (XVIII) (referred to as aerobic oxidation). To the right is given the predicted route for the formation of the keto-carotenoid under anaerobic conditions in *Chromatium warmingii* by a hydroxylation (type 6), followed by a subsequent oxidation of the allylic, secondary alcohol group (type 7).

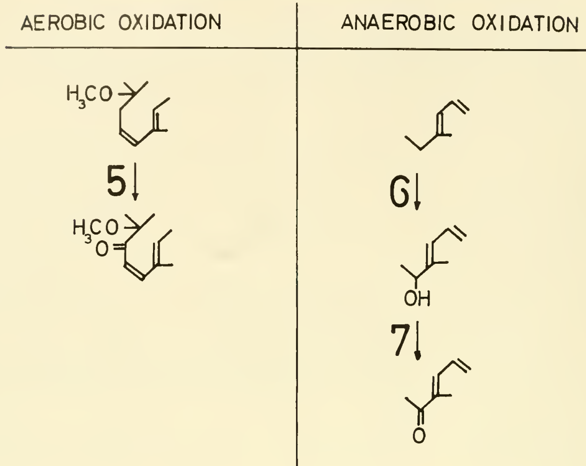

Fig. 5. Hypothetical scheme for the introduction of the conjugated keto-group in carotenoids of photosynthetic bacteria.

The cyclization step is still a major unsolved problem. The bicyclic β-carotene constitutes a minor component of the carotenoid complex of *Rhodomicrobium vannielii* (18,19). The monocyclic γ-carotene has been claimed to be the major carotenoid produced by *Chlorobium limicola* and *Chlorobium thiosulfatophilum;* a hydroxy derivative of γ-carotene present in the two latter bacteria was identified as rubixanthin (3-OH-γ-carotene) by Goodwin and Land (17). A preliminary reinvestigation could not confirm their findings. The major carotenoid of the two *Chlorobium* spp. exhibited an absorption spectrum in visible light analogous to that of γ-carotene, but was more strongly retained on aluminum oxide-containing paper than was synthetic γ-carotene. The hydroxy derivative of γ-carotene from *Chlorobium* spp. can easily be separated from rubixanthin; its hydroxyl group seems to be tertiary. The latter pigment therefore probably represents a 1',2'-dihydro-1'-OH-γ-carotene. We intend to investigate these findings in more detail. However, the occurrence together of cyclic carotenoids and carotenoids with aliphatic end-groups hydroxylated in 1-positions in the two *Chlorobium* spp. and in *Rhodomicrobium vannielii* is interesting. Future studies might reveal whether the latter represent intermediates or alternative side-paths to the cyclic compounds. The cyclization is referred to as reaction type 8 (Fig. 6).

The structural features of the eight reaction types discussed are summarized in Table 3.

In Fig. 7 is presented the postulated pathway of carotenoid biosynthesis in photosynthetic bacteria. The numbers at the arrows refer to the reaction types. The structures of the carotenoids with Roman numerals and those of γ- and β-carotene are known. In the longest vertical column the sequence from the colourless phytoene to spirilloxanthin is depicted. The right-hand branch shows the alternative pathway to spirilloxanthin established in *Rhodopseudomonas gelatinosa*, and to the far right the sequence of the keto-carotenoids in aerated cells of *Rhodopseudomonas gelatinosa* is presented. On the basis of considerations of the distribution pattern in various mutants with deranged carotenoid systems we (23) have earlier assumed that one single enzyme is responsible for the three type 1 dehydrogenations (1a) from phytoene to neurosporene, and that a different enzyme carries out the dehydrogenation 1b. Examination of the quantitative distribution pattern in *Rhodopseudomonas gelatinosa* and *Rhodopseudomonas spheroides* suggests that the 1b reaction is a limiting factor in these organisms.

The keto-carotenoids so far unique for the two *Chromatium* spp. should probably be placed somewhere at the left of the scheme, and the precursors of the cyclic carotenoids (indicated by an enclosure) are not known.

As the number and range of observations is extended, new facts may come to light which will invalidate some of the suggestions made here. I hope, however, that this short review has given the reader a certain insight into the current interpretations of the data available, the tentative speculations still lacking experimental verification, and

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CYCLIZATION STEP

Fig. 6. Possible interpretations of the cyclization step involved in carotenoid biosynthesis.

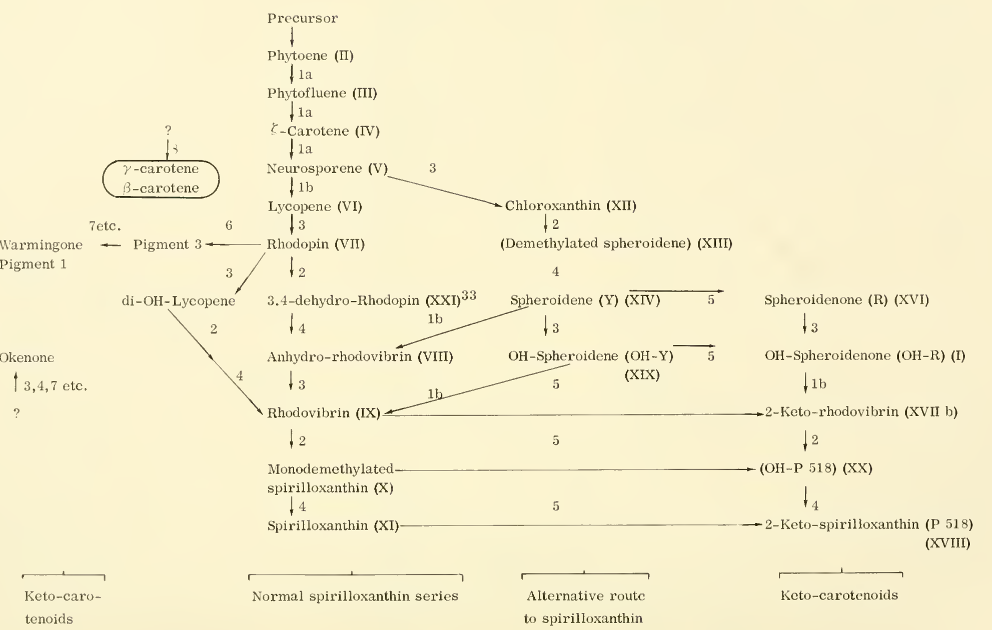

Fig. 7. Postulated pathway of carotenoid biosynthesis in photosynthetic bacteria.

the unsolved problems related to the distribution, chemical structure, and biosynthesis of carotenoids in photosynthetic bacteria.

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TETRAPYRROLES IN PHOTOSYNTHETIC BACTERIA

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Tetrapyrroles in the form of chlorophylls and cytochromes have a vital function in the transformation of radiant energy into a form available for the metabolism of photosynthetic forms of life. It is therefore important to know how photosynthetic organisms synthesize and control the formation of these substances. The significance of this information is not confined solely to photosynthetic organisms. The unique capacity of photosynthetic bacteria and algae to make tetrapyrroles has led to their profitable exploitation by biochemists interested in wider aspects of tetrapyrrole metabolism and now it is not unusual to find cultures of photosynthetic bacteria in laboratories previously devoted to ducks and rabbits.

In this paper the distribution, biosynthesis, and control of formation of tetrapyrroles is discussed with particular emphasis on those areas where information is still sadly lacking.

TYPES OF TETRAPYRROLES FORMED BY PHOTOSYNTHETIC BACTERIA

Chlorophylls.

These comprise by far the major proportion of tetrapyrrole derivatives in photosynthetic bacteria. It seems that bacteriochlorophyll (Fig. 1) is the only form of chlorophyll in the Thiorhodaceae and Athiorhodaceae (see "Note added in proof," p. 52). The structure proposed by Fischer (1) has recently been confirmed (2).

In the green sulfur bacteria two different types of chlorophyll have been identified, designated *Chlorobium* chlorophyll 650 and 660 according to the red absorption maxima of the extracted pigments in ether (3). The structure of these is being currently investigated; the 650 pigment is a derivative of 2-desvinyl-2-α-hydroxyethylpyropheophorbide *a* and the 660 compound is a derivative of δ-methyl-2-desvinyl-2-α-hydroxyethylpyropheophorbide *a*. Both pigments lack the type of cyclopentanone ring typical of other chlorophylls and have no carbomethoxyl groups (4,5). Also, the alcohol side chain differs from phytol ($C_{20}H_{39}OH$) and appears to be trans-trans-farnesol ($C_{15}H_{25}OH$) (6).

Fig. 1. Bacteriochlorophyll (top) and Chlorophyll *a* (bottom).

The amount of bacteriochlorophyll in the Athio- and Thiorhodaceae is of a similar order to that found in algae (Table 1). In the Athiorhodaceae, the conditions under which the organisms are grown can cause quite marked changes in the concentration of bacteriochlorophyll. Apart from factors such as light intensity and oxygen tension (to be discussed later) the carbon source also influences bacteriochlorophyll synthesis (7,8). Iron deficiency causes a marked decrease in bacteriochlorophyll content and this metal seems to be involved in the biosynthesis of all forms of chlorophyll. Formation of *Chlorobium* chlorophyll is diminished by lack of iron (9) and there are numerous examples of iron-deficiency chlorosis in higher plants (10).

TABLE 1

Bacteriochlorophyll content of some photosynthetic bacteria

Organism	Carbon source	Bacteriochlorophyll (mμmoles/mg dry wt)
Athiorhodaceae		
R. rubrum NCIB no. 8255	Malate	13
Rps. spheroides NCIB no. 8253	Malate	12
Rps. palustris 2.1.7	Malate	11
Rps. capsulata 2.3.11	Succinate	22
Thiorhodaceae		
Thiopedia sp.	CO_2	16
Chromatium D	CO_2	24
	Succinate	33

Data from Kornberg & Lascelles (8) and from unpublished personal observations. All cultures were grown anaerobically under a light intensity of about 250 ft-c and estimations were made when the culture density had attained a density of 0.6-1.0 mg dry wt/ml.

Cytochromes and catalase.

The contribution to the total tetrapyrroles made by the prosthetic groups of cytochromes and catalase is slight (about 1%) compared with that made by the chlorophylls (Table 2). Even the high catalase mutant of *Rps. spheroides* (11), of which catalase comprises 5-25% of the dry weight, contains only about 0.5-2.4 mμmole/mg dry weight of tetrapyrrole (calculated as catalase heme). Athiorhodaceae grown in

TABLE 2

Iron porphyrins in photosynthetic bacteria

Organism	Iron porphyrin contributed by			
	Cytochrome b	Cytochrome c	RHP	Catalase
R. rubrum				
Light	0.069	0.11 - 0.18	0.04 - 0.21	<0.001
Dark	0.053	0.04 - 0.12	0.003 - 0.14	<0.001
Rps. spheroides				
Light	-	0.11	0.41	<0.001
Dark	-	0.09	0.06	0.025 - 0.042
Chromatium D				
Light	-	0.03	0.06	-

Values are calculated from the data of Clayton (12), Geller (13) and Bartsch & Kamen (14), and are expressed as mμmoles of iron porphyrin/mg dry wt of cells.

the dark instead of photosynthetically synthesize little or no bacteriochlorophyll and under these circumstances the hemoproteins become the major tetrapyrroles of the cells. Though there is a notable rise in the catalase, particularly in *Rps. spheroides,* the total tetrapyrrole does not approach the level found in light-grown cells (Table 2). It is clear, therefore, that the main business of the tetrapyrrole-forming machinery in photosynthetic organisms is directed towards making chlorophylls.

Porphyrins and derivatives.

In his classical monograph on the Athiorhodaceae, van Niel (15) observed that some species of *Rhodopseudomonas* excreted a pink pigment with absorption bands at 610, 565, and 535 mμ. This was later identified as a mixture of free porphyrins, coproporphyrin III (Fig. 2) being the major component, and is commonly found in the medium of stationary phase cultures (16). The amount of porphyrin varies with the species and strain and is most marked under conditions of iron deficiency. There is an inverse relation between the amount of porphyrin accumulated and the concentration of bacteriochlorophyll in the cells, the latter being favored by addition of iron (16).

When oxygen is rigidly excluded from cultures of Athiorhodaceae, colorless porphyrinogens (Fig. 2) accumulate which are converted to porphyrins by autooxidation in the presence of air (17).

Coproporphyrin III

Coproporphyrinogen III

Protoporphyrin IX

Fig. 2. Coproporphyrin III (top), Coproporphyrinogen III (middle), and Protoporphyrin IX (bottom).

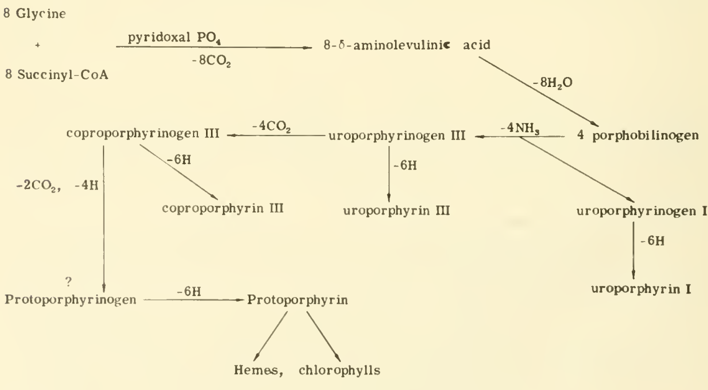
Smaller amounts of magnesium protoporphyrin monomethyl ester have been observed in *Rps. capsulata* and *Rps. spheroides* (18,17, and Lascelles, unpublished observations). Excretion of chlorophyll derivatives by mutant strains of Athiorhodaceae is discussed later.

The accumulation of porphyrins and derivatives by the Athiorhodaceae is connected with the formation of bacteriochlorophyll. It has not been observed in cultures growing aerobically in the dark, when little or no photosynthetic pigments are formed. Also the inverse relation between porphyrin and bacteriochlorophyll, influenced by the iron concentration of the medium, points to an association in the synthesis of these pigments.

BIOSYNTHESIS OF PORPHYRINS AND CHLOROPHYLLS

Pathway to protoporphyrin.

The discovery by Granick (19) that mutants of *Chlorella* blocked in chlorophyll synthesis accumulated free porphyrins gave the first indication that the formation of chlorophylls and hemes proceeded via a common pathway up to the stage of protoporphyrin. The basic outline of the pathway to protoporphyrin is now known (Scheme 1). This has been achieved initially by studies with preparations from avian erythrocytes (reviewed by Granick & Mauzerall (20)) and it is only comparatively recently that bacteria, in particular *Rps. spheroides*, have been exploited (reviewed by Lascelles (16,21)). All the evidence points to a common pathway in animals and photosynthetic bacteria and the exceptional ability of *Rps. spheroides* to form tetrapyrroles has made it a fruitful source for enzymic studies.



Scheme 1. Path of tetrapyrrole synthesis.

There are still major gaps in the pathway to be filled in, particularly in the area between porphobilinogen and protoporphyrin. Uroporphyrinogen and coproporphyrinogen seem firmly established as intermediates and the accumulation of the corresponding oxidized porphyrins in cultures is probably due to spontaneous oxidation of the porphyrinogens. Conversion of porphobilinogen to the uroporphyrinogen III occurs in at least two steps catalysed by different enzyme fractions, one being a porphobilinogen deaminase and the other having an isomerase function (22). An enzyme fraction has been prepared from *Rps. spheroides* which decarboxylates uroporphyrinogen to coproporphyrinogen (23). This conversion is likely to proceed in several stages and the detection by chromatography of traces of porphyrins with five to seven carboxyl groups in enzymic reaction mixtures, as well as in the porphyrin mixtures excreted by whole cells, support this. The conversion of coproporphyrinogen to protoporphyrin has been studied with an enzyme fraction purified twentyfold from beef liver mitochondria (24). The reaction requires oxygen but is not inhibited by cyanide. The mechanism is largely unknown but a tricarboxylic porphyrinogen and protoporphyrinogen are probable intermediates. There is also evidence that an intermediate is, or can become, covalently bound to protein (25).

Under conditions of iron deficiency coproporphyrin III is always the major porphyrin accumulated by photosynthetic bacteria; protoporphyrin and chlorophyll derivatives are not found. With adequate iron, the porphyrin output is considerably less (1 to 10% of that with low iron) but under these conditions protoporphyrin (Fig. 2), magnesium protoporphyrin monomethyl ester, and chlorophyll derivatives predominate. This suggests that iron participates at a stage in the conversion of coproporphyrinogen to protoporphyrin. Such a function for iron is supported by whole cell experiments with *Rps. spheroides* (26,27). Conversion of δ-aminolaevulate (ALA) to coproporphyrin III occurs when iron-deficient cells are incubated anaerobically in the light in the presence of phosphate and Mg^{2+} only; no protoporphyrin is formed. Addition of iron to such suspensions promotes synthesis of protoporphyrin and free heme. Additional evidence for the involvement of iron in protoporphyrin synthesis is provided by the inhibition by *o*-phenanthroline of the mitochondrial enzyme system that converts coproporphyrinogen to protoporphyrin (24). To establish the function of iron in the conversion of coproporphyrinogen to protoporphyrin, further enzymic studies are clearly needed, but so far there has not been much success in preparing extracts of *Rps. spheroides* active in this respect.

Another interesting aspect of the conversion of coproporphyrinogen to protoporphyrin in the photosynthetic bacteria concerns the nature of the oxidant needed for the oxidative decarboxylation. The enzyme systems from animal tissues show an obligatory requirement for oxygen and alternative electron acceptors have not been demonstrated.

Clearly oxygen cannot participate in the reaction in photosynthetic bacteria growing anaerobically and presumably the acceptor is generated by the photosynthetic apparatus. Since *Rps. spheroides* is so active in forming protoporphyrin under appropriate conditions it is a promising candidate for enzyme studies; analysis of this system might clarify the general mechanism by which these bacteria perform other reactions which are obligatorily linked to oxygen in aerobic organisms.

Formation of iron and magnesium protoporphyrins.

Soluble enzyme systems have been purified from animal tissues which catalyse heme synthesis from protoporphyrin and ferrous ions (28,29), and it is probable that similar enzymes are present in bacteria. Burnham (30) has obtained heme synthesis from protoporphyrin and iron citrate with crude extracts of *Rps. spheroides* incubated anaerobically in the light with succinate. Ferrichrome or related iron-binding factors may participate coenzymically in this reaction. In the *Rps. spheroides* systems, ferrichrome replaces iron citrate and experiments with Fe^{59}-labelled ferrichrome have shown that the iron is transferred to protoporphyrin to form labelled heme. Purification of the enzyme is required to establish whether ferrichrome is an obligatory cofactor.

The participation of magnesium protoporphyrin as an intermediate in chlorophyll synthesis was indicated many years ago by the isolation of chlorophyll-less mutants of *Chlorella* which accumulated this metal complex (31). There is no information about the enzymic mechanism of magnesium protoporphyrin synthesis.

Synthesis of chlorophylls.

The pathway from protoporphyrin to chlorophyll and bacteriochlorophyll has received little analysis at an enzymic level and present knowledge is derived mainly from the compounds accumulating in cultures of mutant strains of *Chlorella* and *Rps. spheroides* (Table 3; Scheme 2). An early step is the formation of magnesium protoporphyrin monomethyl ester. The specific incorporation of C^{14}-formate by *Chlorella* into the methyl ester group of chlorophyll suggested that this group is derived from a one-carbon unit (32). In *Rps. spheroides* 1-C^{14}-methionine labels the methyl group of bacteriochlorophyll specifically; inhibition of methyl transfer from methionine by ethionine could account for the inhibition of bacteriochlorophyll synthesis by this analogue (33). Chromatophore preparations from this organism form magnesium protoporphyrin monomethyl ester when incubated with S-adenosylmethionine and magnesium protoporphyrin (39). Neither protoporphyrin nor the corresponding porphyrinogen is methylated in this system, showing that the biosynthetic sequence is:

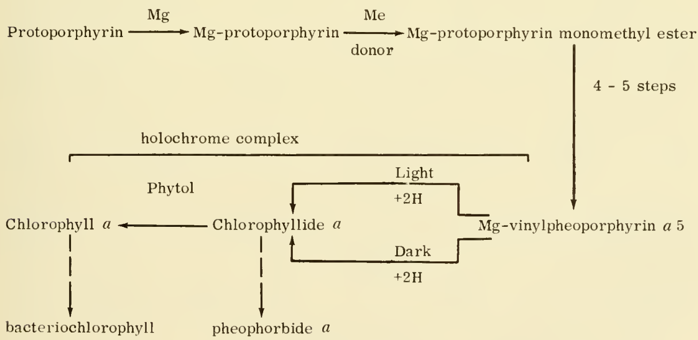
Protoporphyrin ⟶ magnesium protoporphyrin ⟶ magnesium protoporphyrin monomethyl ester

TABLE 3

Chlorophyll derivatives accumulated by photosynthetic microorganisms

Compound	Accumulated by	Reference
Mg-protoporphyrin	Chlorella mutant	(34)
Mg-protoporphyrin monomethyl ester	Chlorella mutant *Rps. capsulata* *Rps. spheroides*	(34) (17) (18)
Mg-vinylpheoporphyrin *a* 5 (protochlorophyllide *a*)	Chlorella mutant	(35)
Protochlorophyll-type pigments	*Rps. spheroides* chlorophyll-less mutants	(36) (37)
Pheophorbide *a*	*Rps. spheroides* carotenoid-less mutants*	(38)

* This mutant excretes a variety of chlorophyll derivatives of which pheophorbide *a* and a closely related compound are the major components; a minor component has the tetrahydropyrrole ring system of bacteriochlorophyll and is probably bacteriopheophorbide.



Scheme 2. Possible pathways from protoporphyrin to chlorophylls.

Four to five intermediate stages between magnesium protoporphyrin monomethyl ester and magnesium vinyl pheoporphyrin a_5 (MgVP; protochlorophyllide a; see Fig. 3) have been postulated by Granick but these are completely unknown. MgVP is accumulated by a *Chlorella* mutant, which, unlike the wild type, does not form chlorophyll unless illuminated. It also accumulates in etiolated leaves treated with ALA in the dark (34). This pigment can exist in three forms (40). Type 1 has an absorption maximum at 631 mμ and is bleached by light; this is the free pigment. Type 2 has an absorption maximum at 650 mμ and is converted in the light to chlorophyllide *a*; this is attached to the holochrome complex studied by Smith (41). Type 3 also has an absorption maximum at 650 mμ but is not transformed by light; this may be attached to a holochrome which lacks a reducing component.

Free MgVP when accumulated by etiolated leaves in the dark from ALA is not utilised for chlorophyll synthesis upon subsequent illumination. For such a transformation to occur the pigment must presumably be attached to the holochrome complex, at least in the higher plants. It appears that free MgVP formed from added ALA cannot enter this complex; it is possible that attachment of the tetrapyrrole component to the holochrome may occur at a stage before MgVP.

The protochlorophyll-like pigment isolated from a strain of *Rps. spheroides*, unable to form bacteriochlorophyll, resembles type 1 MgVP; it has an absorption maximum at 623 mμ and it probably lacks the phytol group (36). A similar, possibly identical pigment has been identified spectroscopically in mutants of the same organism (37).

Perhaps the greatest mystery in chlorophyll synthesis is the nature of the light reaction which results in reduction of ring D of MgVP. The fully functional holochrome complex presumably contains a light-activated reducing system. Most of the simple algae form chlorophyll in the dark and presumably contain an additional enzyme system which catalyses the reduction without the intervention of light. Bacteria of the Athiorhodaceae family must have a similar type of system since they can form bacteriochlorophyll in the dark provided the oxygen pressure is low (42,43). Protein-bound intermediates may participate in the final stages of bacteriochlorophyll synthesis. The obligatory association of pigment formation and protein synthesis or turnover supports this (43,44,45). It seems likely that the enzymes for these later stages are in the chromatophores; this is suggested by the observation of Tait & Gibson (39) that the magnesium protoporphyrin methylating system is confined to the chromatophore fraction.

The accumulation of pheophorbide *a* (Fig. 3) by the blue-green carotenoidless mutant of *Rps. spheroides* provides additional evidence that bacteriochlorophyll and chlorophyll synthesis proceeds by a common path, but the significance of the appearance of this magnesium-free pigment is not clear. A compound similar if not identical with it

Mg vinylpheoporphyrin *a*5

Pheophorbide *a*

Fig. 3. Magnesium vinylpheoporphyrin a_5 (top) and Pheophorbide *a* (bottom).

is accumulated by suspensions of *Rps. spheroides* incubated with 8-azaguanine in the dark under low aeration (unpublished observations). Griffiths (37) has recently isolated a series of mutants of *Rps. spheroides* which do not form bacteriochlorophyll but which accumulate a magnesium-free pigment with a spectrum similar though not identical with pheophorbide *a*. Its absorption maxima correspond with those of *Chlorobium* 650 pheophytin. The slight differences in spectral characteristics from pheophorbide *a* could denote differences in the side chains of the dihydrotetrapyrrole nucleus though the data do not show whether the compound is phytolated.

Esterification with phytol.

This may be the final stage in the synthesis of chlorophylls. In higher plants short exposure to light results in formation of the unesterified chlorophyllide *a*, which is converted to the esterified chlorophyll *a* by subsequent incubation in the dark (46). Accumulation of the unphytolated-protochlorophyll pigment and of pheophorbide *a* by mutants of *Rps. spheroides* suggests that esterification also occurs at a late stage in bacteriochlorophyll synthesis.

It seems likely that the esterification with phytol occurs within the lipoprotein complex of the chromatophore because of the hydrophobic nature of the phytol residue.

CONTROL OF TETRAPYRROLE SYNTHESIS

It is obviously to the advantage of organisms which form chlorophylls to have mechanisms for regulating synthesis of the pigments to fit environmental demands. Not only does the formation of chlorophylls make a considerable drain on glycine and succinyl CoA but organisms making these pigments must also elaborate a formidable array of enzymes for the steps in the biosynthetic pathway. That control mechanisms do exist is shown most clearly in the Athiorhodaceae, which grow either aerobically in the dark or anaerobically in the light and which form bacteriochlorophyll and carotenoids only under photosynthetic conditions. There is now evidence, though far from complete, that synthesis of the pigments is subject to control by negative feedback mechanisms and by enzyme repression.

Iron and hemin.

The excretion of free porphyrins by photosynthetic bacteria under conditions of iron deficiency shows a close resemblance to other biosynthetic systems where breakdown of a negative feedback control occurs due to inability to form a metabolite which inhibits an early enzymic reaction in the biosynthetic pathway (47). Thus, the accumu-

lation occurs only in the later stages of growth and the quantities of porphyrin formed far exceed the amount of tetrapyrrole, as hemes and bacteriochlorophyll, which are formed with adequate iron (26). Iron acts catalytically in preventing porphyrin formation and this could suggest that it is needed to form a compound which controls an early step in the synthesis of porphyrins by negative feedback inhibition. Work with whole cells and partly purified preparations of ALA synthetase of *Rps. spheroides* suggest that heme may exert such a controlling function (48,49). The enzyme is inhibited by low concentrations of hemin (down to 10^{-8} *M*); porphyrin accumulation by intact cells is inhibited by hemin when glycine and succinate are the substrates but conversion of ALA to porphyrin is unaffected. Under normal conditions hemes within the cell are mostly if not entirely present as hemoproteins. Hemoproteins (hemoglobin and myoglobin) also inhibit ALA synthetase and it is in this form that hemes may function in the control mechanism.

Besides inhibiting the action of ALA synthetase hemin also represses synthesis of this enzyme by growing cultures of *Rps. spheroides* (50). The conclusion from these various observations is that the intracellular level of hemes, probably as hemoproteins, participate in the control of tetrapyrrole synthesis by influencing both the synthesis and the action of the synthetase. The effect of hemin on the synthetase does not, however, satisfactorily account for all the effects of iron observed with *Rps. spheroides,* in particular the action of iron in promoting bacteriochlorophyll synthesis.

Bacteriochlorophyll might also act as a controlling factor. Free bacteriochlorophyll does not inhibit ALA synthetase but this may not be significant since in the cell it exists in combination with the chromatophore complex. Gibson *et al.* (33) have suggested that an intermediate between magnesium protoporphyrin methyl ester and bacteriochlorophyll may act as a feedback inhibitor of ALA synthetase and their observations on the effect of ethionine on *Rps. spheroides* support this. This analogue inhibits bacteriochlorophyll synthesis, probably by interfering with the methylation step, but stimulates the accumulation of coproporphyrin.

Since the path of heme and chlorophyll synthesis is common up to the protoporphyrin stage and since photosynthetic organisms must form both types of tetrapyrrole for photosynthetic development, control mechanisms might be expected to operate at the branch joint leading to iron and magnesium protoporphyrins. Information about this must await knowledge of the enzyme systems catalysing the insertion of the metals into the tetrapyrrole structure.

Light intensity and oxygen.

On teleological grounds it is an advantage for an organism, which relies on light for energy, to be able to increase or decrease its chloro-

phyll content in response respectively to decreased or increased light intensity. This is analogous to the response of animals which form more hemoglobin under diminished oxygen tensions.

Such adjustments in chlorophyll content occur in the plant kingdom as shown by the higher levels in shade leaves compared with sun leaves, while unicellular algae grown in dim light are richer in chlorophyll than those grown in bright light (10).

The elegant experiments of Cohen-Bazire *et al.* (42) have shown that synthesis of photosynthetic pigments by cultures of Athiorhodaceae is regulated by light intensity. In *Rps. spheroides* the rate of synthesis of bacteriochlorophyll is inversely proportional to the light intensity and the pigment content of cells grown in dim light (50 ft-c) is about eight times higher than in those grown in bright light (5000 ft-c). On transfer from dim to bright light or *vice versa* cultures rapidly adjust their pigment level by preferential synthesis or by transient repression of pigment formation.

In the Athiorhodaceae oxygen exerts a spectacular control over pigment synthesis as shown by the almost complete absence of bacteriochlorophyll and carotenoids in organisms grown aerobically in the dark. Introduction of oxygen into cultures growing in the light results in an immediate arrest of pigment synthesis and this is reversed by restoration of anaerobic conditions (42). These experiments suggest that absence of pigment in dark-aerobic cultures might be due to repression of their synthesis by oxygen rather than to an obligatory requirement for light. This was confirmed by showing that Athiorhodaceae can indeed form bacteriochlorophyll and carotenoids in the dark provided that the oxygen tension is reduced (43). With suspensions of *Rps. spheroides* forming bacteriochlorophyll in the dark the oxygen tension which permits synthesis is critical and must presumably be sufficient for general metabolism (e.g. to supply ATP by oxidative phosphorylation) yet insufficient to cause repression of pigment formation.

In an attempt to understand the mechanism by which oxygen represses bacteriochlorophyll synthesis, attention has been given to the key intermediate, succinyl CoA. In organisms such as *Rps. spheroides* with a tricarboxylic acid cycle, ALA synthetase has to compete for succinyl CoA with enzymes which would pull it through the cycle; utilisation via the cycle might be favored by high oxygen tensions since there is evidence that oxidation of succinate becomes rate-limiting under anaerobic conditions (51). Increasing the level of the synthetase could favor diversion of the succinyl CoA towards tetrapyrrole synthesis. There is in fact a strong correlation between level of the synthetase and ability to form bacteriochlorophyll; the enzyme is five to ten times higher in *Rps. spheroides* grown anaerobically in the light than when grown aerobically (43). In addition, synthesis of the enzyme is repressed by high oxygen tensions, though, like bacteriochlorophyll, it is formed at a maximum rate under low oxygen

tension in the dark (43,50). ALA dehydratase is also repressed by oxygen, suggesting that co-ordinate repression by oxygen occurs in the tetrapyrrole pathway.

Regulation of bacteriochlorophyll synthesis by oxygen and by light intensity may operate by a similar mechanism (42). In support of this the levels of ALA synthetase and dehydratase in *Rps. spheroides* are affected by the light intensity just as they are influenced by the oxygen pressure; their rates of formation in growing cultures are inversely proportional to the light intensity (50).

These observations suggest that one of the ways in which oxygen and light may influence the formation of bacteriochlorophyll is by repressing synthesis of enzymes concerned in early stages of the biosynthetic pathway. They tell us nothing of the mechanism by which the repressing effect is exerted. Nor do they fully account for all the observed effects of oxygen. Control by enzyme repression only would result, upon the introduction of oxygen, in a *gradual* fall in the differential rate of bacteriochlorophyll synthesis by cultures growing in the light; this would occur as the enzymes already present became diluted out. In fact, oxygen produces an immediate and complete stoppage of pigment synthesis (42,50). This suggests that oxygen is inhibiting the action of one or more enzymes on the biosynthetic path. Since porphyrins do not accumulate in oxygen-repressed cultures it seems that an early stage is either directly or indirectly inhibited by oxygen.

In addition to the effects of oxygen and light on tetrapyrrole formation, consideration must be given to their action on the carotenoids. These respond to the environment in the same way as bacteriochlorophyll, yet the biosynthetic pathways have nothing in common, except perhaps for the phytol residual of the bacteriochlorophyll.

A full understanding of the response of the pigment system to the environment can only come when we know more about the steps in the formation of the chlorophylls and carotenoids and about the stage where their synthesis becomes interwoven with the lipoproteins of the chromatophore and chloroplast structures.

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Note added in proof (page 35). It has been learned that another pigment designated as bacteriochlorophyll *b*, has been identified spectroscopically in a *Rhodopseudomonas* sp. Its structure is unknown (Eimhjellen, Aasmundrud, and Jensen, *Biochem. Biophys. Res. Commun., 10,* 232, 1963).

A NOTE ON THE EFFECT OF INHIBITORS OF ELECTRON TRANSPORT AND PHOSPHORYLATION ON PHOTOPIGMENT SYNTHESIS IN *RHODOPSEUDOMONAS SPHEROIDES*

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INTRODUCTION

The general features of the control of photopigment synthesis in the photosynthetic bacteria are well known (1,2). The hypothesis originally proposed (1) to account for the changes in the bacteriochlorophyll and carotenoid pigment contents of cells grown at different light intensities or oxygen tensions is adequate to a first approximation at least. It does not, however, account satisfactorily for all the kinetic observations (2). The hypothesis assumed that pigment synthesis is controlled by the ratio of the oxidized to the reduced form of an electron carrier. When the carrier becomes oxidized, for example by a sudden increase in light intensity, pigment synthesis is inhibited; conversely, when the carrier is reduced, pigment synthesis is accelerated.

More recent experiments have shown that at constant light intensity the bacteriochlorophyll content of a culture depends directly upon its growth rate. For example, when *Rhodopseudomonas spheroides* is maintained in a chemostat the pigment content of the cells depends upon the dilution rate. If it is assumed that the rate of reduction of the electron carrier which controls pigment synthesis is proportional to the growth rate, then our original hypothesis can account for these results also.

It seemed possible that the site of control could be determined by studying the effect of inhibitors of electron transport and phosphorylation on bacteriochlorophyll synthesis. This paper describes in a preliminary way some experiments along these lines. The results indicate that this will be a fruitful approach and suggest that pigment synthesis is controlled by the ratio of reduced to oxidized DPN.

RESULTS AND CONCLUSIONS

All experiments were conducted with *Rps. spheroides*, strain Ga. Medium B of Sistrom (3) was used. The cultures were aerated with 5% CO_2 in N_2. A light intensity of 600 fcs was used throughout. Two layers of red cellophane were used as a filter to avoid photoreactions with pigmented inhibitors. Bacteriochlorophyll and neurosporene were

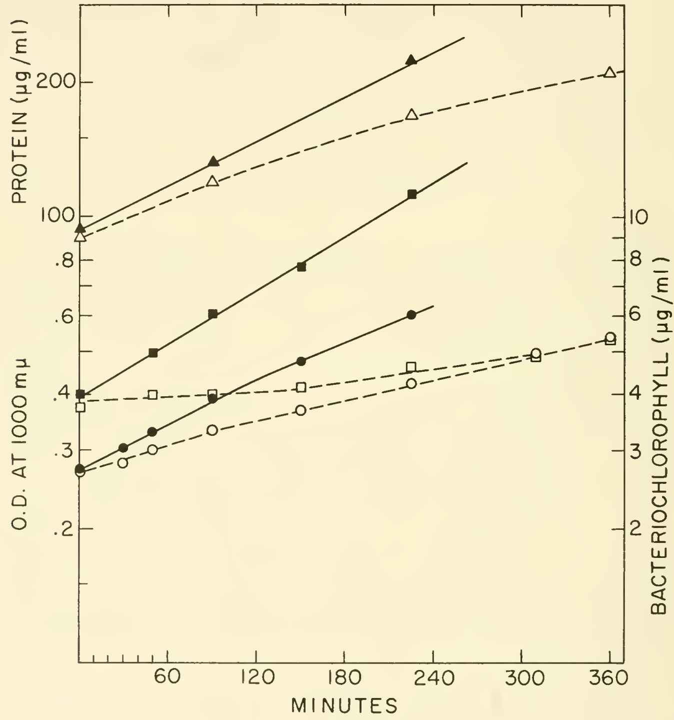

Fig. 1. Effect of dicoumarol on growth and bacteriochlorophyll synthesis. A culture was grown overnight at a light intensity of 800 fcs; one hour before the start of the experiment the culture was diluted and 90 ml portions placed in rectangular lucite growth vessels and illuminated with 600 fcs. At zero time dicoumarol (6×10^{-5} *M*) was added to one vessel (open points); the other served as a control (filled points). Bacteriochlorophyll (µg/ml), □ and ■; cell mass (OD at 1000 mµ), ○ and ●; protein (µg/ml), △ and ▲.

estimated as described previously (1); protein was determined by the Folin-Lowry method. Except in experiments with dinitrophenol, increase in protein paralled increase in turbidity at 1000 mμ, and in some experiments protein was not determined.

Experiment 1. The effect of dicoumarol on growth and bacteriochlorophyll synthesis is shown in Fig. 1. The growth rate is reduced to about 50% of the control. Bacteriochlorophyll synthesis is immediately inhibited but eventually recommences.

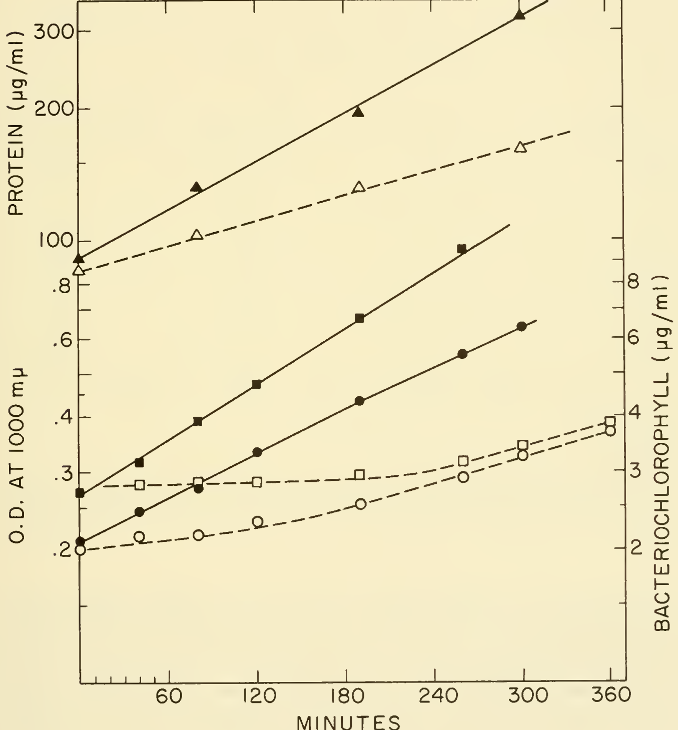

Fig. 2. Effect of dinitrophenol (DNP) on growth and bacteriochlorophyll synthesis. The experiment was conducted as described in the legend of Fig. 1, except that at zero time one culture received DNP (4×10^{-5} *M*). Symbols as in Fig. 1.

Experiment 2. The effect of dinitrophenol (DNP) (6×10^{-5} *M*) is similar to that of dicoumarol (Fig. 2). When lower concentrations of either dicoumarol or DNP were used bacteriochlorophyll synthesis was inhibited but to a lesser degree (Table 1).

Experiment 3. Amytal at a concentration of 1.6×10^{-3} *M* inhibits bacteriochlorophyll synthesis to a lesser extent than growth (Fig. 3); consequently, the specific bacteriochlorophyll content increases. This is in marked contrast to the results with DNP and dicoumarol.

TABLE 1

Comparison of the effects of certain inhibitors on growth and bacteriochlorophyll synthesis.

Inhibitor	Molarity	Relative growth rate	Relative bacterio-chlorophyll content
none	—	100	100
DNP	3.0×10^{-5}	62	79
"	4.0×10^{-5}	56	71
"	6.0×10^{-5}	60	60
dicoumarol	2.5×10^{-5}	72	80
"	5.0×10^{-5}	64	71
amytal	1.0×10^{-3}	71	111
"	1.6×10^{-3}	65	126
atebrin	4.0×10^{-5}	55	84
DL-5-methyl-tryptophan	2.5×10^{-4}	64	64

In order to avoid the effects of self-shading, the bacteriochlorophyll content was determined in each case when the bacteriochlorophyll concentration was 4 μg/ml.

Experiment 4. Cohen-Bazire and Kunisawa (4) have reported that the specific bacteriochlorophyll content is reduced in cultures grown at a low temperature. Fig. 4a shows the results of an experiment in which a culture was subjected to a sudden decrease in temperature. Bacteriochlorophyll formation is inhibited, but not completely as it is after addition of DNP or dicoumarol. Essentially the same result is obtained when growth is inhibited by 5-methyltryptophan (Fig. 4b).

Fig. 5 shows the differential rate of bacteriochlorophyll synthesis in the presence of amytal and of 5-methyl-tryptophan. In Table 1 are shown the relative growth rates and bacteriochlorophyll contents obtained with inhibitors.

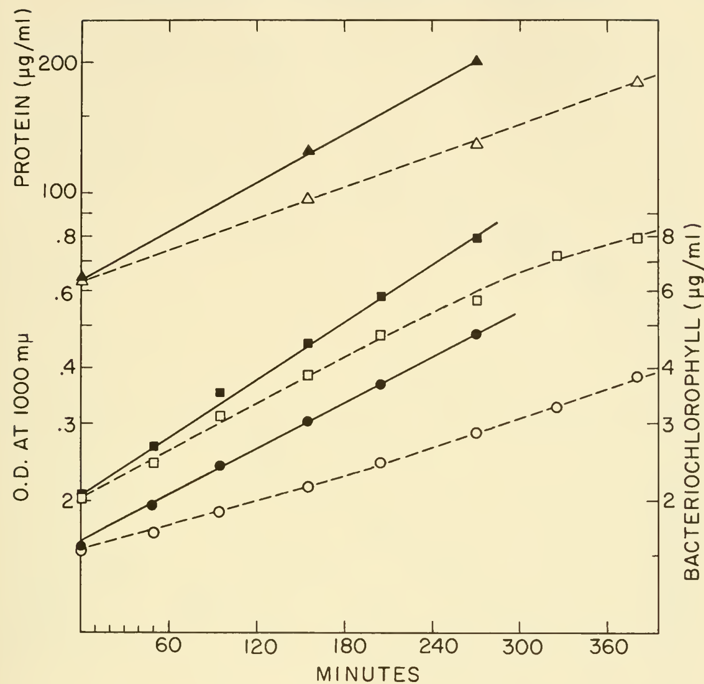

Fig. 3. Effect of amytal on growth and bacteriochlorophyll synthesis. The experiment was conducted as described in the legend of Fig. 1, except that at zero time one culture (open points) received amytal (1.6×10^{-3} *M*). Symbols as for Fig. 1.

These results can be summarized by saying that amytal mimics the effect of a sudden *decrease* in light intensity, while DNP and dicoumarol mimic the effect of a sudden *increase* in light intensity. Bacteriochlorophyll synthesis is completely, although temporarily, inhibited by the latter compounds. When the growth rate is reduced by lowering the temperature or by addition of 5-methyl-tryptophan there is only a partial inhibition of bacteriochlorophyll formation.

Dicoumarol and DNP presumably uncouple phosphorylation and electron transport; consequently, the electron transport chain becomes more oxidized and bacteriochlorophyll synthesis is stopped. However, the specific pigment content will decrease because of continued growth

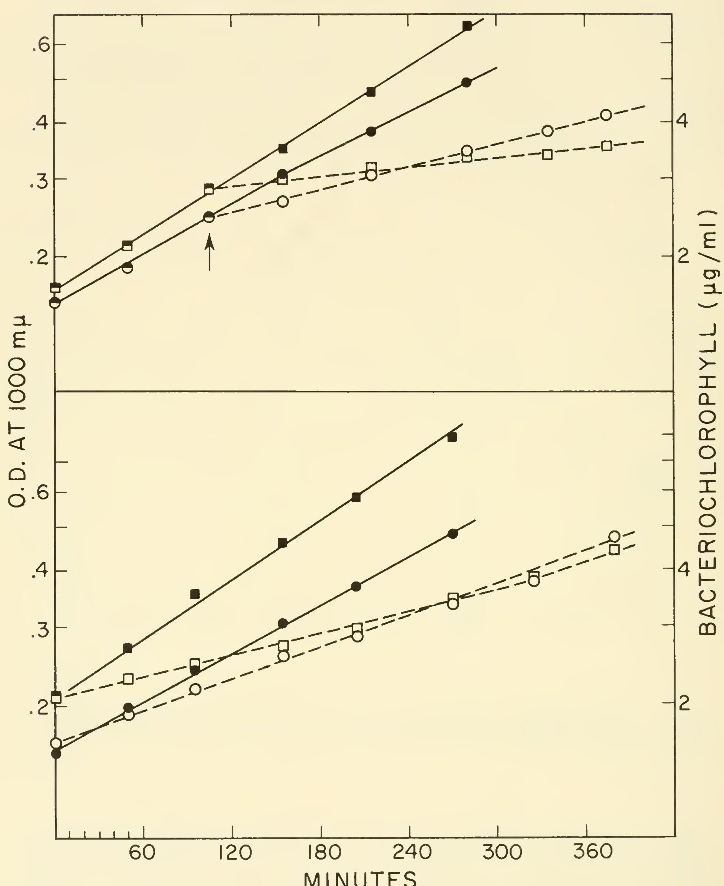

Fig. 4. Effect of temperature shift and of 5-methyl-tryptophan on growth and bacteriochlorophyll synthesis. (a) Two cultures were grown at 34°, at the time indicated by the arrow one culture (open points) was transferred to 23°, the second culture (filled points) remained at 34°. (b) This experiment was conducted as described in the legend for Fig. 1; at time zero DL-5-methyl-tryptophan (50 μg/ml) was added to one culture (open points). Bacteriochlorophyll (μg/ml), □ and ■; cell mass (OD at 1000 mμ), ○ and ●.

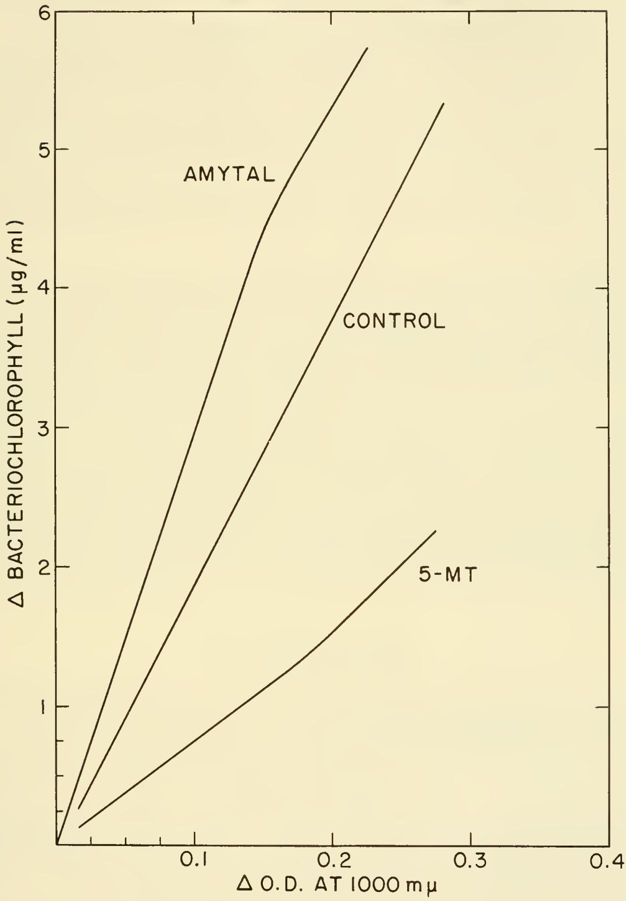

Fig. 5. Differential rate of bacteriochlorophyll synthesis in the presence of amytal and of 5-methyl-tryptophan (5-MT). The data of Figs. 3 and 4b have been replotted to show the increase in bacteriochlorophyll relative to increase in cell mass (OD at 1000 mμ).

of the cells, the supply of photooxidant will accordingly fall and the electron carriers will return to their initial level of reduction and bacteriochlorophyll synthesis will recommence.

Amytal is reported to be a poor inhibitor of cyclic photophosphorylation in *Rhodospirillum rubrum* chromatophores (5); however, amytal is known to block the oxidation of DPNH by flavoprotein in mitochondria (6). It therefore seems reasonable to ascribe both the inhibition of growth and the stimulation of the differential rate of bacteriochlorophyll synthesis by amytal to a decrease in the rate of DPNH oxidation. In other words, the electron carrier which controls pigment synthesis is DPN. Preliminary results indicate that 2-*n*-nonyl-hydroxyquinoline-N-oxide and SN5949 have the same effect on bacteriochlorophyll synthesis as amytal.

Atebrin inhibits photophosphorylation in *R. rubrum* chromatophores (7) and DPNH oxidation in mitochondria (8). In each case it is likely that the inhibition is on a flavoprotein. It would be expected that atebrin should have the same effect as amytal. As can be seen from Table 1, this is not the case: atebrin inhibits growth and reduces the specific bacteriochlorophyll content. However, it should be noted that the pigment content is reduced by only 15%, although growth is inhibited by almost 50%. This is in contrast to the results with DNP, dicoumarol and 5-methyl-tryptophan. It is possible that atebrin inhibits growth not only by interfering with oxidation of DPNH but also in some other fashion unrelated to electron transport. The small reduction in specific bacteriochlorophyll content which is observed may represent the net result of these contrary tendencies.

ACKNOWLEDGMENTS

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THE HEME PROTEINS OF PHOTOSYNTHETIC BACTERIA *

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INTRODUCTION

The area of research I have been asked to discuss was inaugurated by an observation of Dr. L. P. Vernon when he and I were engaged in collaboration (1,2) on energy-storage reactions in chromatophore preparations in 1952. It is a source of particular satisfaction a decade later to be here at this session sponsored by the C. F. Kettering Foundation and its new research director, Dr. Vernon.

The heme proteins of the photosynthetic bacteria have been considered in the past primarily from the standpoint of function. This is so well documented now (3), even if still incompletely understood, that I propose in this paper to adopt another approach—that is, to consider the heme proteins as objects of intrinsic biochemical interest. To paraphrase President Kennedy's famous exhortation—"Let us not ask what heme proteins have done for photosynthesis, but what photosynthesis has done for heme proteins!"

CLASSIFICATION

The index of progress in any science can be indicated by the status of classification schemes. In the case of heme proteins, and natural tetrapyrrole proteins in general, classifications based on function have maintained a certain priority over those based on structure. This follows, obviously, because it is usually easier to make functional assignments than to solve structural problems, especially for important macromolecules of biological importance, such as proteins. Thus, in the tetrapyrrole-conjugated proteins, which include the mag-

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nesium and iron chelates, functions which are readily apparent, such as photosensitization for the chlorophylls (magnesium chelates) and oxidation catalysis for heme proteins (iron chelates), are familiar to all of us.

The structural classification of heme proteins must be made on the basis of imperfectly understood chemical properties and behavior, as deduced from magneto-chemical, spectrochemical, and physical organic studies on the proteins and on model compounds. A simple summary of present classification schemes can be presented, based on four generally accepted criteria:

(1) The basic chelate structure. This is the beta-substituted tetrapyrrole structure, associated with type III porphyrins. Three possible variations occur. The first, and most frequent, is based on the porphyrin skeleton, in which two of the pyrrole moieties possess double bonds which do not participate in the conjugated macrocyclic resonance system. This we can call the "P" class. The second is the dihydroporphyrin, or chlorin, in which one of these double bonds is removed by hydrogenation. This occurs primarily among the magnesium chelates (chlorophylls), but one example is known in the heme proteins—namely, the D-type cytochrome (4) (5), formerly called "cytochrome a_2." This we can call the "D" class. Thirdly, we must entertain the possibility that the tetrahydroporphyrin structure, in which no nonparticipating double bonds are left, may also be found to occur for heme chelates, as it does for the magnesium chelates (bacteriochlorophyll). This gives a third class, which we can term "T."

For the present, we can assume that general structures, other than type III, will not occur. One contribution from the study of bacterial cytochromes is already apparent in that we are prepared to accept the possibilities of classes "D" and "T."

(2) Ligand interactions. The special effects created by the introduction of ligand groups from the protein into the extraplanar positions of the tetrapyrrole ring cannot be defined completely on the basis of any single chemical criterion, but the magnetochemical behavior is as adequate as any. This criterion leads to specification of three subclasses for each of the main classes established by criterion 1. These are "high spin" (H), "low spin" (L), and "mixed spin" (M). The M subclass is actually derivative from the other two because it is probable that no absolutely pure H or L state exists. However, as a practical matter, the totality of heme proteins known at present falls easily into three such classes.

(3) Binding type. The chelate may be bound to the protein by simple acid-base linkages which may be split easily by the usual acid-acetone treatment; or it may be bound by covalent linkages (thioether, ester, carbon-carbon bonds, etc.), which resist such treatment. These two cases can be distinguished by the letters "F" ("free") and "C" ("covalent").

(4) Degree of saturation of side chains. The presence of electron-withdrawing side-chains (unsaturated groups) provides one further subdivision, which may be termed "U," whereas their absence gives a subdivision, termed "S" (saturated).

It follows that thirty-six possible combinations exist, twelve for each of the three main classes; *i.e.*, for the P class there are the following subclasses: HFU, HFS, HCU, HCS, LFU, LFS, LCU, LCS, MFU, MFS, MCU, MCS.

The conventional classes of heme proteins, derived from studies of aerobic tissues (beef, yeast, etc.) are bunched into a relatively few of the above subclasses. Thus, the oxygen storage and transport proteins (myoglobins, hemoglobins, chlorocruorins) fall into the PHFU class. So do the catalases and some peroxidases. Myeloperoxidase probably belongs in the class PHCU, as does lactoperoxidase. The oxidation catalysts, cytochromes *b* and *c*, appear in the classes PLFU and PLCS, respectively. The oxidases, or "*a*" type cytochromes, appear to belong to the class, PMFU. Thus, just five combinations appear to include all the conventional heme proteins.

The bacterial heme proteins supply a few more. Thus, the so-called "a_2" and "a_4" cytochromes, from *E. coli* and *Acetobacter* sp. (4) (5), belong somewhere in the "D" class. In addition, a diheme protein which occurs in *Pseudomonas* sp. (6) appears to fall into both classes "D. . ." and "PLCS".

The photosynthetic bacteria, together with the plant systems, provide representatives of the "*b*" and "*c*" type cytochromes, as well as peroxidases and catalases. In addition, the purple photosynthetic bacteria have a type of oxidase—called "cytochrome *o*" (7) (8)—which appears to be a particle-bound member of the same class as the peroxidases. It is uncertain whether it is similar in spin type, so it may conceivably fall into a "PM" category, like the "*a*" type cytochromes. Finally, there is the authentic new class of heme proteins—provisionally called "cytochromoids" and known previously as "RHP"-type proteins (3e)—which falls into the class PMCS. Thus, we see that the bacteria have added at least three new combinations, on the basis of structure alone. More than this, they have alerted biochemists to the possibility that new combinations may exist, not only in bacteria and in plants, but also in animal tissues.

PROPERTIES AND FUNCTIONS

I wish now to summarize briefly present notions about the structures and functions of heme proteins in photosynthetic bacteria. I shall deal with these in terms of the various general classes known.

(a) General considerations. First, I should bring to your attention certain general considerations which apply to all the work which has been done. In researches with bacteria as source material, amounts of protein available rarely exceed a few milligrams. Even this minute amount is obtained only after rather arduous culture and isolation procedures. For instance, to obtain 10 milligrams of the pure *c*-type cytochrome from *Rhodospirillum rubrum*—a microorganism very rich in this heme protein—10 grams dry weight of bacteria are required. Most work on protein fine structure—such as sequence determination, x-ray analyses—demands the sacrifice of literally kilograms of material. Another uncertainty, less often encountered in conventional aerobic systems where function is rather obvious, is the determination of function in bacterial metabolism. Bacterial systems employ heme proteins in a variety of ways which are quite different from those commonly associated with the aerobic processes of mammals. Hence, assays for activity cannot always be applied.

Future researches will tend to the development of procedures for bulk culture so that greater amounts of material are available. In additon, intensified enzymic analyses of particle, or insoluble, electron transport systems coupled to energy storage and reduction of substrates other than molecular oxygen will be likely.

(b) *c*-Type cytochromes. These occur most frequently in the greatest concentrations of all the bacterial heme proteins. Pure specimens have been obtained in varying amounts, ranging from a few micrograms up to hundreds of milligrams, from all species of the photosynthetic bacteria available. In general, they show no marked variance from cytochrome *c*, as indicated by the usual spectrochemical criteria, sequence, and other structural studies, but as a rule are wholly inactive in the classic cytochrome *c* oxidase system of mitochondrial tissues. Moreover, they usually exhibit acid isoelectric points, owing to a preponderance of aspartic and glutamic acid residues. Another interesting difference is that none of the bacterial proteins show an acetylated N-terminal group, as in cytochrome *c*. There are great variations in size, oxidation potentials, and associated properties. Some data (9,10,11) which relate to the composition of *c*-type cytochromes are exhibited in Table 1.

Functionally, they differ completely from the conventional cytochrome *c*, in that they appear to be associated wholly with the photooxidase system of chromatophores. That is, they do not act as terminal catalysts in the reduction of molecular oxygen. The most informed guess, at present, based on the very extensive accumulation of data from enzymic studies, dynamic spectrophotometric and flash spectrophotometric observations (3,12-15), implicate the *c*-type cytochromes (including the analogous heme proteins of plant chloroplasts) as substrates for the primary photochemistry of the photosynthetic process. The reaction is not certain, but involves one of two alterna-

TABLE 1

*Composition of c-Type Cytochromes**

Amino Acid Composition

Per Heme:

R. rubrum - (Lys_{17} His_2 Asp_{14} Thr_8 Ser_6 Gly_9 Pro_3 Gly_9 Ala_{16} Val_6 $Ileu_2$ Leu_8 Met_2 Cys_2 Tyr_5 Phe_5 Try_1); 9 amides

Chromatium - (Lys_{20} His_7 Arg_8 Asp_{24} Thr_{12} Ser_{15} Glu_3 Pro_{18} Gly_{29} Ala_{35} Val_{19} $Ileu_{17}$ Met_7 Cys_5 Tyr_{13} Phe_{10} Try_3)

Rps. Palustris - (Lys_{12} His_2 Asp_{15} Thr_8 Ser_2 Gly_8 Pro_3 Gly_{12} Ala_{16} Val_9 $Ileu_2$ Leu_8 Cys_2 Tyr_2 Phe_5 $Try_?$)

Heme Peptide Sequence:

R. rubrum - H_2N-Ser-Lys-Cys-Leu-Ala-Cys-His-Thr-Phe-Asp-Glu-Gly-Ala-Asp NH_2 -Lys-COOH (Residues 14-28)

End-Group Sequences;

```
                       Glu
R. rubrum - H2N-Glu-Gly-Asp-Ala-Gly-Ala - - - - - Lys-COOH
                      (NH2)
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Chromatium - H_2N-Glu

* These data have been taken both from published articles (9,10,11) and unpublished observations by K. Dus, H. de Klerk, and M. D. Kamen.

tives—either the excited chlorophyll oxidizes the cytochrome, after loss of an electron, or it oxidizes the cytochrome before loss of an electron. Either possibility is consistent with the data now at hand.

(c) *b*-Type cytochromes. Very little is known about these heme proteins, except that they exist in amounts which may approach those of the *c*-type cytochromes. Only one instance of a solubilized specimen has been reported (16). Attempts to link the *b*-type cytochromes of the purple photosynthetic bacteria with a conventional function in the electron transport chain, coupled to phosphorylation either in dark or light, have been frustrated by the simultaneous occurrence of the *o*-type cytochromes and cytochromoids, spectrochemical characteristics of which mask expected spectral shifts which might be ascribed to *b*-type cytochromes.

(d) Catalases and peroxidases. Practically nothing is known about these types of heme proteins, except that their presence is evident

in chromatophore preparations, even those from strict anaerobes. Some qualitative observations indicate the catalase of *Chromatium* to be a heme protein (17). Proto-heme, the usual prosthetic group, can be extracted by acid-acetone (18), but this may arise wholly from the *b*-type cytochrome which is present.

(e) "*o*-Type" cytochromes. The dark oxidase activity of *R. rubrum* seems to require the presence of a particle-bound heme protein with spectrochemical characteristics like those of a cytochromoid (see below), in the visible range. No isolation of these proteins has been reported. Researches, based on analysis of spheroplasts, obtained by lysis of dark-grown cells, and other systems enriched in the aerobic phosphorylation system, are needed.

It may be remarked here that not a single bacterial oxidase, let alone the "*o*-type" proteins, has been characterized as yet.

(f) Cytochromoids (3e). This class illustrates best the surprises which may be in store for biochemists, when they begin to take bacterial heme proteins more seriously. Again, we owe to Leo Vernon the original observation which led to the discovery of these proteins. He noted in 1953 that trichloracetic acid extracts of *R. rubrum* contained a heme protein, other than a cytochrome of the "*c*" type, with an absorption spectrum like that of myoglobin, or hemoglobin (2). Since then, our laboratory has been engaged in continuous research in an effort to elaborate the nature and function of what we now call "cytochromoids." Parenthetically, I may add that the existence of cytochromoids is a surprise only to those who persist in ignoring possibilities of combinations such as those given in the classification scheme I have described above.

The official definition, as proposed by the Commission on Enzymes of the International Union of Biochemistry (5), is "heme proteins with a hemoglobin-like spectrum and a reactivity with ligands which do not react with cytochrome *c*." Cytochromoids are essentially heme proteins in which the normal heme prosthetic group retains the high-spin or mixed-spin character of "open" type heme proteins (19), while being bound covalently as in cytochrome *c*. The mixed-spin character used in classification emerges only in the oxidized forms of cytochromoids so far studied. There are just two specimens—one from *R. rubrum* (I), the other from *Chromatium* (II). Another specimen, recently isolated from *Rps. palustris* (2), shows the same spectral characteristics and ligand behavior as I and II, but differs in that it has a high oxidation potential ($E_{m,7}$ 250 mv) and is not autoxidizable, whereas I and II have low oxidation potentials ($E_{m,7} \sim -8$ to -5 mv) and are rapidly autoxidizable (3e). Thus, with just three specimens examined thoroughly, the cytochromoids appear already to exhibit a wide range of physicochemical character, just as do the "*c*" cytochromes.

Both I and II have molecular weights close to 28,000, and contain two heme groups per mole. Sequence analysis for the diheme-

containing moiety of II has been accomplished and results are consistent with the assumption that one heme is attached by thioether bonds to two cysteinyl residues, separated by two residues, as in cytochrome *c*. The placement of the other heme is uncertain, as only one more cysteine is available for linkage of the covalent type. Larger quantities of protein will be needed to permit further elaboration of the peptide structure as well as that for the whole protein.

The nature of the second heme group is still in question, also. It is certain that it does not differ in oxidation potential appreciably from the value found by titration of the protein.

The primary sequences for I and II, beginning at the terminal end, show interesting correlations (see Table 2). Thus, for II, the sequence

TABLE 2

Composition of Cytochromoids

Amino Acid Composition

Per Heme:

R. rubrum - $(Lys_{16}\ His_{2}\ Arg_{1.5}\ Asp_{9.5}\ Thr_{7}\ Ser_{10}\ Glu_{12.5}\ Pro_{4}\ Gly_{7}\ Ala_{29}\ Val_{4.5}\ Ileu_{6.5}\ Leu_{8}\ Tyr_{3}\ Phe_{4}\ Met_{2}\ Cys_{2}\ Try_{1})$

Chromatium - $(Lys_{9}\ His_{1}\ Arg_{4}\ Asp_{12}\ Asp(NH_{2})_{2}\ Thr_{6}\ Ser_{3}\ Glu_{17}\ Glu\ NH_{2\ 2}\ Pro_{3}\ Try_{1}\ Gly_{14}\ Ala_{24}\ Val_{10}\ Leu_{4}\ Ileu_{5}\ Met_{2}\ Cys_{2}\ Tyr_{3}\ Phe_{5})$

Rps. Palustris - $(Lys_{12}\ His_{1}\ Arg_{2}\ Asp_{11}\ Thr_{4}\ Ser_{4}\ Glu_{8}\ Pro_{3}\ Gly_{6}\ Ala_{17}\ Val_{4}\ Ileu_{5}\ Leu_{8}\ Met_{(0.5?)}\ Cys_{2}\ Tyr_{(0.5?)}\ Phe_{1}\ Try_{(?)})$

Heme Peptide Sequence

Chromatium - H_2N-Phe-Ala-Gly-Lys-Ser-Glu NH_2 -Cys-His-Thr-Leu-Val-Ala-Asp-Glu-Gly-Ser-Ala-Lys-Cys-His-Thr-Phe-Asp-Glu-Gly-Ser-COOH

End-Group Sequences

R. rubrum - H_2N-Ala-AspNH_2-Val-Ala-Gly - - - - - - - Glu-COOH

Chromatium - H_2N-Ala-Gly-Leu/Ileu-Ser-AspNH_2 --- Ala-COOH

is: H_2N-Ala·Gly·Leu(Ileu)·Ser·$AspNH_2$. . .; for I it is: H_2N-Ala·$AspNH_2$ ·Val·Ala·Gly. . . Thus, both end groups are identical; there are simple cross-overs between the asparagine residues and the glycine residues, while leucine-valine, and serine-alanine are comparable pairs. The carboxyl end group for I is glutamic acid, and for II is alanine. It is of interest that the two residues which separate the cysteinyl groups, presumably holding one of the hemes in II, are serine and glutamine, just as in chicken cytochrome *c*. In all other mammalian and animal *c*-type cytochromes, these two residues are alanine and glutamine.

These are only a few of the results at hand; space prohibits presentation of others, which in any case are still very preliminary. I have included the results shown only to provide examples of the sort of information coming out of our present studies.

The most remarkable properties of cytochromoids are exhibited in their behavior with ligands (2,21), with which they would normally be expected to react; such reagents as azide, cyanide, hydrosulfide, methylimidazole, nitrosobenzene, etc., fail to attach to the central iron atom even at extremes of pH. The proteins in the oxidized state appear to be accessible only to protons, CO and NO, and not even to protons when in the reduced state (21,22). Reactions with NO occur with both reduced and oxidized forms, but only marginally. Affinities are many orders of magnitude less than for usual NO-heme interactions.

CO reacts more strongly than NO and, of course, only with the reduced form, with which it forms an easily photodissociable complex. The fact that cytochromoids attach NO less firmly than CO is anomalous, and so is another finding—that the photodissociability is pH-dependent.

These ligand interactions are sufficiently weak so that taken with the absolute lack of reaction with ligands in general they weight the similarity to "*c*" cytochromes greater than to myoglobins or peroxidases, as based on the spectral and magnetic susceptibility data. The sequence studies provide more firm evidence for the close relation to the "*c*" cytochromes.

Finally, the testimony of data on functional involvement of cytochromoids as intermediates in the photoactivated electron-transport chain coupled to photophosphorylation (23,24,25), rather than as oxidases or peroxidases, completes the similarity to the "*c*" cytochromes. The relevance of the term "cytochromoids" is obvious.

Other counts against an oxidase function for cytochromoids are: (1) One of them occurs, as in (II), in a strict anaerobe; (2) *R. rubrum* oxidase from dark-grown aerobic cultures contains no extractable cytochromoids and shows little (26) or no (27) detectable bound cytochromoids; (3) The oxidase activity of *R. rubrum* is inhibited by approximately 10^{-5} M cyanide (2), whereas cytochromoid I does not combine with cyanide even at a cyanide concentration of 10^{-2} M (2,21). There have been data on action spectra for the relief of the CO inhibi-

tion of oxidase activity in dark-grown aerobic *R. rubrum* cultures (7, 28) which show very good correlations between such spectra and the CO, reduced-minus-reduced difference spectra of cytochromoid I, but these apparently only prove the identity of these spectra for "cytochrome *o*" and cytochromoid I.

CONCLUDING REMARKS

I have not burdened you with many data in this presentation because it is certain that neither space nor time allotted permitted a detailed discourse, and because I feared the general outlines which have emerged in this area of research might not be visible through the flood of tables and figures which would have resulted from any attempt to document my remarks. I have included references which I hope will aid those of you who wish to inquire further into the subject matter I have presented.

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THE STRUCTURE OF THE PHOTOSYNTHETIC APPARATUS IN THE GREEN AND PURPLE SULFUR BACTERIA[1]

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INTRODUCTION

The concept of the chromatophore as the basic structural unit of bacterial photosynthesis has grown steadily since this structure was first described by Schachman, Pardee, and Stanier (1) in 1952 and subsequently shown to be functional in photosynthetic phosphorylation by Frenkel (2). However, obvious exceptions to this rule have been observed in recent years. Under most conditions of growth the cytoplasm of the photosynthetic bacterium *Rhodomicrobium vannielii* contains a lamellar system which in all probability is the photosynthetic apparatus of the cell (3). It also has been reported that lamellar structures are characteristic of *Rhodospirillum molischianum* (4,5) and in some cases can be observed in *R. rubrum*. Recently Cohen-Bazire and Kunisawa (6) have shown that in cells of *R. rubrum* grown at high light intensity chromatophore synthesis is suppressed and that the chromatophores, if present at all, are localized in the peripheral areas of the cell. They suggest further that the photochemical apparatus of this organism has its origin in the bacterial cytoplasmic membrane. Boatman and Douglas have indications of similar relationships in subaerobically grown cultures of *R. rubrum* (7).

Electron microscopy of thin sections of cells of the purple bacterium *Chromatium* strain D reveals the presence of circular vesicular chromatophores throughout the cell. Disruption of the cells by various means yields a preparation containing stable, uniform chromatophores approximately 300 Å in diameter, with a molecular weight of approximately 15,000,000; these are capable of catalyzing photosynthetic phosphorylation without the addition of artificial electron transport carriers (8). It has not been possible to obtain a functional subunit of this structure. This organism has therefore served well as a model

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system for investigations dealing with the nature of the bacterial chromatophore.

A much simpler unit of photochemical activity has been shown to be present in the green sulfur bacterium *Chlorobium thiosulfatophilum* (9). The cytoplasm appears granular and contains small (100 Å) particles. After cell disruption, similar particles can be isolated and are functional in carrying out photosynthetic phosphorylation. These particles have a molecular weight of about 1,500,000 and contain chlorophyll, cytochromes, quinones, carotenoids, and lipids in amounts that seem to indicate that this isolated structure may indeed represent the minimal structural unit necessary for photosynthetic phosphorylation (10,11).

Thus, it now seems clear that there really are significant differences in the architecture of the photochemical apparatus of the various photosynthetic bacteria.

THE STRUCTURE OF THE GREEN SULFUR PHOTOSYNTHETIC BACTERIUM *CHLOROBIUM THIOSULFATOPHILUM* STRAIN L

As previously stated, *Chlorobium* appears to be an exception among the photosynthetic organisms studied to date in that thin sections of the cells observed by electron microscopy indicate the absence of any form of visicular or lamellar structure known to be associated with a photochemical apparatus. However, the authors have not yet made a detailed analysis of the cell with electron microscopy.[2]

Following disruption of the cell, either by ultrasonic treatment or with the Hughes press (which is not subject to the criticism of breaking up internal membranes as is ultrasonic treatment), a sedimentable fraction containing most of the pigment of the cell could be obtained. Examination of the pellet by electron microscopy did not disclose any vesicular or membrane associations, in agreement with our observations on cells of *Chlorobium thiosulfatophilum* (strain L).

These photochemical macromolecules behave differently from the chromatophores obtained from other photosynthetic bacteria in several ways. The pigments are more readily dissociated from them than from the chromatophores of the purple bacteria and, indeed, the particles appear to be perhaps the equivalent of subunits or chromatophore fragments of a more highly organized system. Therefore, for this and many other reasons, we prefer not to regard this pigmented component of the cell as a chromatophore. Fig. 2 illustrates some of the physicochemical properties of this macromolecular system and the degree of

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[2] Cohen-Bazire, in this volume, shows thin sections of other strains of *Chlorobium* which show ultrastructural differentiation which may or may not be associated with the pigment-bearing structure.

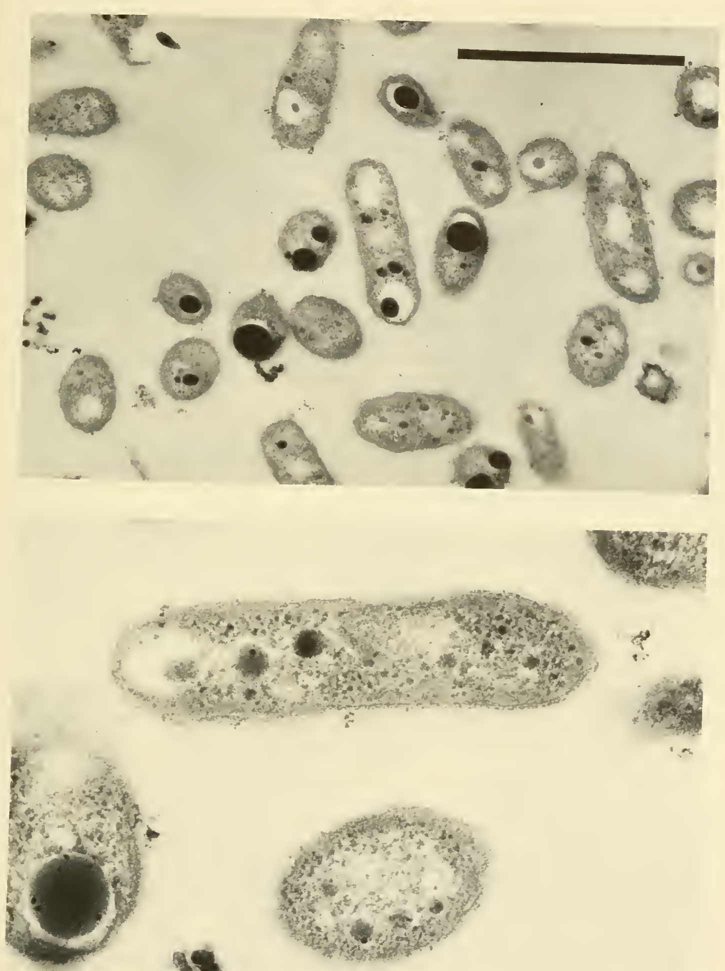

Fig. 1. Electron micrograph of *Chlorobium thiosulfatophilum* strain L. The large electron dense intracellular inclusions are polymetaphosphate granules. Cytoplasm appears granular and is devoid of lamellar or vesicular inclusions. Top - 56,000 x (Bar = 1 μ). Bottom - enlargement to 140,000 x.

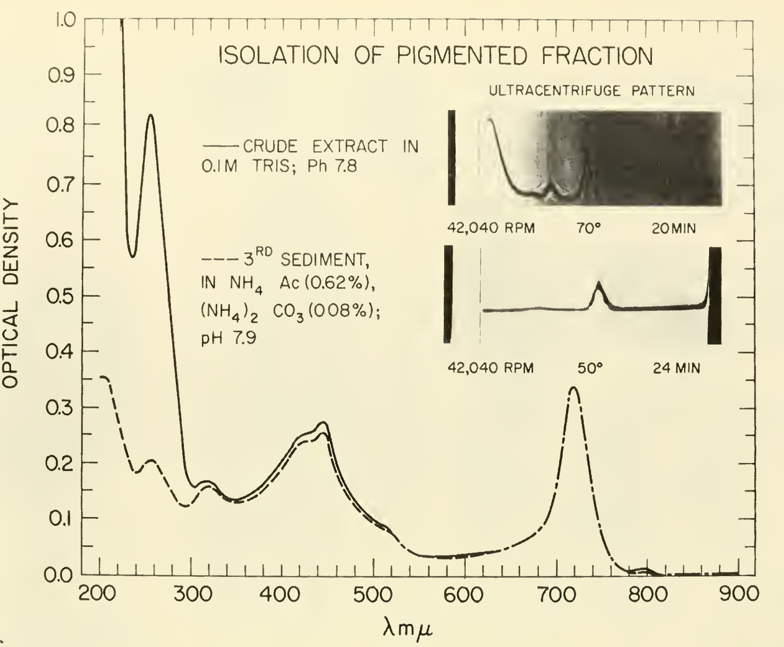

Fig. 2. Record of changes in the sedimentation diagram (Spinco Model E) and adsorption spectrum (Cary Model 14) during isolation of the pigmented component from *Chlorobium thiosulfatophilum*. There are three major components in the crude extract. The color due to the photosynthetic pigments is associated with the component (50 S) which sediments most rapidly. The adsorption at 260 mμ due to nucleic acid is greatly reduced as the slower components are eliminated.

homogeneity of fractions after isolation and purification. With minimal purification, the pigmented fraction shows a strong absorption maximum at 260 mμ, indicating the presence of nucleic acids, but upon repeated sedimentation and purification in buffered systems a single ultracentrifugal peak can be obtained. It is clear that the photochemical pigment system is in an unaltered form and is free of both nucleic acids and slower sedimenting components. The estimated molecular weight of this particle from the above data is one and one-half million. This is by far the simplest defined photochemical system described to date which occurs in a natural state.

Chemical analysis shows that the particulates isolated from *Chlorobium* contain carotenoids, chlorophyll, cytochromes, quinones, and phospholipids; the data of Hulcher and Conti (12), as shown in Table 1, give a reasonable analysis of the particles. It is interesting to note that the quinone associated with these particles is a new one and very similar to plastoquinone (13). Olson and Romano (14) have

TABLE 1

Estimation of Cytochromes and Other Components in Chlorophyll-containing Particles of Chromatium *and* Chlorobium

Constituent	*Chromatium* (mμmoles/1.0 ml suspension)	*C. thiosulfatophilum* (mμmoles/1.0 ml suspension)
Cytochromes:		
type 555, 552	0.91×10^{-5}	0.85×10^{-5}
type 612	none	1.66×10^{-5}
type 630-640	0.78×10^{-5}	none
Total Cytochrome (ext. coef.)	1.69×10^{-5}	2.51×10^{-5}
Total Cytochrome (pyridine hemochromogen)	3.18×10^{-5}	1.59×10^{-5}
Chlorophyll	7.10×10^{-4}	2.34×10^{-3}
Carotenoids	4.00×10^{-4}	4.48×10^{-4}
Protein[1]	4.56 mg/ml	24.0 mg/ml
Molar ratios:		
Chl:carotenoid:cytochrome[2]	40:20:1	100:20:1
Chl:carotenoid:cytochrome	20:10:1	150:30:1

[1] Protein determined by the spectrophotometric method of O. Warburg and W. Christian, *Biochem. Z.*, *310*, 384 (1941).

[2] Calculated from total cytochrome, determined from extinction coefficients. From Hulcher and Conti, *Biochem. Biophys. Res. Commun.*, *3*, 497 (1960).

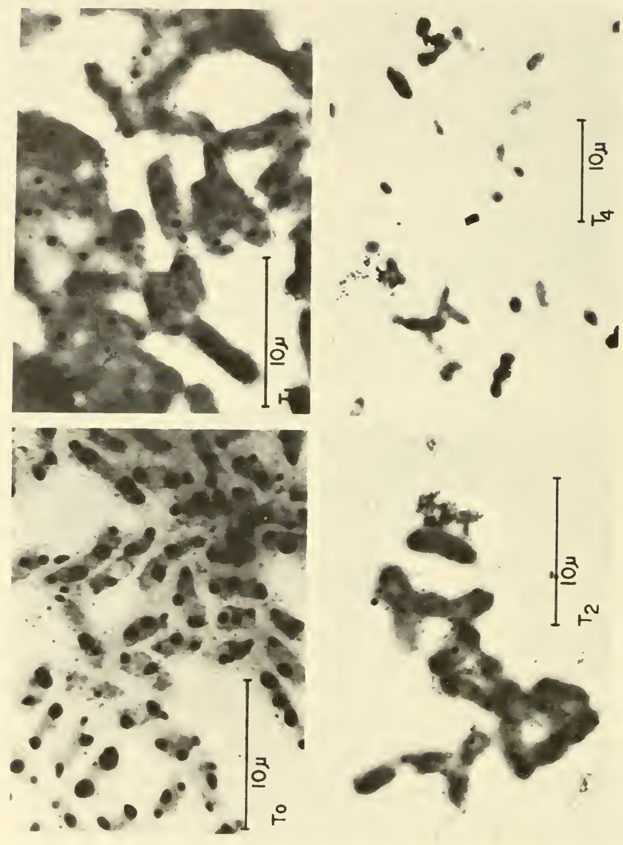

Fig. 3. The effect of serial transfer of *Chlorobium thiosulfatophilum* in the absence of inorganic phosphate in the growth medium, on the content of polyphosphate in the cells. (T_0 ---- T_4 = 4 transfers). Growth was relatively unaffected through three such transfers (T_3) but polyphosphate was essentially depleted.

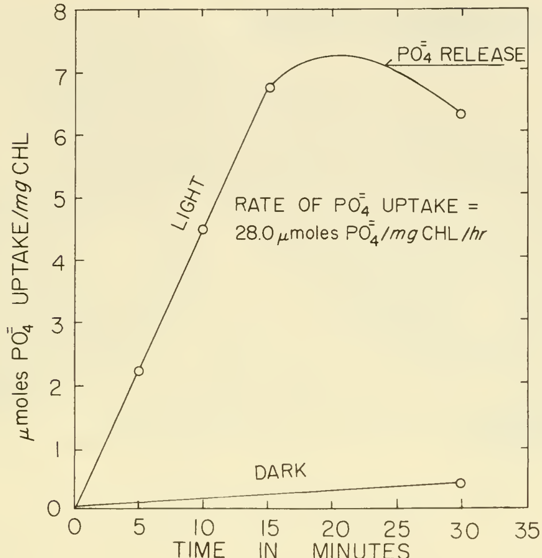

Fig. 4. Phosphorus uptake by a particulate fraction from *Chlorobium*. The fraction consisting of pigmented particles was incubated in light and dark in Warburg vessels containing 0.8 ml extract; 10 μmoles $MgCl_2$; 10 μmoles Kh_2PO_4; 100 μmoles Tris pH 7.0; 7.5 μmoles ADP; 0.5 ml Hexokinase (Sigma), and water to a total vol. of 2.5 ml. 0.5 ml of TCA (10%) was added to stop the reaction and the precipitate removed by centrifugation. Rates of phosphate uptake are expressed as μmoles orthophosphate esterified/hr/mg chlorophyll.

recently shown that bacteriochlorophyll is also associated with the particle from *Chlorobium* and have suggested that *Chlorobium* chlorophyll may act as an accessory pigment to the photochemically active bacteriochlorophyll.

It is only recently that we have been able to show that the small particle is indeed an active photochemical system. During experiments designed to measure light-dependent phosphate uptake, inorganic phosphate was always released into the medium, making measurements of photophosphorylation difficult. It has recently been shown by Hughes *et al.* (11) that *Chlorobium thiosulfatophilum* accumulates large amounts

of polymetaphosphate in discrete intracellular granules, from which inorganic phosphate is released by an ADP-dependent and light-independent reaction. The polymetaphosphate granules represent a large proportion of the intracellular material. Fig. 3 illustrates the effect of continued serial transfers on growing cultures of *Chlorobium* in a phosphate deficient medium. It can be readily seen that the size of the polymetaphosphate granules decreases with each transfer. Cells essentially free of these polymetaphosphate granules were used to study photosynthetic phosphorylation.

The results shown in Fig. 4 indicate a rapid (and reproducible) rate of photosynthetic phosphorylation over short periods of time. There is still an indication of phosphate release which can mask the photophosphorylation during longer periods of incubation. However, it now seems clear that these functional macromolecules can catalyze the light-dependent esterification of inorganic phosphate into ATP in the absence of any artificial electron transport carriers. It is interesting to note that Levine (15) recently has been able to demonstrate photosynthetic phosphorylation by *Chlamydomonas* chloroplasts for the first time by growing cells deficient in polymetaphosphate.

THE STRUCTURE OF THE PURPLE SULFUR PHOTOSYNTHETIC BACTERIA

As stated previously, electron microscopy of thin sections of cells of the purple bacterium *Chromatium* strain D has always revealed the presence of circular vesicular chromatophores throughout the cell.

Fig. 5 is a thin section of the bacterium indicating the classical picture of the internal contents of the cell packed with spherical chromatophores.

The relationship of these chromatophores to the cytoplasmic membrane in the photosynthetic bacteria has been a subject of speculation for some time. In the succeeding article in this volume by Cohen-Bazire, this discussion is extended in great detail in regard to the nonsulfur photosynthetic bacterium *R. rubrum*. It has been possible to obtain a separation of the photosynthetic pigments and enzymatic properties ordinarily associated with the bacterial cell membrane, such as succinic dehydrogenase, in *Chlorobium thiosulfatophilum* (16). However, such a separation has not been successfully achieved in the purple bacteria. Recent experiments in our own laboratory in association with Dr. R. Bennett have indicated that succinic dehydrogenase activity is always associated with purified chromatophores, but can be separated from the major fraction containing hexosamine which is a constituent of the cell wall. Therefore, chromatophores appear biochemically related to the cell membrane and not to the cell wall. However, the difficulties involved in electron microscopy of such

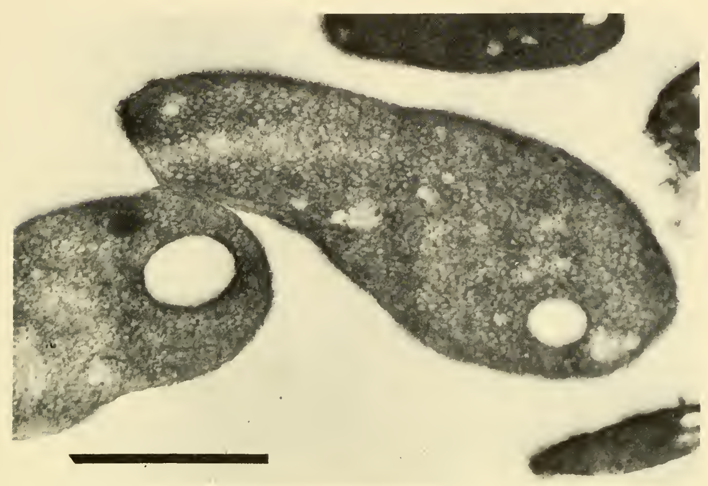

Fig. 5. Electron micrograph of thin section of *Chromatium* strain D grown at low light intensity (see text).

small chromatophores as appear in *Chromatium* have limited us in further speculation concerning a structural association.

Variations in the far-red region of the absorption spectrum of bacteriochlorophyll have also been a subject of considerable speculation. The wide diversity of the *in vivo* spectrum of this pigment in a variety of photosynthetic bacteria is shown in Fig. 6. The divergence in spectra of *Chromatium* is caused by different nutritional conditions of growth (see below). Wassink *et al.* (17) have reported variations in the far-red chlorophyll spectrum of *Chromatium*, and it was suggested that the multiplicity of absorption maxima represented varieties of bacteriochlorophyll-protein complexes produced under different conditions of growth. Cohen-Bazire, *et al.* (18) and Bergeron and Fuller (19) demonstrated changes in the *in vivo* spectrum of bacteriochlorophyll that were associated with carotenoid deficiency. Wassink and Kronenberg (20), however, were able to grow carotenoid deficient cells of *Chromatium* with a relatively normal spectrum. Bril (21) has recently confirmed Wassink's observations and points out that these divergent experimental results are not readily explained. One point is clear, *viz.*, that alteration of the fine structure in the far-red spectrum is not due directly to carotenoid deficiency. Frenkel and Hick-

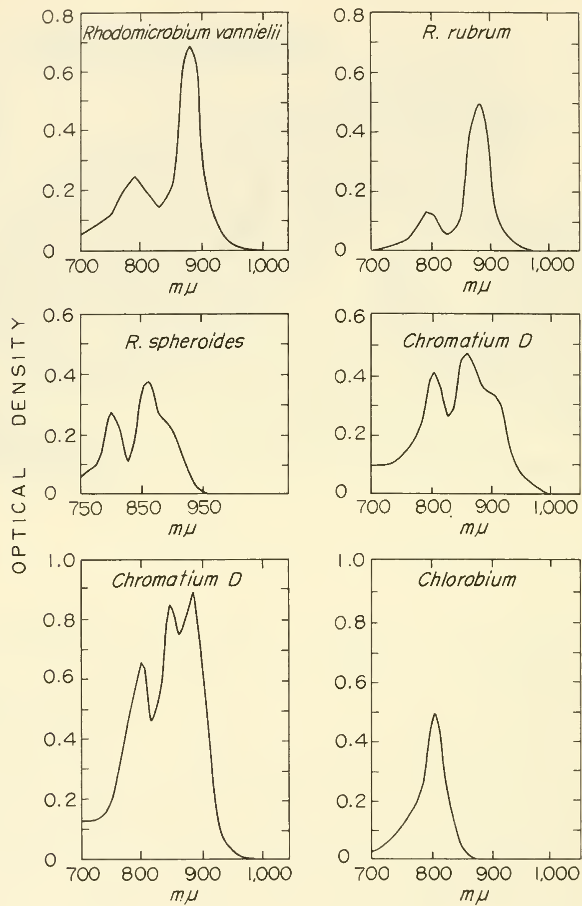

Fig. 6. Diagramatic representation of various far-red presentations of the spectrum of bacteriochlorophyll associated with the photochemical apparatus in a variety of bacteria. The 800 mμ peak is always present and the variation occurs in the region from 800–900 mμ. The simplest spectrum, with a single peak at 800 mμ, occurs in a pigmented lipoprotein from *Chlorobium* (14). The divergent spectra in *Chromatium* D are dependent on nutritional conditions and light intensity during growth, which may well affect the photochemical structures (see text).

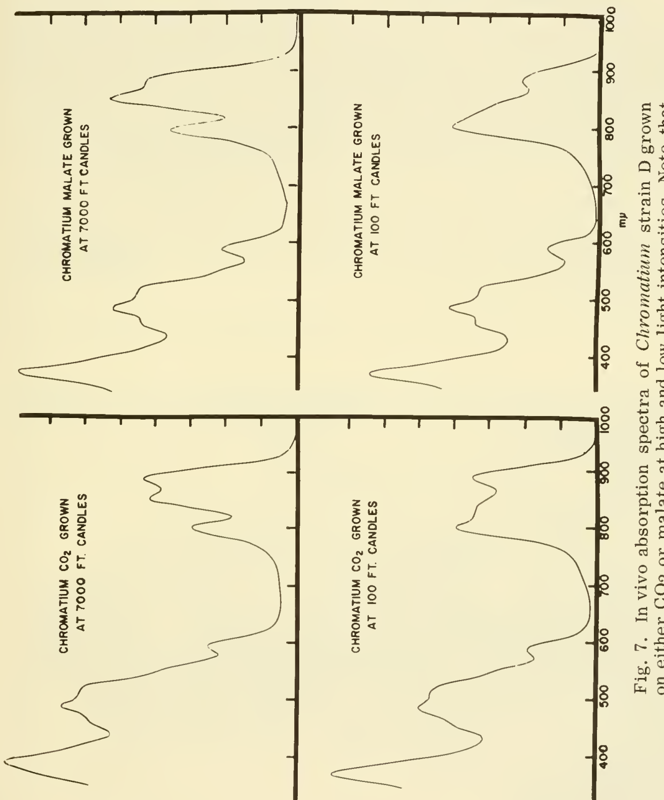

Fig. 7. In vivo absorption spectra of *Chromatium* strain D grown on either CO_2 or malate at high and low light intensities. Note that the 800 mμ peak remains relatively constant and that primary variations occur in the 850 mμ and 890 mμ regions.

man (22), Bril (21), and Fuller (10) have noted that chromatophore structure in a single species might be dependent on such factors as growth conditions and age of cells and that the far-red fine structure of the spectrum of bacteriochlorophyll may be related to altered structures. These conflicting observations can now be reconciled.

Fig. 7 illustrates the dramatic changes that occur in the far-red region of the *in vivo* absorption spectra; these appear to be dependent upon the light intensity to which the cells are exposed during growth. High light intensity seems to enhance the 850 mμ and especially the 890 mμ absorption maxima. Heterotrophic conditions of growth seem to depress this effect to some extent. Other carbon sources such as acetate or succinate yield the same spectrum as malate. In previous experiments, where diphenylamine was used to suppress carotenoid synthesis, red cellophane was placed between the light source and the culture bottles to depress the light-catalyzed breakdown of diphenylamine. Inadvertently this reduced the effective far-red incident light. Although growth did not appear to be affected, the spectrum was altered in a manner similar to that shown for cells grown at low light intensities as seen in Fig. 7.

STRUCTURAL ALTERATIONS

Chemical analysis of the cells grown at 7000 and 100 foot-candles of incident light was undertaken, and the results are shown in Table 2.

TABLE 2

Chemical Analysis of Chromatium *Cells Grown at High and Low Light Intensities*

	7000 Ft.-Candles	100 Ft.-Candles
CONSTITUENTS	% of Dry Wt.	% of Dry Wt.
CHLOROPHYLL	0.3	1.0
PROTEIN	38.0	35.0
LIPIDS (Including Carotenoid Pigments)	19.0	29.6
CARBOHYDRATES	26.2	20.0
NUCLEIC ACIDS	6.0	6.5
INORGANIC ASH	11.7	9.0
TOTALS	101.2	101.3
CHL/PROTEIN RATIO	.008	.02

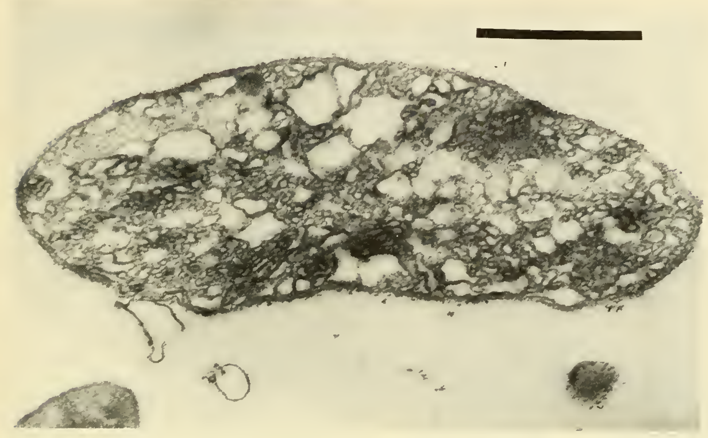

Fig. 8. Electron micrograph of a thin section of *Chromatium* strain D grown at 7000 foot candles of incident light. Bar equals 1μ. (See text for details).

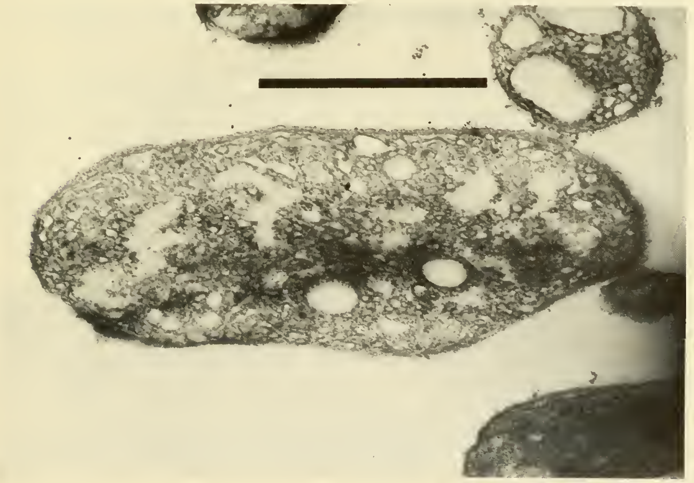

Fig. 9. Electron micrograph of a thin section of *Chromatium* strain D grown at 7000 foot candles of incident light. Bar equals 1μ. (See text for details).

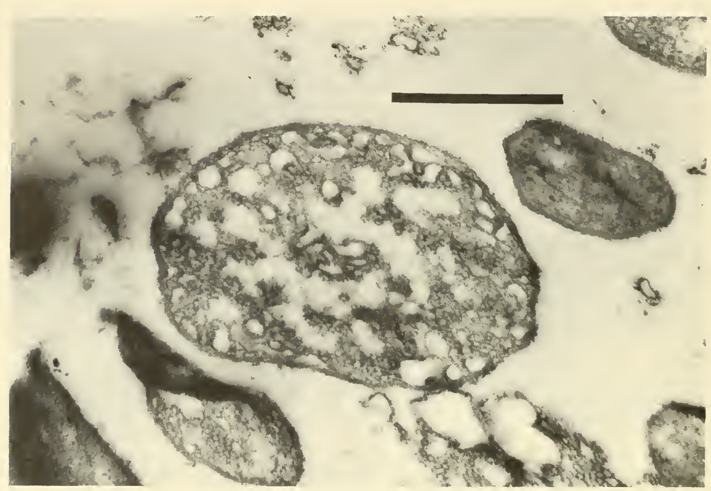

Fig. 10. Electron micrograph of a thin section of *Chromatium* strain D grown at 7000 foot candles of incident light. Bar equals 1μ. (See text for details).

A striking change in the chlorophyll to protein ratio of the cells can be seen. At low light intensities the amount of chlorophyll per mg protein doubles. These data are similar to those obtained by Cohen-Bazire *et al.* (18), Sistrom (23), and Lascelles (24), who noted an increase in the chlorophyll content of cells of *Rhodopseudomonas spheroides* when grown at low light intensities. Of equal interest is the rather striking increase in total lipids in cells grown at low light intensity. This increase may represent structural lipids associated with the chlorophyll-bearing structure.

Although *Chromatium* strain D has always appeared to contain chromatophores at all stages of growth, electron microscopy of cells exhibiting spectral and chemical differences was undertaken to ascertain if the changes in the spectrum and chlorophyll-protein ratios might be reflected in the structure of the photochemical apparatus.

Fig. 5 illustrates the appearance in electron micrographs of *Chromatium* cells grown under conditions of low light intensity. The presence of chromatophores (~300 Å in diameter) throughout the cell is in conformity with the observations of other investigators. In contrast to this, cells grown under conditions of high light intensity (7000 foot-candles incident light) show marked structural alterations (Figs. 8 and

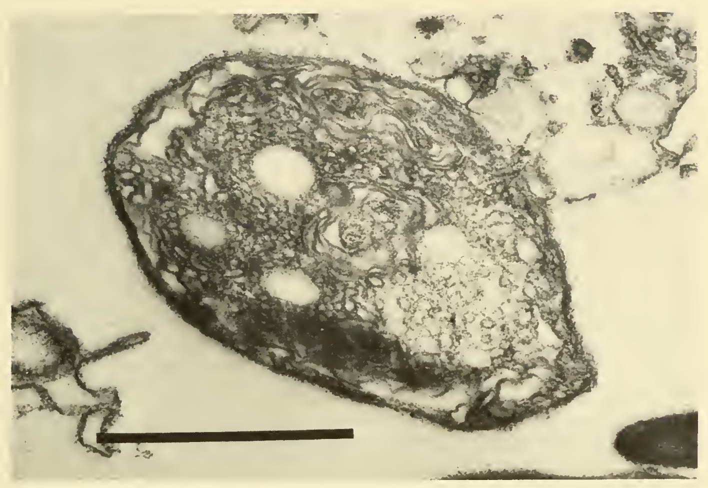

Fig. 11. Electron micrograph of a thin section of *Chromatium* strain D grown at 7000 foot candles of incident light. Bar equals 1μ. (See text for details).

9). Although chromatophores are still present, an intracytoplasmic membrane system can also be observed. These intracytoplasmic membranes are observed most frequently in the peripheral areas of the cells. Close inspection of the plates reveal that these membranes are paired. Each membrane (~100 Å thick) corresponds to a unit membrane, but this has not yet been clearly demonstrated by high resolution studies. Fig. 10 is another micrograph of a cell grown under conditions of high light intensity. It appears that chromatophore-like structures are formed at the terminal ends of the invaginated paired membranes (see arrow). Occasionally, cells grown under conditions of high light intensity appear to have the intracellular membranes organized in a lamellar system (Fig. 11).

It was observed that when cells are grown under conditions which induce the formation of intracellular membranes and cell-free extracts prepared, the majority of the pigment-bearing particulate material sediments at relatively low centrifugal forces as compared to the situation encountered with "normal" cells.

SUMMARY

Thin sections of the green photosynthetic bacterium *Chlorobium thiosulfatophilum* strain L are unlike other photosynthetic organisms in that no lamellar or chromatophore-like structures appear in the cell. A homogeneous fraction containing the photosynthetic pigments can be isolated and shows the capacity to catalyze photosynthetic phosphorylation. This particulate fraction is by far the simplest photochemical system thus far described. The intracellular origin of this fraction, particularly in relation to the bacterial cytoplasmic membrane, is not clearly understood at this time.

In the purple sulfur bacterium *Chromatium* strain D, where the classical chromatophore structure has always been observed in sections of cells, a complex intracytoplasmic membrane system is produced under certain environmental conditions. The pigment and lipid contents of the cell increase sharply when low light intensities are used for growth. The structure of the photochemical apparatus and the physical and chemical environment of the bacteriochlorophyll—reflected by the fine structure of its far red spectrum—are under control of both incident light intensity and metabolic conditions of growth.

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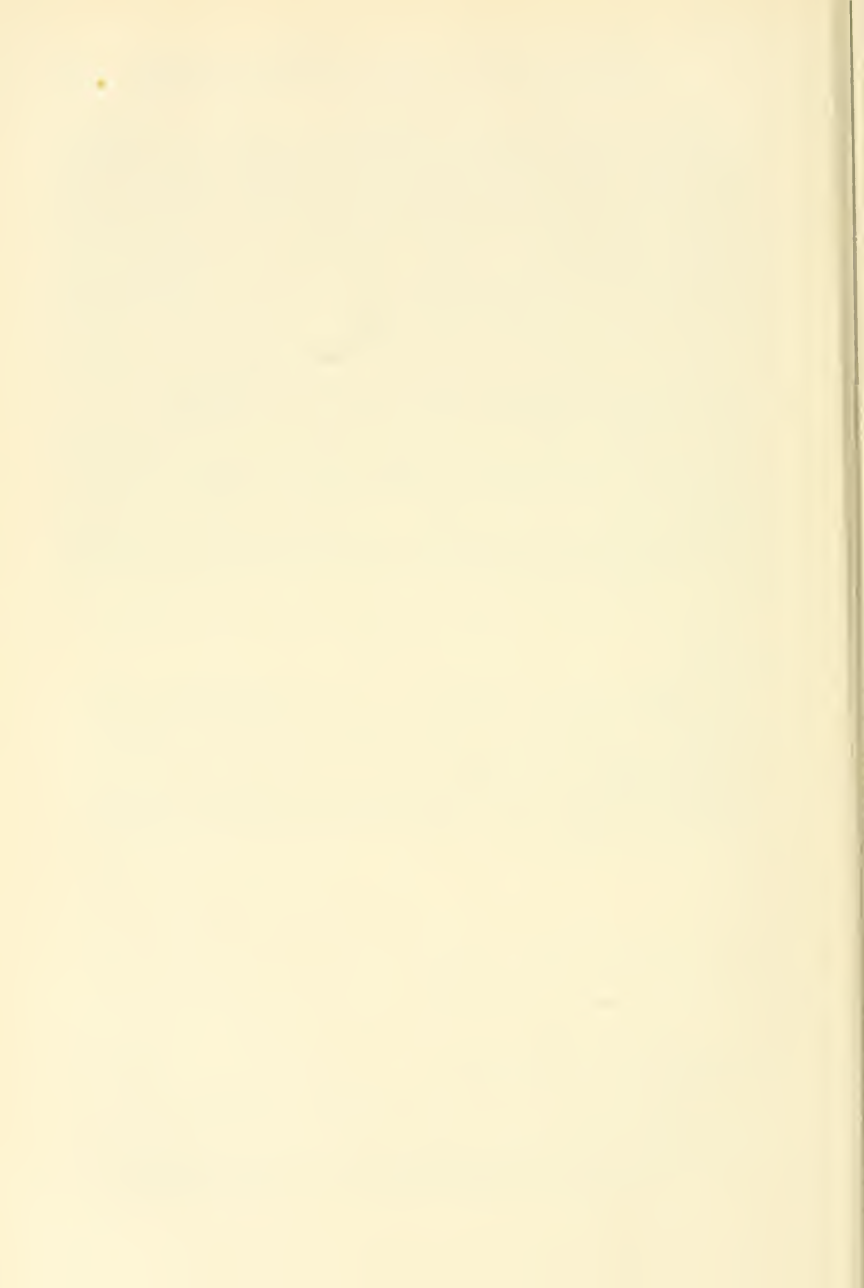

SOME OBSERVATIONS ON THE ORGANIZATION OF THE PHOTOSYNTHETIC APPARATUS IN PURPLE AND GREEN BACTERIA[1]

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The light microscope does not have the resolving power necessary to reveal the structure and organization of the photosynthetic apparatus in cells of procaryotic organisms. Only one conclusion could be drawn from the observations on photosynthetic bacteria and blue-green algae by light microscopy: namely, that even the largest members of these groups were devoid of organelles having the typical structure of chloroplasts. Our first positive information about the structure of the photosynthetic apparatus in these groups was accordingly derived from physico-chemical studies on the pigment-bearing elements that could be isolated from extracts of broken cells. As early as 1938, French (1) showed that the native pigment complex can be extracted from purple bacteria in water-soluble form and is bound to protein, as shown by its precipitability with ammonium sulfate. In 1952, Schachman, Pardee, and Stanier (2) demonstrated that the pigment complex in extracts of *R. rubrum* prepared by mechanical abrasion or sonic oscillation can be readily sedimented by high-speed centrifugation. By applying a series of differential centrifugations, they succeeded in isolating a physically homogeneous particulate fraction which had the typical *in vivo* absorption spectrum of this bacterium. The particles were isodiametric, and about 600 Å in diameter. They were designated as chromatophores. No fraction of corresponding size and uniformity could be isolated from aerobically grown cells of *R. rubrum*, which are essentially devoid of photosynthetic pigments.

Soon after, Frenkel (3) made the important discovery that chromatophores could perform photophosphorylation, and the investigation of their biochemical properties was undertaken in several laboratories. The finding that chromatophores possess significant photochemical functions lent support to the belief that they are the structures responsible for the performance of photosynthesis in the bacterial cell.

[1] This work was supported by a grant from the National Science Foundation to Professor Michael Doudoroff.

It is, therefore, not surprising that when ultra-thin sections of photosynthetic bacteria were eventually examined in the electron microscope the interpretation of the observed intracellular structures was strongly influenced by earlier work on isolated chromatophores. For example, Vatter and Wolfe (4), who performed the first extensive study of fine structure in purple bacteria, stated that: ". . . the cytoplasm [of *Rhodospirillum rubrum*] is organized into discrete units which appear less dense than the cytoplasm and are surrounded by a membrane. Since these structures are of the size of the isolated chromatophores of Schachman *et al.*, they are believed to be identical with them and to represent the chromatophore arrangement which exists within the cell."

The studies of Vatter and Wolfe (4), Bergeron (5), and Drews (6) established the presence of abundant membrane-bounded vesicles of low electron opacity in the cytoplasmic region of photosynthetically grown cells of several purple bacteria: *R. rubrum, Rhodopseudomonas spheroides,* and *Chromatium* strain D. However, this is not a universal feature of internal structure in purple bacteria. As shown by Drews (6), Vatter, Douglas and Wolfe (7), and Boatman and Douglas (8), *Rhodospirillum molischianum* and *Rhodomicrobium vannielii* do not contain such elements; instead, thin sections reveal the presence of regularly disposed lamellae, the intracellular arrangement of which is characteristic for each species. These structures resemble the lamellar systems found in the cytoplasm of blue-green algae, which likewise assume special patterns characteristic of the different species, as shown by the work of Ris and Singh (9).

When Schachman *et al.* (2) first isolated and described bacterial chromatophores, it was not recognized that ultrasound and mechanical abrasion do not simply break bacterial cells open; these treatments also comminute, to a greater or lesser extent, the internal structures. The comminutive effects of these methods of cellular breakage could be clearly appreciated only after the effects of the much gentler procedure of osmotic lysis on cell structure had been explored, a field that was opened by the work of Weibull in 1954 (10). In the light of subsequent knowledge about bacterial cell structure, it is by no means evident that the chromatophores as defined by Schachman et al. do in fact exist as discrete structural entities in the intact cell; they could be physical artifacts, produced by the fragmentation of larger structural elements during the treatment employed for breakage of the cells. This possibility was first recognized by Karunairatnam *et al.* (11), as a result of their observation that lysed spheroplasts of *R. rubrum* retain essentially all of the photosynthetic pigment system. At about the same time, we independently performed similar experiments with *R. rubrum* and *Rhodopseudomonas spheroides*, with similar findings. In the case of *Rhodopseudomonas spheroides,* we observed that the cellular inclusions of poly-β-hydroxybutyric acid, which are

easily visible by phase contrast microscopy, are liberated from lysed spheroplasts under conditions where the release of pigment is negligible. Clearly, therefore, the lysed spheroplasts could not physically retain individual structural elements of the dimensions of chromatophores.

The gaps and apparent contradictions in our present knowledge about the internal organization of the cell in photosynthetic bacteria have led us to undertake an extensive study of fine structure in this group. The present communication constitutes a progress report.

EXPERIMENTAL PROCEDURES

These have been described in a recent paper (12). Here, it is sufficient to say that we have used, throughout, the preparative technique for electron microscopy of bacteria developed by Ryter and Kellenberger (13), with two minor modifications. The period of main fixation has been reduced in recent work from 16 to 2 hours, since this appears to give equally good, if not superior, preparations. Secondly, most of the sections shown have been post-stained with lead hydroxide (14), a treatment which greatly sharpens the definition of wall and membrane structure. Negative staining with phosphotungstic acid was performed as described by Huxley and Zubay (15). All electron micrographs were taken with a Siemens Elmiskop I, operating at 80 KV.

THE FINE STRUCTURE OF *RHODOSPIRILLUM RUBRUM* AND *RHODOPSEUDOMONAS SPHEROIDES*

Physiological studies in our laboratory have shown that environmental conditions profoundly affect the chemical composition of the cell in nonsulfur purple bacteria (16,17). In particular, the cellular content of photosynthetic pigments can vary over a wide range, even under conditions of strictly photosynthetic growth (anaerobiosis), in response to such factors as light intensity and temperature. In facultatively aerobic strains, pigment synthesis can be completely suppressed by growth in the presence of air; if oxygen access becomes limited, photosynthetic pigment synthesis resumes, even in the absence of light (18). The magnitude of these environmental effects on the pigment content of *R. rubrum* and *Rhodopseudomonas spheroides* is shown by the representative data assembled in Table 1.

Accordingly, our first goal in the cytological study of these bacteria was to compare the fine structure of cells with different pigment contents. Typical results are shown in Figs. 1 to 7. It can be seen that the

TABLE 1.

Bacteriochlorophyll content of nonsulfur purple bacteria growing under different environmental conditions

Rhodospirillum rubrum	
Photosynthetic growth at light intensity of:	Chlorophyll content of cells, μg/mg cellular protein.
50 foot-candles	25.0
2000 foot-candles	10.2
6000 foot-candles	5.6
Respiratory growth conditions:	
Full aeration	0.2
Full aeration, then limiting oxygen for 3 hr.	3.3
Rhodopseudomonas spheroides	
Photosynthetic growth at light intensity of:	
50 foot-candles	66
9500 foot-candles	5.9
Aerobic growth	0.14

classical profile of a photosynthetically grown cell with a vesicle-filled cytoplasmic region, as first described for these two species by Vatter and Wolfe (4), is characteristic only for cells which have been grown at very low light intensities, and therefore have a high specific chlorophyll content (Figs. 1 and 5). In sections of cells grown at progressively higher light intensities, the abundance of vesicles declines systematically, and their location in the cell becomes increasingly peripheral. In cells grown anaerobically at light intensities of 5000 foot-candles or higher, most of the cytoplasmic region in sections is filled with a dense array of ribosomes, and the relatively rare membrane-bounded vesicles occur exclusively in the neighborhood of the cytoplasmic membrane (Figs. 2 and 6). Two other important structural features become evident in such cells. Firstly, the typical unit membrane which bounds each vesicle is identical in thickness and fine structure to the cytoplasmic membrane. Secondly, the membrane that encloses a vesicle is sometimes sectioned in a plane that reveals its continuity with adjacent regions of the cytoplasmic membrane. The central, transparent area of such vesicles opens through a narrow aperture into the space that lies between wall and membrane, and is consequently external to the cytoplasm proper. Contrary to the report

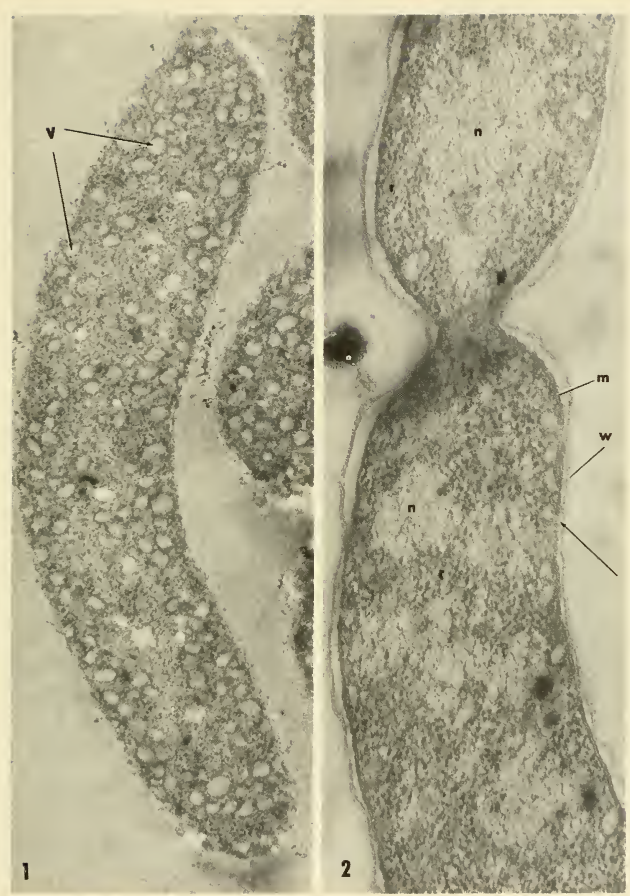

Fig. 1. Section of *R. rubrum* growing exponentially at a light intensity of 50 foot-candles. The membrane bounded vesicles (v) extend deep into the cytoplasm, but are most abundent at the periphery of the cell. Main fixation 18 hours, no post staining. X 84,000.

Fig. 2. Section of a dividing cell of *R. rubrum* growing exponentially at a light intensity of 6000 foot-candles. Membrane-bounded vesicles are sparsely and irregularly distributed around the periphery of the cell. At the point indicated by an arrow, a portion of the vesicular membrane is continuous with the cytoplasmic membrane and the light central area of the vesicle opens into the space that lies between the cell wall (w) and the cell membrane. Nucleoplasm (n) and ribosomes (r) are particularly well preserved in this section. Main fixation 2 hours, post staining with lead hydroxide. X 120,000.

Fig. 3. Section of *R. rubrum* growing exponentially under strictly aerobic conditions in the dark and containing only traces of photosynthetic pigments. The membrane-bounded vesicles are extremely rare in this type of cell. Arrows indicate two such vesicles. Main fixation 2 hours, followed by a short treatment with ribonuclease; post staining with lead hydroxide. X 120,000.

Fig. 4. Section of *R. rubrum* from a culture grown aerobically in the dark, and then allowed to synthesize photosynthetic pigments for 3 hours under semiaerobic conditions in the dark. Peripheral membrane-bounded vesicles are more numerous than in cells grown strictly aerobically. Compare with Fig. 3. Treatment as described for Fig. 3. X 120,000.

Fig. 5. Section of *Rhodopseudomonas spheroides* growing photosynthetically at a light intensity of 50 foot-candles. The cytoplasm is packed with membrane-bounded vesicles (v) of relatively uniform dimensions, 500 Å in diameter. Main fixation 2 hours, post staining with lead hydroxide. X 120,000.

Fig. 6. Section of *Rhodopseudomonas spheroides* growing exponentially under photosynthetic conditions at a light intensity of 9500 foot-candles. Internal membranes (arrows) can be distinguished at the periphery of the cell; in this section, only one irregularly shaped, membrane-bounded vesicle (v) is visible. The very electron-dense structure in the middle of the cell is polymetaphosphate. β indicates areas originally occupied by poly-β-hydroxbutyric acid. Several deposits of glycogen (g) are also visible in this section. Main fixation 2 hours, post staining with lead hydroxide. X 120,000.

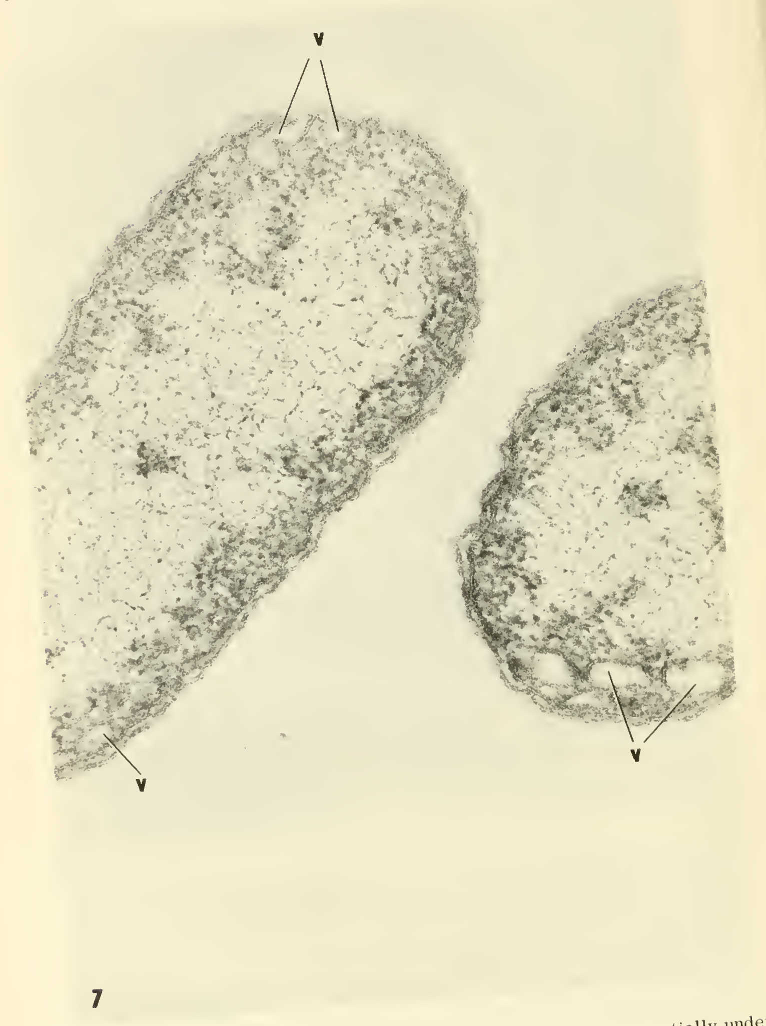

Fig. 7. Sections of *Rhodopseudomonas spheroides* growing exponentially under strictly aerobic conditions in the dark. Note peripheral membrane-bounded vesicles (v). Main fixation 2 hours, post staining with lead hydroxide. X 120,000.

of Vatter and Wolfe (4), vesicles are not completely absent from aerobically grown depigmented cells (Figs. 3 and 7), although they are rare, particularly in *R. rubrum*.

These cytological studies lead us to the following interpretation of the organization of the photosynthetic apparatus in the two species so far discussed. The photosynthetic pigment system is incorporated into a continuous unit membrane, which arises from the cytoplasmic membrane and can intrude into the cytoplasm to a greater or lesser extent, depending on the specific pigment content of the cell. Since structurally indistinguishable membranous intrusions occur in aerobically grown cells with a negligible pigment content, these intrusions are not necessarily always associated with the presence of a functional photosynthetic apparatus. However, recent fine structure studies have shown that a variety of membranous intrusions derived from the cytoplasmic membrane may occur in nonphotosynthetic, aerobic bacteria (19). The "simple intrusives" discovered by Murray (19) in the nonphotosynthetic organism *Spirillum serpens* are quite similar in appearance and intracellular position to the sparse, peripheral vesicles characteristic of *R. rubrum* and *Rhodopseudomonas spheroides* grown aerobically in the dark or anaerobically at high light intensities. A general functional interpretation of these intrusions in aerobic and photosynthetic bacteria has been offered by Stanier (20).

On the basis of a recent study of the fine structure of *Rhodospirillum molischianum*, Giesbrecht and Drews (21) have proposed a very similar interpretation of the organization of the photosynthetic apparatus in this species. In cells sectioned after osmotic lysis, the physical connection between the characteristic lamellar bundles and the cytoplasmic membrane was clearly evident.

THE FINE STRUCTURE OF SOME NEWLY ISOLATED PURPLE SULFUR BACTERIA

Thus far, fine structure studies on the sulfur purple bacteria have been confined to a single strain, *Chromatium* strain D, the only representative of this group which has been generally available in pure culture. Recently, Dr. Norbert Pfennig has succeeded for the first time in isolating and growing in pure culture a number of other types, including *Chromatium okenii* and *Thiospirillum jenense* (22). Through his kindness, we have been able to examine the fine structure of some of the strains in his collection. *C. okenii* and *T. jenense* were of particular interest, since their cells are much larger than those of any other photosynthetic bacteria, with a volume about a thousand times that of the cell of *Rhodopseudomonas spheroides* or *R. rubrum*. Figs. 8 and 9 show typical thin sections of these two large purple sul-

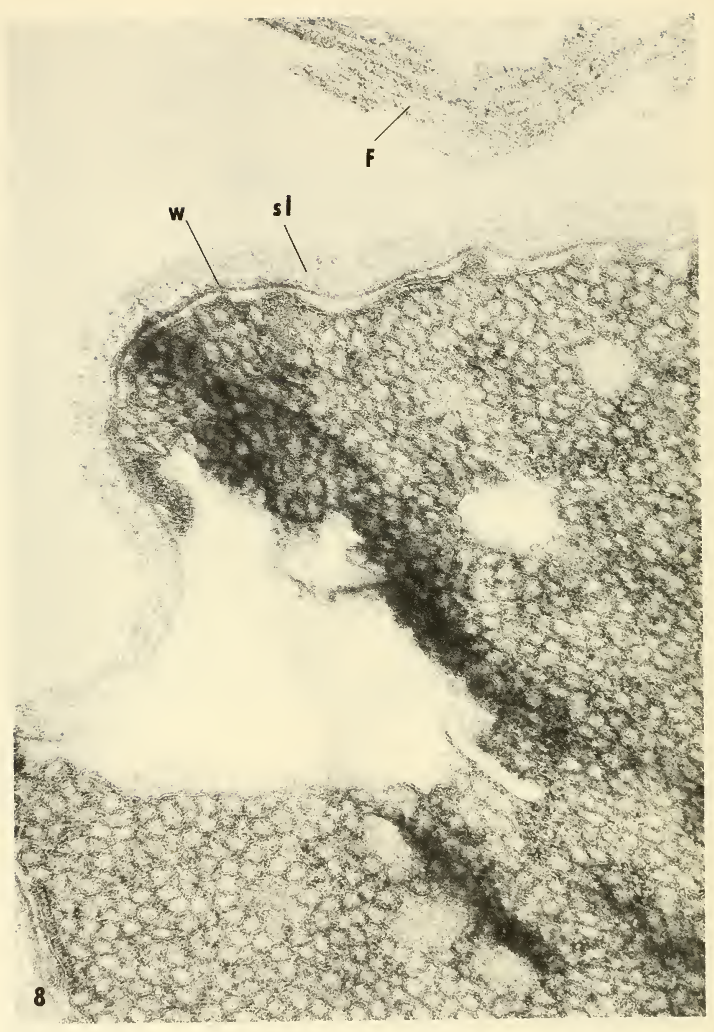

Fig. 8. Part of a section of a cell of *Chromatium okenii* grown at low light intensity (less than 40 foot-candles) and depleted of sulfur inclusions. The cytoplasm is filled with membrane-bounded vesicles. The cytoplasm has retracted from the cell wall at the pole of the cell. Several slime layers (Sl) are present around the cell wall (w). The figure includes also a longitudinal section of the flagellar tuft (F) of this bacterium. Main fixation, 2 hours, post staining with lead hydroxide. X 100,000.

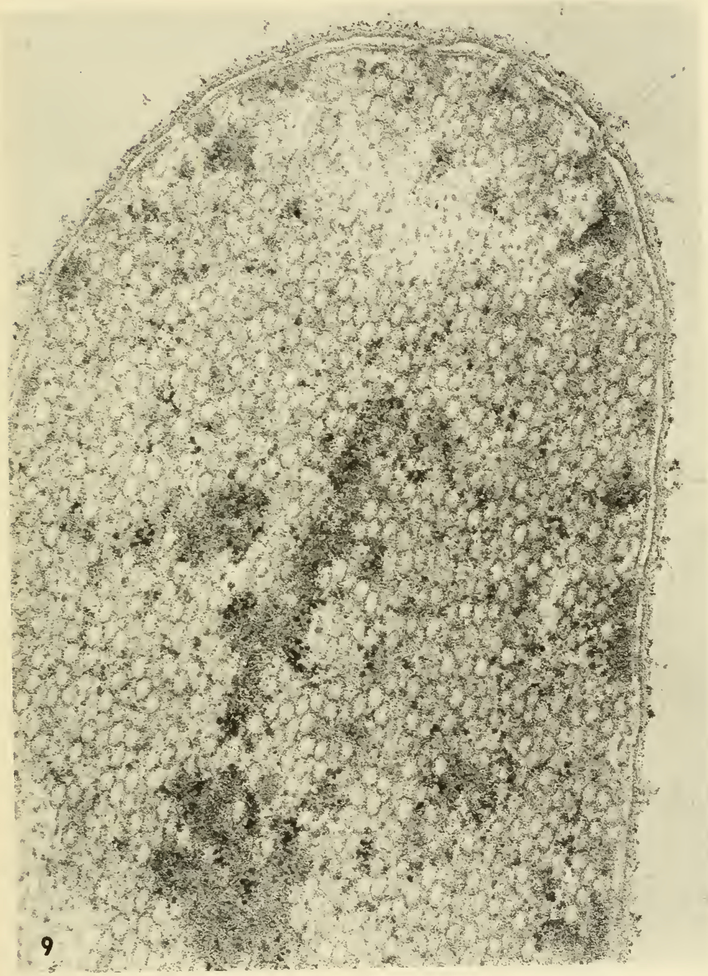

Fig. 9. Part of a longitudinal section of *Thiospirillum jenense* grown at low light intensity. As in *Chromatium okenii* (Fig. 8), the cytoplasm is almost filled with membrane-bounded vesicles. The cell wall is surrounded by a thin slime layer. Main fixation 2 hours, post stained with lead hydroxide. X 120,000.

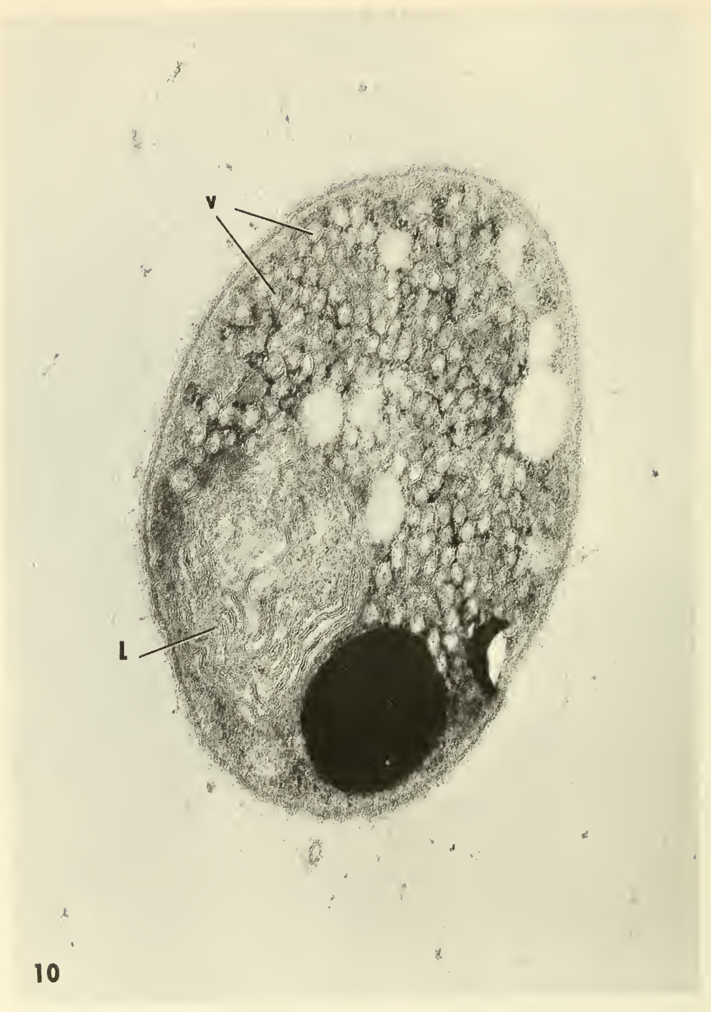

Fig. 10. Relatively thick section of a small sulfur purple bacterium, *Thiocapsa*, grown at low light intensity. The cytoplasm contains typical membrane-bounded vesicles (v), as well as a large inclusion of paired lamellae (L). The very electron-dense areas represent polymetaphosphate deposits. The clear areas are probably deposits of glycogen. Main fixation, 18 hours, post staining with lead hydroxide. X 120,000.

fur bacteria; the sections were prepared from cells grown in very dim light (<40 foot-candles) and depleted of internal sulfur deposits prior to fixation. The cytoplasmic region is in each case densely filled with vesicles bounded by unit membranes. The individual vesicles are approximately 500Å in diameter and are thus structurally indistinguishable from the vesicles of *R. rubrum* and *Rhodopseudomonas spheroides*.

A markedly different internal membrane structure occurs in the small, spherical cells of *Thiocapsa* (Fig. 10). These cells were also taken from a culture grown in very dim light. Just as in the case of the larger purple sulfur bacteria, much of the cytoplasm is filled with typical 500Å vesicles surrounded by unit membranes; however, in many sections, a large area is occupied by an extensive system of parallel paired lamellae.

THE STRUCTURE OF ISOLATED MEMBRANE FRACTIONS

The interpretation which has been offered for the structure of the photosynthetic apparatus in purple bacteria implies that the chromatophores which can be isolated from broken cells constitute fragments derived from an initially continuous membrane system, no doubt frequently contaminated with associated wall material. In the case of *R. rubrum*, the presence of numerous wall fragments in crude chromatophore preparations can be readily established by the examination of material negatively stained with phosphotungstic acid, since the wall of this species has the distinctive fine structure characteristic of the wall of *spirilla*. This probably explains the observation of Newton (23) that antisera prepared against chromatophore material are capable of agglutinating intact cells.

If one avoids methods of cell breakage that cause considerable comminution, and fractionates the extract by sucrose gradient centrifugation, it is possible to obtain from *R. rubrum*, though in small yields, membrane fractions that are essentially devoid of wall material. Sections show that the unit membrane structure is well preserved in such material (Fig. 12). The geometric form of these membrane fragments is revealed by negative staining with phosphotungstate. Fig. 11 shows our best preparation of this kind to date, made with membrane material prepared from a large *Chromatium* species provided by Dr. Pfennig. Similar preparations from *R. rubrum* give a virtually indistinguishable picture. To judge from such preparations, most of the membrane fragments have the form of cups or hemispheres, flattened to a greater or lesser extent by drying. We have thus far been unable to detect any fine structure in them.

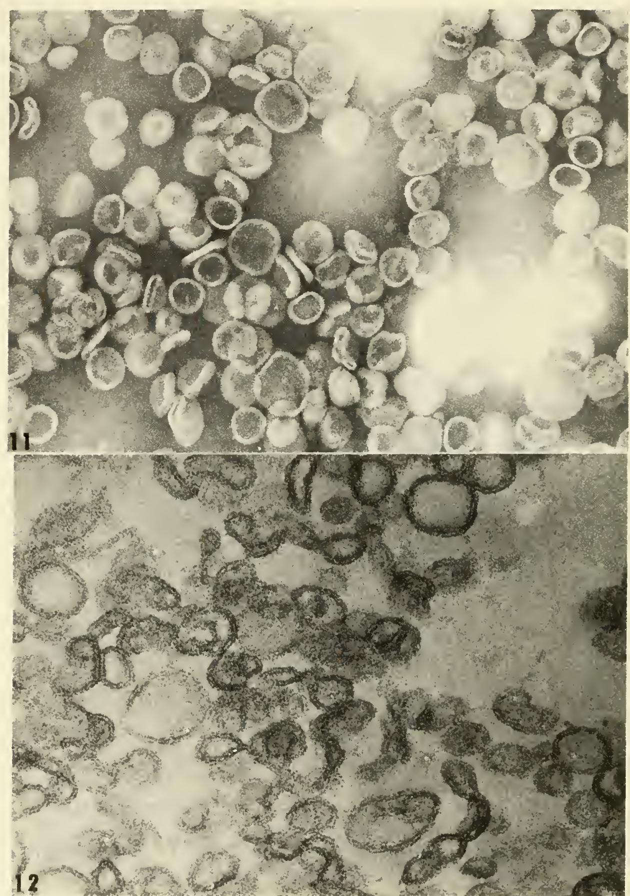

Fig. 11. Preparation of pigmented membrane fraction isolated from a large *Chromatium* (strain Tassajara) negatively stained with phosphotungstate. X 180,000.

Fig. 12. Thin section of pigmented membrane fraction isolated from *R. rubrum* grown photosynthetically at 50 foot-candles. Main fixation 2 hours, post staining with lead hydroxide. X 180,000.

THE INTERNAL ORGANIZATION OF THE *CHLOROBIUM* CELL

Fine structure studies on green bacteria have so far been very limited. Vatter and Wolfe (4) published one micrograph of a thin section of *Chlorobium limicola*. The cytoplasm contained numerous very electron-dense bodies, which the authors equated with chromatophores, despite the fact that these bodies showed no resemblances to the vesicular elements of purple bacteria. They could perhaps be more reasonably interpreted as deposits of polyphosphate. Bergeron and Fuller (24) have published a micrograph of a thin section of *Chlorobium thiosulfatophilum*, which shows, according to the authors, that the fine structure of this bacterium is essentially indistinguishable from that of an ordinary nonphotosynthetic true bacterium such as *Escherichia coli*. Bergeron and Fuller (24) also made the first detailed study of the location of the pigment system in cell-free extracts of a green bacterium. They broke the cells of *C. thiosulfatophilum* by a highly comminutive treatment: sonic oscillation of cells suspended with a fine synthetic sapphire abrasive. The bulk of the pigment system in such extracts was associated with particles about 150 Å in diameter, difficultly separable from ribosomes, and having a molecular weight of approximately 1.5 million. Since the most conspicuous cytoplasmic elements observable in their thin sections were also particles with an approximate diameter of 150 Å, Bergeron and Fuller assumed the identity of their isolated "holochrome" with these cellular elements.

We have recently collaborated with Dr. Norbert Pfennig on a study of the fine structure of five strains of *Chlorobium*. The strains included representatives of the *limicola* and *thiosulfatophilum* physiological types, and also of the two sub-groups which can be distinguished on the basis of the chlorobium chlorophyll that they contain. They are thus representative of the group as a whole.

Although our work on these organisms is still in progress, the findings to date, with respect both to the structure of the intact cell and to the properties of the pigment system in extracts, differ in major respects from the findings of Bergeron and Fuller (24). The green bacteria that we have examined all share a highly distinctive and complex fine structure, quite unlike that found in purple bacteria—or, indeed, in any other type of bacterium so far studied by modern techniques of electron microscopy. Thin sections of three different strains are shown in Figs. 13-15.

The cell wall has the double-layered structure characteristic of Gram-negative bacteria, with a thin, sharply defined inner layer, and a thicker, less dense outer layer. In some strains, it is ornamented by rod-shaped extensions about 300 Å wide, with a helically patterned surface structure (Figs. 13 and 14); the presence of these elements on the wall seems to be characteristic of strains that give a slimy type of growth in liquid media. Underlying the wall is a complex membrane

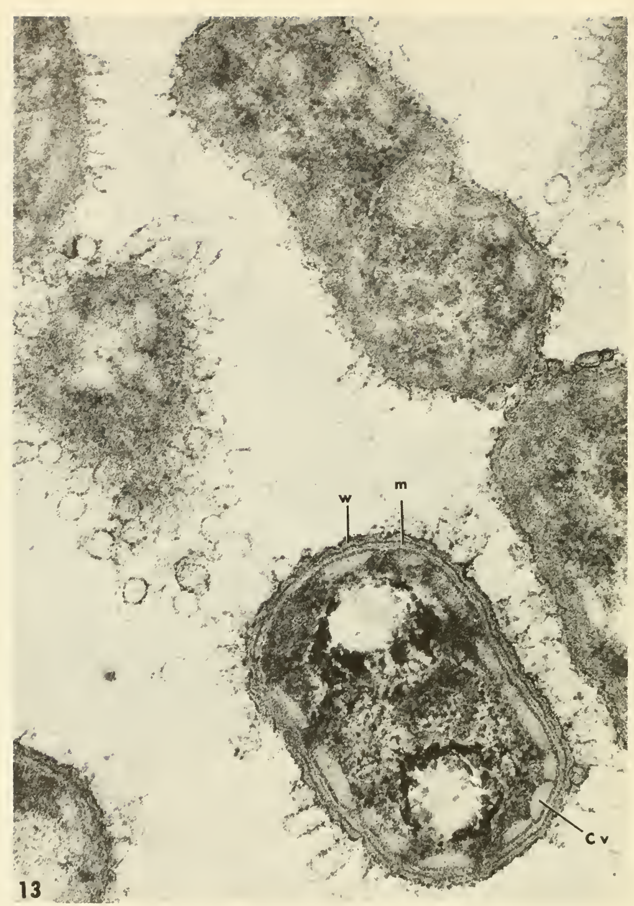

Fig. 13. Sections of *Chlorobium limicola* strain R grown at low light intensity. The median section at the lower right shows clearly the complex wall (w), with its rod-shaped extensions and the cell membrane (m) of this organism. The large oblong chlorobium vesicles (Cv) surrounded by an electron-dense membrane are clearly seen in the median section. Their general distribution through the cortex of the cell is well shown by the tangential section at the upper right. At upper left, a tangential section has passed transversely through some of the rod-shaped extensions from the cell wall. Main fixation 2 hours, post staining with lead hydroxide. X 135,000.

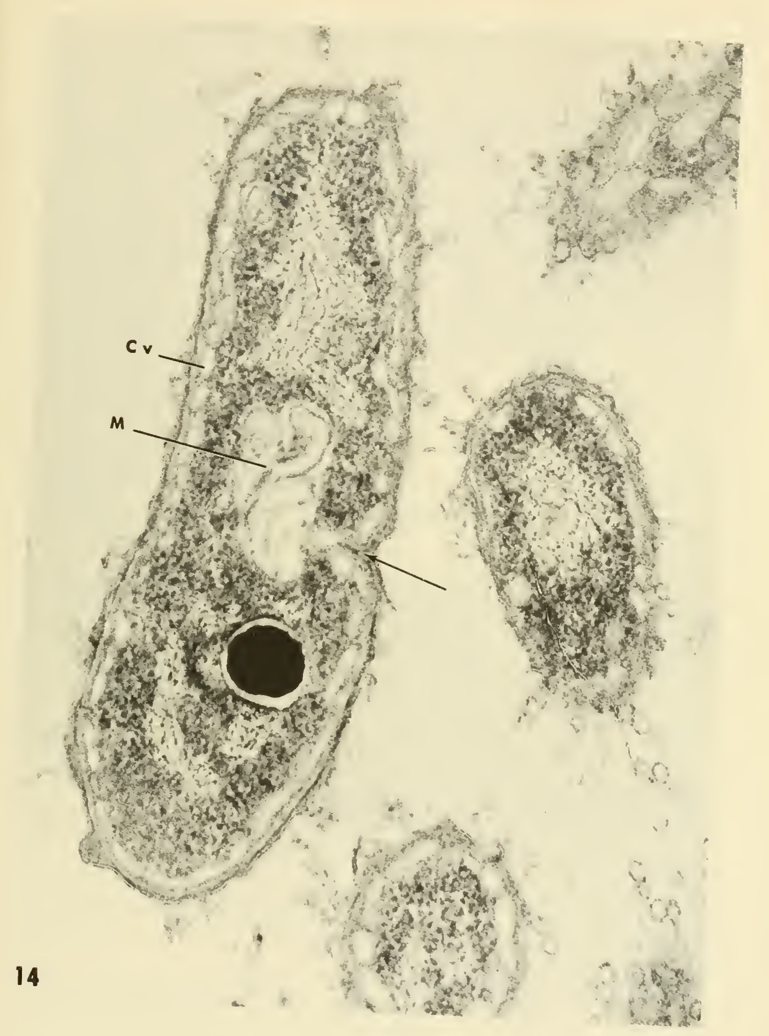

Fig. 14. Sections of *Chlorobium thiosulfatophilum*, strain Tassajara, grown at low light intensity. The general cell structure is very similar to that of *C. limicola*, strain R. A large mesosomal structure (M) connected to the cell membrane (arrow) is visible in the center of one cell. Note also the very electron-dense deposit of polymetaphosphate. Main fixation 2 hours, post staining with lead hydroxide. X 100,000.

system, the detailed structure of which is not yet fully interpretable; we have the impression that it may not be organized in the same fashion in every strain. Immediately within the inner layer of the cell wall, there are either one or two unit membranes. Parallel and adjacent to this unit membrane system is a thinner, electron-dense membrane, some 50 Å thick. This thin membrane appears to surround and enclose the series of large, clear oblong areas which line the cortex of the cytoplasm between the surface membrane system and the ribosomal region. Every strain that we have examined contains these characteristic oblong structures, the shape and disposition of which are particularly well shown in tangential sections (Figs. 13 and 14). We shall term them *chlorobium vesicles*. They are relatively large, and of somewhat variable dimensions, 1000-1500 Å long and 300-400 Å wide. In vestopal-embedded cells (Figs. 13-15), the chlorobium vesicles are transparent to the electron beam, and hence might be interpreted as empty. However, in epon-embedded cells, they appear much denser than the adjacent cytoplasm.

All the strains of green bacteria that we have examined also contain large, conspicuous, and complex membranous intrusions similar in structure and derivation to the so-called "mesosomes" found in a variety of nonphotosynthetic true bacteria. There are relatively few (1 to 3) in the cell; they commonly lie deep in the cytoplasm, sometimes intruding into the nuclear region. In favorable sections, the mesosomal membranes can be seen to connect with the inner layer of the cytoplasmic membrane (Fig. 14).

In this anatomical labyrinth, where is the photosynthetic apparatus located? We are not yet prepared to give a categorical answer to this question, and will simply describe a few observations which bear on it. The first point to be emphasized is that all the strains of green bacteria we have examined have a very high specific chlorophyll content—far higher than that of purple bacteria growing at the same light intensity. This is shown by the data in Table 2. In view of our cytological experience with purple bacteria, it seems reasonable to assume that the photosynthetic apparatus of a procaryotic organism with such a high chlorophyll content should occupy a substantial volume of the cytoplasmic region of the cell. For this reason, it is unlikely that the mesosomal elements constitute the sole, or even the major, site of the photosynthetic pigment system.

The cells of green bacteria can be readily broken in the French pressure cell, or (in the case of cultures in the stationary phase) by osmotic lysis, following treatment with lysozyme and versene. After osmotic lysis, followed by DNAse treatment to reduce the viscosity of the lysate, all the chlorophyll and carotenoid in the extract is sedimented at low gravitational fields, in association with the lysed spheroplasts. Such behavior would not be expected of a holochrome with a molecular weight of 1.5 million. In another experiment, cells were

TABLE 2.

Chlorophyll content of green bacteria grown at low light intensity (<40 foot-candles)

Strains[2]	*Chlorophyll*[1] *content of cells, μg/mg cellular protein*
Chlorobium limicola R (660)	142
Chlorobium limicola ML (650)	120
Chlorobium limicola 17 CR (650)	118
Chlorobium thiosulfatophilum, B (660)	115
Chlorobium thiosulfatophilum Tassajara (660)	190
Chlorobium thiosulfatophilum 6 CR (660)	100

1 The absorption coefficients for chlorobium chlorophylls 650 and 660 in methanol were taken from the publication of R. Y. Stanier and J. H. C. Smith, The chlorophylls of green bacteria, *Biochim. Biophys. Acta, 41,* 478 (1960).

2 The values in parentheses represent the type of chlorobium chlorophyll present in each particular strain.

broken in the French pressure cell, and after a low-speed centrifugation to remove residual intact cells and large fragments, the extract was centrifuged at 100,000 x g for 2 hours. The pigment system was completely sedimented. A very small fraction of the total pigment was contained in a loose layer overlying the pellet. After removal of this layer, the pellet was resuspended and subjected to centrifugation through a linear sucrose gradient (0.5-2.0 M) for 2 hours at 25,000 rpm. At the end of this period, most of the pigment was contained in a broad, deep-green band. This material had a considerably higher specific chlorophyll content (325 μg/mg protein) than the original cells (190 μg/mg protein). It proved to be structurally heterogeneous upon examination in the electron microscope after negative staining with phosphotungstate (Fig. 16). However, the bulk of the material consisted of vesicles 1000-1800 Å long and 500-750 Å wide. They are accordingly similar in form and dimensions to the peripheral chlorobium vesicles which are such a constant feature of thin sections of intact cells. Although this evidence certainly cannot be considered conclusive, it suggests that the chlorobium vesicles could be the major site in the cell of the photosynthetic pigment system. If this supposition is confirmed by subsequent work, the 150 Å holochrome particles isolated by Bergeron and Fuller must either be contained within the vesicles or else derived from them by comminution.

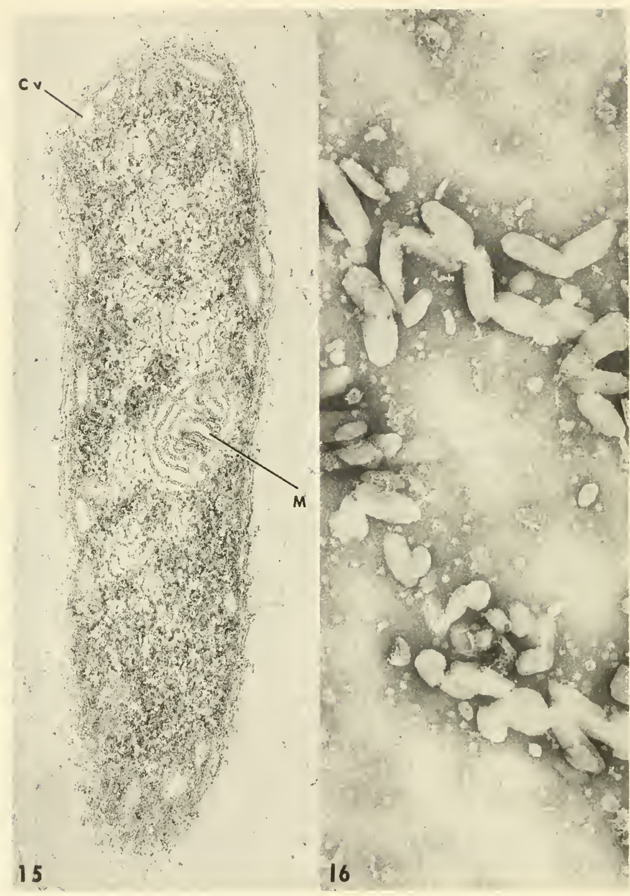

Fig. 15. Section of *Chlorobium thiosulfatophilum*, strain 6CR. The cell wall of this strain does not show the rod-shaped extensions characteristic of the strains illustrated in Figs. 12 and 13. The general structure of the cell is very similar to that of *C. thiosulfatophilum*, strain Tassajara. Chlorobium vesicles (Cv), mesosomal element (M). Main fixation, 2 hours, post staining with lead hydroxide. X 120,000.

Fig. 16. Negatively stained preparation of the main pigmented fraction isolated from extracts of *C. thiosulfatophilum*, strain Tassajara (see text).

ACKNOWLEDGMENTS

This work owes much to the generous help and advice given by Dr. J. H. McAlear and his associates on the staff of the Electron Microscope Laboratory. We are particularly indebted to Mr. Lloyd Thibodeau and Mr. Philip Spencer for their technical assistance. Much valuable help in the performance of experiments was given by Miss Riyo Kunisawa.

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ISOLATION OF PHOTOCHEMICALLY ACTIVE CHROMATOPHORES FROM *RHODOSPIRILLUM MOLISCHIANUM*[1]

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A bacteriochlorophyll-containing particulate fraction capable of carrying out light-induced phosphorylation has been isolated from cells of *Rhodospirillum molischianum*.[2] The cells from which these particles are derived contain lamellar structures in the cytoplasm (Fig. 1) showing the same characteristics as those described by Drews (1) and by Giesbrecht and Drews (2). When such cells are subjected to sonic disintegration followed by fractionation in the ultracentrifuge according to the procedures described earlier for *R. rubrum* (3), a fraction is obtained which contains well-defined particles as indicated in Fig. 2 A. The chromatophores of *R. molischianum*, when compared with those isolated from *R. rubrum* (Fig. 2 B), usually are of greater diameter and also reveal more surface detail in phosphotungstate-treated preparations. Judging from the appearance of sectioned cells, the chromatophores of *R. molischianum* are disc shaped, while those of *R. rubrum* appear to be nearly spherical. Giesbrecht and Drews (2) have described chromatophores in *R. molischianum* as consisting of stacks of 5-15 lamellae. The chromatophores described here (Fig. 2 A) appear to be subunits of such stacks.

Rates of light-induced phosphorylation observed with isolated chromatophores from *R. molischianum* (150-200 μmoles orthophosphate esterified as ATP per hour per μmole bacteriochlorophyll) are comparable to those reported by us for *R. rubrum* (3). Thus far we have not been able to elicit photoreduction of pyridine nucleotides by these particles.

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[1] This study was supported by grants E-2218 and E-3989 from the National Institutes of Health.

[2] We wish to thank Dr. C. B. van Niel for the culture of *Rhodospirillum molischianum*.

[3] Holder of Graduate Fellowship from the Charles F. Kettering Foundation.

Fig. 1. Section of *Rhodospirillum molischianum* from a 3-day-old anaerobic light grown culture.

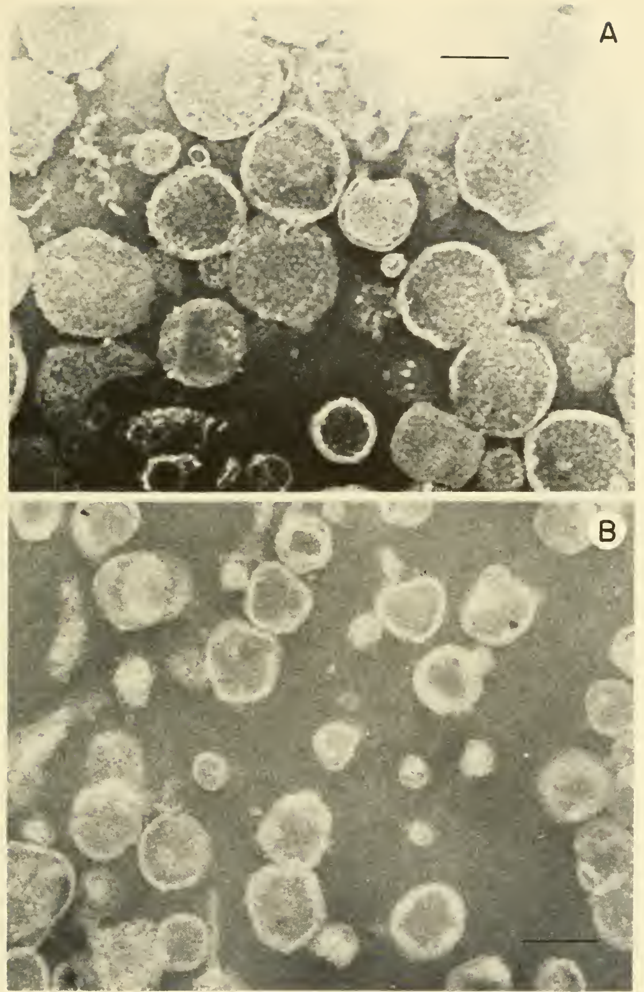

Fig. 2. Isolated chromatophores. Negative staining with neutral phosphotungstate. A. From cells of 3-day-old culture of *Rhodospirillum molischianum* comparable to those in Fig. 1. B. From cells of 2-day-old culture of *Rhodospirillum rubrum* grown anaerobically in the light. Length of black bar represents 0.1μ.

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ISOLATION OF BACTERIOPHEOPHYTIN-CONTAINING PARTICLES FROM *RHODOSPIRILLUM RUBRUM*[1]

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The existence of two or three red peaks in the absorption spectra of members of the Athiorhodaceae and Thiorhodaceae has given rise to much speculation as to their identity. A partial fractionation of detergent-treated chromatophores has been achieved by Bril (1); the resulting material, however, was unstable and was not further identified.

When two-day-old, actively growing cultures of *R. rubrum* are extracted with distilled water or with phosphate buffer (pH 7.0) at 5°C, dilute colloidal extracts are obtained whose ratios of extinction at 800 mμ to that at 880 mμ are relatively high as compared with the absorption characteristics of whole cells or of isolated chromatophores. By differential centrifugation it is possible to obtain a preparation which, in the red portion of the spectrum, shows only one peak at 794 mμ and none at 880 mμ (Fig. 1). This material can be sedimented by centrifuging at 60,000 x g for 1 hour and is free of cytochrome-550. The spectrum of the resuspended B_{794} fraction (Fig. 1) also shows a pronounced peak at 364 mμ, but little if any carotenoid absorption.

When this material is extracted with methanol and the pigment transferred to ethyl ether, it becomes apparent that the spectrum of the extract corresponds to that of bacteriopheophytin obtained through the acidification of chromatographically purified bacteriochlorophyll. This spectrum also is in agreement with published spectra (Fig. 2, 3) of bacteriopheophytin (2). It does not appear likely that the B_{794} material represents a decomposition product as it can be obtained most abundantly from cultures of rapidly growing cells and is more difficult to obtain in any quantity from older cultures. The origin and possible role of these particles remains to be determined.

Methanol extracts of intact cells or of isolated chromatophores from *R. rubrum*, when prepared in the presence of $CaCO_3$ and separated chromatographically, also yield a trace of bacteriopheophytin. We do not

[1] A preliminary communication. This investigation has been supported by grants from the National Institutes of Allergy and Infectious Diseases (E-2218) and the U. S. National Science Foundation (G-9888).

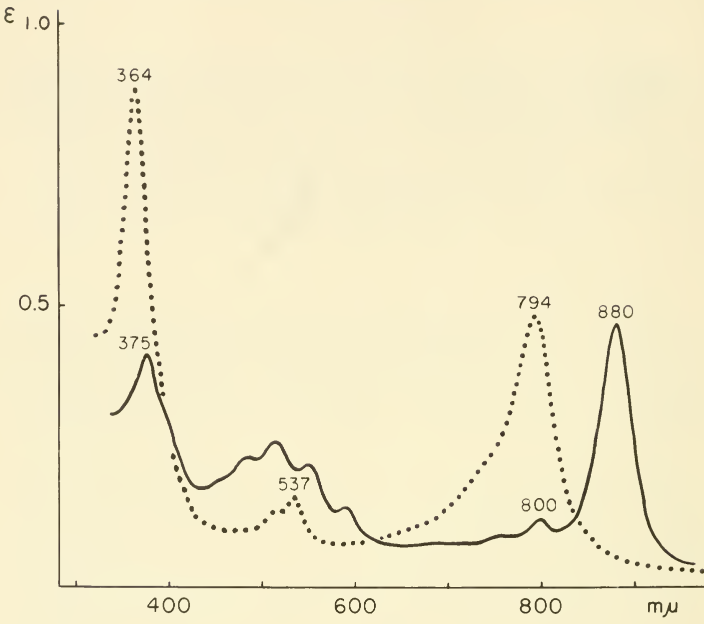

Fig. 1. ——————Absorption spectrum of "normal", washed chromatophores from *R. rubrum* in 0.1 M K glycylglycine pH 7.7.
. . . . Absorption spectrum of B_{794} particles (obtained from 2-day-old cultures) after removal of 880 mμ absorbing material by differential centrifugation; in 0.1 M K glycylglycine pH 7.7.

believe this to be an extraction artifact since bacteriopheophytin does not form readily from bacteriochlorophyll even in the presence of dilute acids, and the isolation procedures have been carried out under conditions which should prevent the accumulation of acids in the extraction mixtures.

The cause for the discrepancy in the position of the *in situ* absorption peaks near 800 mμ in the two types of particles may be ascribed to the presence or absence of the B_{880} bacteriochlorophyll. The B_{880} material has a higher extinction at 800 mμ than at 794 mμ; thus, in the presence of excess B_{880} the absorption peak of the presumed bacteriopheophytin would tend to be shifted toward longer wavelengths (*i.e.*, from 794 mμ toward 800 mμ). A more quantitative evaluation of this spectral shift will be attempted.

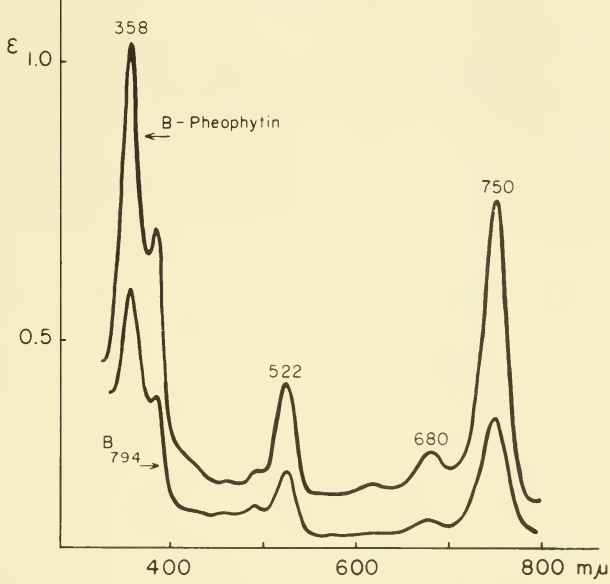

Fig. 2. Upper curve: Absorption spectrum of bacteriopheophytin prepared from purified bacteriochlorophyll by acidification with aqueous acetic acid in acetone. Resulting bacteriopheophytin extracted with ethyl ether. Spectrum measured in dry ethyl ether after removal of acetic acid and acetone.
Lower curve: Spectrum of B_{794} extract in dry ethyl ether.

An additional observation points to the possible identity of the material giving rise to the 800 mμ peak in "normal" chromatophores and the 794 mμ peak in the "abnormal" particles: the treatment of both types of preparations with dilute KOH (pH 10) leads to a shift of the 800 mμ peak or of the 794 mμ peak to 754 mμ; the position of the latter peak is identical in the two preparations.

Work is in progress to establish a difference spectrum between "normal" chromatophores and B_{794} particles in an effort to obtain an approximation of the *in situ* absorption spectrum of B_{880} alone. Also, we hope to be able to separate particles containing only the 880 mμ peak and thereby obtain the *in situ* spectrum of B_{880} directly.

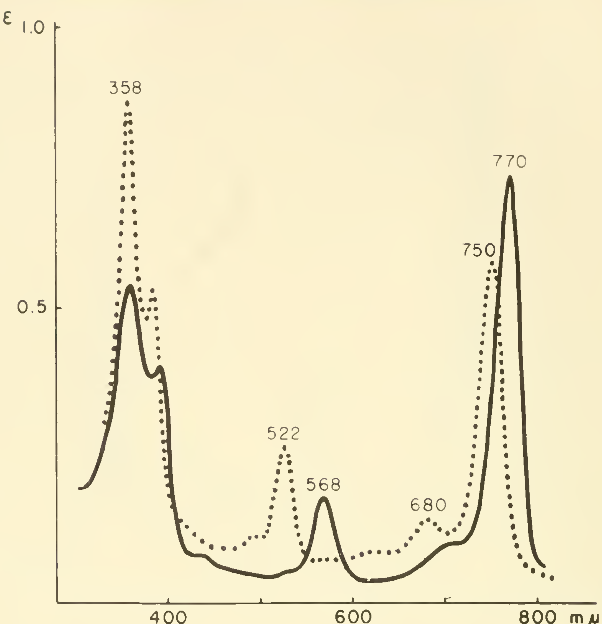

Fig. 3.——————Absorption spectrum of bacteriochlorophyll in dry ethyl ether.
. . . . Absorption spectrum of bacteriopheophytin in dry ethyl ether.

We have tested the B_{794} particles for their capacity to photoreduce pyridine nucleotides but with negative results thus far. It would, however, be most surprising if these particles did show any capacity to carry out partial photosynthetic reactions in the absence of true bacteriochlorophyll.

CONCLUSIONS

A particulate material which has only one red absorption peak at 794 mμ has been isolated from actively growing cultures of *R. rubrum*. The pigment responsible for the absorption peak has been characterized as bacteriopheophytin on the basis of its spectral characteristics.

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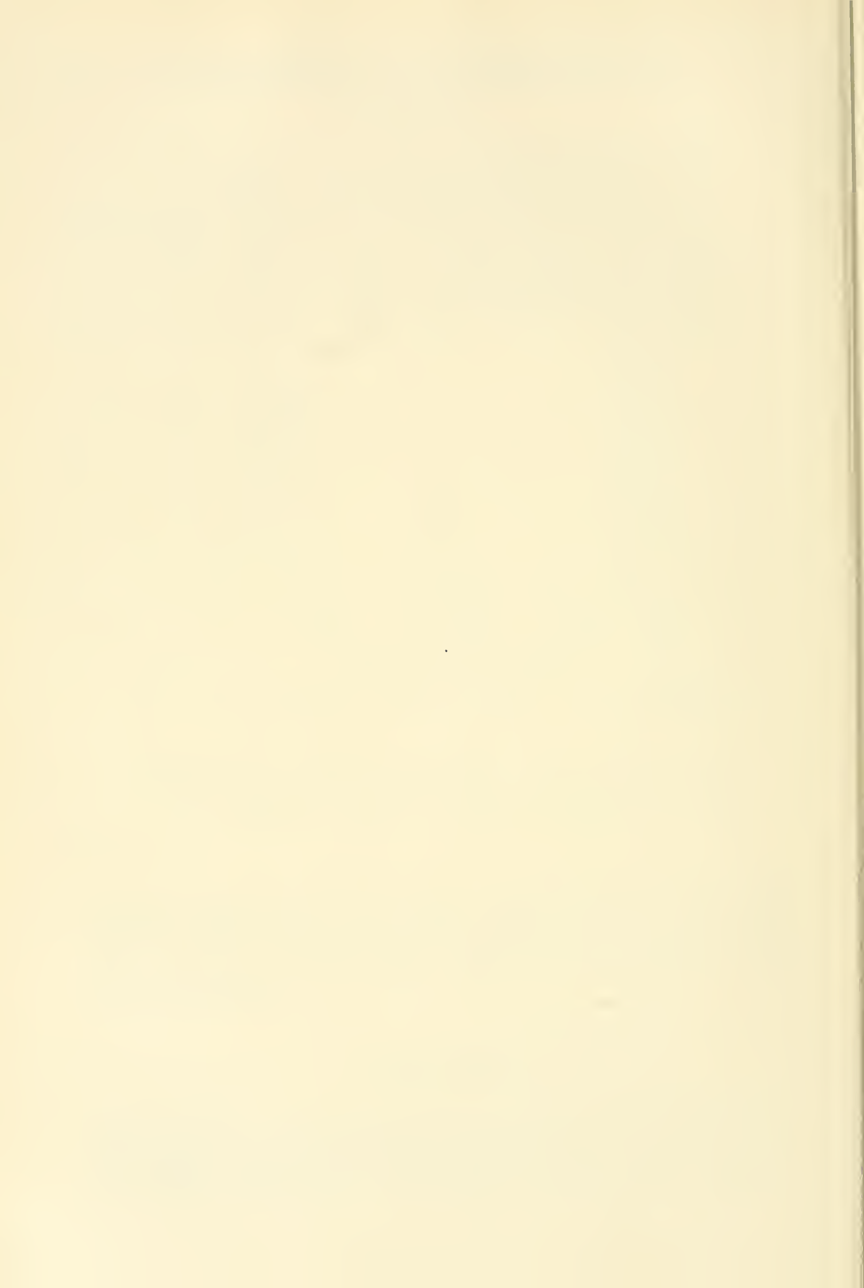

STRUCTURE AND FUNCTION IN BACTERIAL PHOTOSYNTHESIS[1]

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In the concluding section of his recent monograph on *Synthesis and Organisation in the Bacterial Cell*, E. F. Gale (1) comments: "The title should also include 'disorganisation,' as most of the results discussed in these pages have been concerned with the consequences of disorganisation—frequently on so drastic a scale that it is a miracle that anything of significance survives."

During the past decade, our understanding of bacterial photosynthesis has been greatly increased through the study of biochemical activities which survive the violent cell-disruption procedures commonly used (sonic oscillation, grinding with alumina, French pressure cell). In fact, the discovery (2) that pigmented subcellular particles ("chromatophores") from *R. rubrum* and similar bacteria catalyze an anaerobic light-dependent phosphorylation of ADP has led to a profound revision of views on the fundamental features of the overall bacterial process. On the other hand, reflection on the recent history of analysis of other multi-component processes of comparable complexity (*e. g.*, mitochondrial electron transfer and oxidative phosphorylation) suggests that it would indeed be remarkable if particles obtained by the methods noted retained all of the biochemical capacities characteristic of the photochemical "apparatus" in its native state.

The foregoing considerations provided the stimulus for earlier studies (3,4) on isolation of the particulate photochemical system by milder procedures. In particular, osmotic lysis of protoplasts appeared to represent a potentially ideal approach. The initial experiments (3) surprisingly revealed that upon osmotic lysis of *R. rubrum* protoplasts[2] (prepared by treatment with lysozyme + EDTA) the pigment system was not released but, rather, was retained in the membranous "ghost" structures. In sharp contrast with particles obtained by drastic methods of cell breakage, the pigmented "ghosts" are sedimentable

[1] Supported by grants from the U. S. Public Health Service (E-2640) and the National Science Foundation (G-9877).

[2] This term is used with the understanding that the cell-forms referred to are not entirely analogous to the protoplasts of Gram-positive bacteria.

at low centrifugal force. As would be expected, washed "ghost" preparations photophosphorylate ADP when supplemented with appropriate cofactors (4).

Pigmented membranous structures which catalyze photophosphorylation can also be obtained by disrupting cells of *R. rubrum* through the sequential action of lysozyme and polymyxin B, in the absence of an osmotic stabilizer (4). Polymyxin B is believed to react with lipid components of the cytoplasmic membrane of sensitive Gram-negative bacteria, causing disorganization of the membrane and an attendant loss of specific permeability properties (5). It is significant that the structures produced by the action of lysozyme + polymyxin B on *R. rubrum* cells do not contain the large opaque granules characteristically seen in this organism when it is grown in certain media. This observation reinforced the conclusion (4) that retention of the pigment system in the "ghosts" was not due to physical entrapment of "chromatophores" by a limiting (damaged) membrane. "Chromatophores," however, are readily released when the relatively fragile protoplasts or "ghosts" are further disintegrated, *e.g.*, by shaking with Ballotini beads in the Mickle apparatus or by exposure to surface-active agents such as sodium lauryl sulfate (4).

The results briefly summarized above were the first indications[3] that the system of pigments and electron carriers responsible for photochemical generation of ATP in *R. rubrum* is normally integrated with the cytoplasmic membrane or intracytoplasmic extensions of the membrane. This conception (3,4) received additional support from a cytological investigation by Giesbrecht and Drews (7) with another species of *Rhodospirillum (molischianum);* their electron micrographs indicate that "chromatophores" arise by invagination of the cytoplasmic membrane and may, in fact, remain attached to the latter by tubular stalks. A recent study (8) with *R. rubrum* has provided similar cytological evidence for an association of the photochemical apparatus with the cytoplasmic membrane.

It is well known that the properties of "particulate" enzymes can be greatly influenced by the state of the structure with which they are combined. Accordingly, in a photosynthetic bacterium such as *R. rubrum,* disengagement of the pigment system and its associated catalysts from the complex *in vivo* matrix might be expected to result in significant alteration—or even total loss—of certain biochemical properties (4). It appears that the normal redox balances of electron

[3] Immunochemical studies conducted with *Chromatium* at about the same time by J. W. Newton (6) led him to conclude that "the photosynthetic apparatus of purple bacteria is not necessarily a unique, discrete intracellular entity, since it contains macromolecular configurations common to the cell surface on one hand and certain 'intracellular' proteins on the other. It seems advisable, therefore, provisionally to consider it a part of a more complex organizational state within the cell."

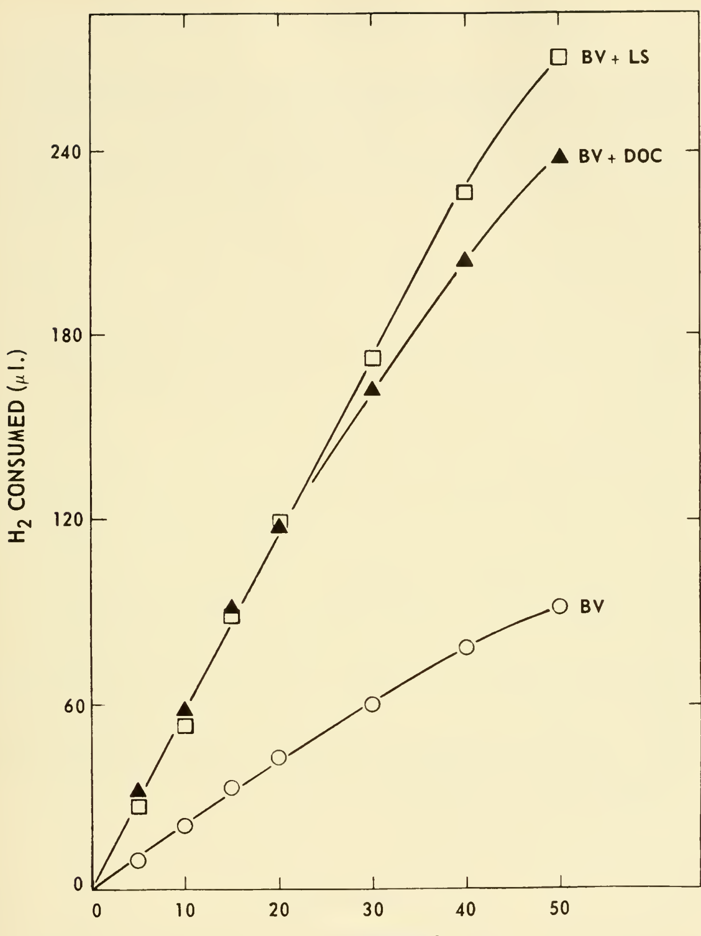

Fig. 1. Effect of lipid-dispersing agents on hydrogenase activity of *R. rubrum* particles.

Washed *R. rubrum* cells, grown in a malate + glutamate medium, were suspended in 0.05 M potassium phosphate buffer pH 7.1 and sonicated (Raytheon 10 KC) under hydrogen for 3 minutes. Pigmented particles were collected by centrifugation at 144,000 x g for 60 minutes and resuspended in 0.05 M phosphate buffer pH 7.6. The Warburg vessels contained, in a final fluid volume of 2.0 ml: particles equivalent to 0.75 mg bacteriochlorophyll; potassium phosphate pH 7.6, 275 μmoles; benzyl viologen (BV), 100 μmoles (added at zero time); where indicated, 10 mg sodium deoxycholate (DOC) or 10 mg sodium lauryl sulfate (LS). Gas phase, H_2 (KOH in center well); temperature 30°C.

transfer carriers involved in photophosphorylation are significantly disturbed by disruption of the native structure. Thus, optimal phosphorylation by isolated pigmented particles is observed only if the overall redox potential is experimentally adjusted to a favorable region by addition of suitable reductants or "redox buffers" (9,10).

The process of photochemical H_2 evolution apparently is even more dependent on maintenance of *in vivo* structural integrity. Despite many attempts, this reaction has not yet been definitively demonstrated in a cell-free system using components exclusively derived from active cells of photosynthetic bacteria. In this connection, it is noteworthy that conversion of intact cells of *R. rubrum* to protoplasts using the lysozyme technique causes almost complete loss of H_2-evolution activity (3). This could evidently be due to a direct inhibitory effect of lysozyme or to loss of a required enzyme, or cofactor, from the protoplasts. It is also conceivable that a relatively minor disturbance of a delicate organizational state may be responsible for the disappearance of activity.

Although pigmented particles from *R. rubrum* catalyze light-dependent phosphorylation and certain oxidation-reduction reactions, it seems possible that the properties of such particulate fragments may be substantially modified by spontaneous architectural changes which occur during their release from the cell. For example, structural lipids or lipoproteins organized in some sort of membranous fabric may envelop, or partially overlay, normally accessible enzymatic components. This possibility is suggested by the effects of deoxycholate and other surface active agents on hydrogenase activities of pigmented particle preparations. As shown in Fig. 1, lipid-dispersing agents can cause marked acceleration of hydrogenase activity with an artificial electron acceptor (activity with ferricyanide is also stimulated). Similarly, hydrogenase activity of *R. rubrum* particles with FMN or FAD is completely dependent on the presence of deoxycholate or sodium lauryl sulfate (11).

CONCLUSIONS

The data now available from experiments with photosynthetic bacteria and previous experience with other complex particulate systems indicate the desirability of exploring the photo- and biochemistry of bacterial photosynthesis in subcellular preparations produced by milder methods of cell rupture than are usually employed. Ideally, one could hope to develop procedures which preserve the native surface structures (cell "envelope" or "hull") and intracytoplasmic membranes essentially intact but which effectively eliminate the perme-

ability barriers of the cell.[4] Studies with such preparations possibly will provide evidence that the cellular organization of photosynthetic bacteria resembles that of other types of cells in the sense that ". . . electron transport-coupled generation of ATP and ATP-dependent energy and ion transport may be specialized manifestations of a common enzymic function, inherent in cellular membrane structures. . ." (15).

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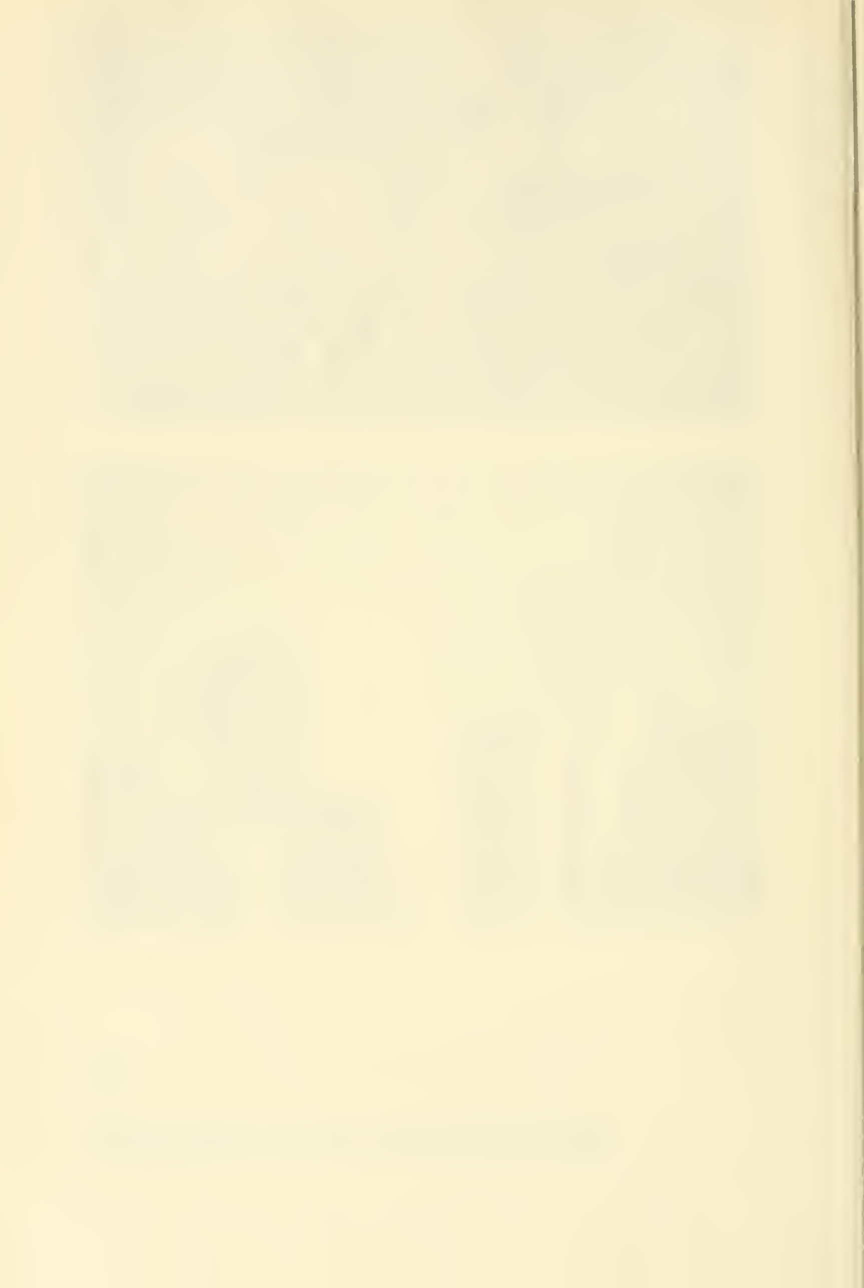
4 Possible approaches are described in refs. 12-14.

Top: W. Wiesner, D. I. Arnon; *bottom:* B. F. Burnham, J. Lascelles.

II

METABOLISM and PHYSIOLOGY



METABOLIC ASPECTS OF BACTERIAL PHOTOSYNTHESIS[1]

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The overall metabolism of photosynthetic bacteria is clearly distinguished from that of green plants in at least two particular respects (Table 1). These properties have been known for many decades and much effort has been expended in obtaining evidence for hypotheses designed to reconcile the "apparent" differences in bacterial and green plant photosynthesis. With increasing awareness of comparative biochemical correlations, it became natural to suppose that the light-

TABLE 1

Distinctive features of autotrophic metabolism in photosynthetic bacteria and green plants

	Photosynthetic bacteria	Green plants
Requirement for an "accessory" hydrogen (electron) donor	+	−
Production of oxygen	−	+

dependent metabolism in all photosynthetic organisms must be "basically similar." Thus, the discordant properties of the bacteria as compared with higher plants could be viewed as the result of relatively minor variations on a basic theme (1,2).

Since the time of Pasteur, the power of comparative biochemistry in rationalizing common principles in the framework of metabolism has been amply demonstrated. It is undoubtedly safe to say that at present the comparative biochemical approach is so ingrained that it has become a stock-in-trade aspect of our expanding methodology for

[1] Research of the author is supported by grants from the National Science Foundation (G-9877) and the U. S. Public Health Service (E-2640).

exploring metabolic phenomena. This is illustrated by the fact that we routinely mix subcellular components isolated from very different organisms in order to reconstruct certain complex metabolic processes; at least, to serve as models. At the same time, a number of investigators working in different fields of biology have expressed disquieting attitudes on the general aims of comparative biochemistry and have also criticized current tendencies in its usage. This has resulted in some debate as to whether comparative biochemistry should *now* be concerned primarily with the study of common chemical principles shown by different forms of life, or with the origin and nature of biochemical variability from patterns common to many organisms. It can be argued that this difference in viewpoint represents a trivial philosophic confusion concerning two sides of the same coin, but I am inclined to believe that the debate is ultimately inspired by important questions which have not as yet received the scrutiny they deserve.

One can legitimately ask whether undue emphasis on a particular apparent correlation may not have the effect of obscuring (or delaying the recognition of) a more profound relationship or a significant instance of biochemical "disunity." This possibility has led Ernest Bueding (3) to caution that excessive attention to biochemical unity, at the expense of adequate consideration of biochemical diversity, may lead to a distorted picture. In a very perceptive recent essay (4) relating to this topic, Seymour Cohen concludes that "the notion of the 'unity of biochemistry' has been advanced in an overly simplified form and reflects a primitive stage in the development of the discipline." Furthermore, he cites the area of photosynthesis as providing an example "in which the predilection for simplicity has impeded the development of understanding."

Research advances during the past decade or so have sharpened the focus on the comparative biochemistry of photosynthesis considerably, but it is my opinion that a really satisfactory conception has not yet been achieved. The remainder of this paper is concerned with general metabolic properties of photosynthetic bacteria and with the discussion of several questions which are of particular significance for reevaluation of the comparative problem.

Carbon metabolism and its ramifications

From the standpoint of carbon nutrition, photosynthetic bacteria can grow anaerobically under two markedly different sets of conditions (2,5). On the one hand, CO_2 can serve as the sole or primary source of carbon, provided an inorganic hydrogen donor is present. On the other hand, many types can grow luxuriantly in a completely synthetic medium containing a single organic compound, such as malate, in place of CO_2 and the inorganic "accessory" hydrogen donor. Certain

photosynthetic bacteria (e.g., *Chromatium*) can grow under both sets of circumstances (see Table 2).

The term "autoheterotrophic" (6) is suggested as a convenience to designate the growth pattern when an organic compound constitutes the carbon source, in cognizance of an autotrophic mechanism for the energy supply and a heterotrophic carbon metabolism. Fixation of CO_2, liberated during the metabolic conversions of added organic compounds, also occurs during autoheterotrophic metabolism, but with most substrates the extent of this process is unknown and undoubtedly varies considerably depending on the nutritional conditions. In this connection, it is noteworthy that certain anaerobic heterotrophs require surprisingly large quantities of CO_2 for optimal growth (9,10). An example is given by the myxobacterium *Cytophaga succinicans*, whose CO_2 requirement has been shown (11) to be related to the energy metabolism of the organism. In this instance, CO_2 is essential in sub-

TABLE 2

Sources of carbon, reducing power, and energy for anaerobic growth of photosynthetic bacteria

	Mode of growth: "autotrophic"	"autoheterotrophic"
Carbon:	CO_2	organic compounds
Reducing power:	H_2, $S^{=}$ *etc.*	organic compounds
Energy:	light	light

strate amounts because it is a precursor of oxaloacetate (through condensation with phosphoenolpyruvate), which in turn functions as a major oxidant for $NADH_2$ generated during the fermentative breakdown of glucose. Accordingly, the CO_2 is eventually converted to the carboxyl group of succinate and in essence has served as an electron acceptor.

It seems quite possible (12,13) that CO_2 may be used, to some extent, in a similar way during autoheterotrophic metabolism of photosynthetic bacteria (i.e., simply as an "accessory electron acceptor"). In fact, some thirty years ago F. M. Muller (14) concluded that this probably was the case during growth of purple sulfur bacteria on organic substrates such as succinate, acetate, and butyrate. Muller also appreciated the autoheterotrophic character of the purple bacteria, which is clearly evident from the following statements made in his well-known paper published in 1933:

> In the meantime it has to be kept in mind that the experiments referred to above yielded another fact of primary importance, *viz*. that also in this heterotrophic metabolism the cooperation of radiant energy is an essential factor. We must conclude from this fact that photochemical processes play a part in the conversion of the organic substrates, which is a most remarkable phenomenon. Besides the purple sulphur bacteria and the *Athiorhodaceae*, no heterotrophic organisms are known in whose metabolism radiant energy plays such an important part.

The potential significance of heterotrophic carbon metabolism in bacterial photosynthesis was also recognized by others (e.g., see 15, 16,17), leading to the suggestion (16,18) that CO_2 fixation may be bypassed or suppressed during photometabolism of certain organic substrates. This point of view, however, was not generally considered or discussed for some time, apparently because of preoccupation with the concept that CO_2 reduction was a central feature of all bacterial photosyntheses. Combined autotrophy and heterotrophy in the sense used here is not confined to photosynthetic bacteria and may actually be more widespread in nature than is commonly supposed. An interesting example is found in the organism *Desulfovibrio desulfuricans* (Hildenborough strain). Mechalas and Rittenberg (19) have shown quite conclusively that this bacterium can use the "autotrophic" oxidation of H_2 with sulfate as the energy source for "heterotrophic" growth on assimilable organic compounds. They suggest that other "heterotrophs" which contain hydrogenase, such as *Escherichia coli*, *Azotobacter*, etc., may also be capable of using "autotrophic" oxidation of H_2 (e.g., with O_2) as an energy supply for "heterotrophic synthesis."

There has been considerable discussion in the literature on the fundamental differences between autotrophs and heterotrophs, and it is becoming increasingly difficult to make satisfactory definitions. Since the discovery of CO_2 fixation in heterotrophs, many other distinctions which were once thought to be decisive have diminished in significance. In addition, the list of facultative autotrophs and organisms in the "grey zone" between the extremes constantly increases in length. This is really another way of saying that we are gradually seeing an enlarged spectrum of similarities and this, in itself, poses problems for comparative biochemical interpretations. It is interesting that this development was clearly anticipated by B. C. J. G. Knight in his early classic monograph (20) on bacterial nutrition. Knight not only emphasized the arbitrariness of the classification of organisms into the two categories of autotrophic and heterotrophic, but also held the view that this separation had "serious disadvantages from the point of view of the use of comparative physiology as a guide to further investigation."

It was noted earlier that certain photosynthetic bacteria (e.g., *Chromatium* and *R. rubrum*) can grow using either CO_2 or a single organic compound as the sole, or primary, carbon source. There is

little doubt that there must be numerous qualitative and quantitative metabolic differences between cells (of the same strain) growing under the two sets of conditions. First of all, we can expect that different pathways of carbon metabolism will predominate. The types of changes that might be anticipated are illustrated by the studies of H. L. Kornberg and his colleagues (21) with the facultative chemosynthetic autotroph *Micrococcus denitrificans*. Their results indicate that when the micrococcus grows autotrophically the reductive pentose cycle operates as a primary mechanism. On the other hand, cells cultivated as heterotrophs on acetate do not contain significant quantities of ribulose diphosphate carboxylase or other enzymes of the cycle for which tests were made. In place of the pentose cycle, cells growing on acetate apparently utilize the glyoxylate cycle as an important means of obtaining carbon skeletons for biosynthesis of cellular constituents. One of the key enzymes of the glyoxylate cycle is isocitratase, and this enzyme is found in high concentration in acetate-grown cells of *M. denitrificans* and other organisms which use this particular cycle. Formation of isocitratase in such bacteria is ordinarily greatly suppressed when the carbon source is a C_4 dicarboxylic acid, and this effect is believed to involve repression or related mechanisms of regulatory control.

We can confidently predict that in the photosynthetic bacteria the transition from autotrophic to autoheterotrophic growth, or *vice versa*, will also evoke a number of enzymatic changes governed by repression, derepression, or induction mechanisms. Although the data available are relatively limited, it appears that appreciably different patterns of carbon metabolism are found in different photosynthetic bacteria and, consequently, alteration of the carbon source used for growth leads to varied responses. Autotrophically grown cells of *Chromatium* contain a high level of ribulose diphosphate carboxylase, but the isocitratase content is low (22). Growth on acetate is characterized by a striking increase in isocitratase and a significant decrease in the carboxylase level. It seems that when acetate is the carbon source for autoheterotrophic growth a modified type of glyoxylate cycle becomes a prominent pathway in *Chromatium* (22). The nonsulfur purple bacteria *Rhodopseudomonas palustris* and *Rhodopseudomonas capsulatus* resemble *Chromatium* (and *M. denitrificans*) in that they also contain isocitratase in large amount when grown on acetate (23). On the other hand, only traces of this enzyme are found in acetate-grown cells of *Rhodopseudomonas spheroides* and *R. rubrum* (23); in these particular organisms, the glyoxylate cycle evidently does not function to a quantitatively significant extent.

The general picture emerging from studies on carbon pathways is that the mechanisms used by photosynthetic bacteria may vary significantly, depending on the nutritional conditions, and may range from

predominance of one particular sequence to a balanced mixture of alternative pathways. These appear to include, in whole or in part, most of the major pathways now recognized, such as the reductive pentose cycle, the citric acid cycle (13,24), and the glyoxylate cycle. It is relevant to note that the reductive pentose cycle is only one of several mechanisms by which photosynthetic bacteria can reduce substantial quantities of CO_2 to cell material. For example, there is some evidence (25,26) that, in *Rhodospirillum*, CO_2 may condense with a C_2 fragment to form a C_3 compound such as pyruvate, and it is possible that under certain conditions this type of reaction is of importance in net synthesis (see also ref. 27). Another type of CO_2 assimilation is encountered in the metabolism of *Rhodopseudomonas gelatinosa*. Acetone serves as a carbon source for photosynthetic growth of this organism, but only if CO_2 is also provided. Siegel's studies (28-32) on this system suggested the following sequence of carbon conversions:

$$CO_2 + \text{acetone} \longrightarrow \text{acetoacetate} \longrightarrow 2 \text{ acetate} \longrightarrow \text{cell materials}$$

The initial step is an endergonic carboxylation and Siegel's investigations were particularly instructive in that they clearly indicated the required energy can be supplied alternatively by: light-induced phosphorylation, dark oxidative phosphorylation, or substrate-level phosphorylation coupled with the dark anaerobic fermentation of added acetoacetate.

An important question that arises in connection with enzymatic alterations caused by variation of the carbon source is whether such changes will be primarily limited to enzymes specifically concerned with carbon transformations. It seems very unlikely that this would be the case, since the carbon conversions which occur during growth are obviously intermeshed or connected in some way with a multitude of other types of reactions. These interconnections provide a potential basis for an amplification of alterations, which may eventually attain relatively major proportions.

In growing cells, particularly close relationships exist between carbon and nitrogen metabolism and, in the purple bacteria, the nature of the nitrogen growth source exerts a profound influence on the ultimate fate of organic compounds supplied in the environment. This has been studied particularly in *R. rubrum*, but the available data suggest that many other photosynthetic bacteria behave similarly. Let us compare gross metabolic events in cultures of *R. rubrum* growing anaerobically in the light on malate, with either an ammonium salt or an amino acid such as glutamate as the nitrogen source. With the ammonium salt, the bacterium grows rapidly and, considering that it is an anaerobe, the cell yield is remarkably high (33); aside from CO_2, metabolic byproducts are not found in the medium in appreciable

quantity. With glutamate serving as the nitrogen source, the growth rate is somewhat reduced and an additional metabolic product is now observed, *viz.*, molecular hydrogen (34). The source of this hydrogen and the mechanism of its formation are problems of great interest because of their relationships with the energy metabolism of photosynthetic bacteria.

Hydrogen production by *R. rubrum* is dependent on light and, except for cells which contain readily expendable endogenous reserves, on the addition of oxidizable organic substrates, e.g., citric acid cycle intermediates (16,34). Maximal yields of gas are observed with truly resting cells and the quantities produced closely approximate those predicted on the basis of complete conversion of the organic compound to H_2 and CO_2 (24), i.e.:

Acetate:

$$C_2H_4O_2 + 2H_2O \longrightarrow 2CO_2 + 4H_2$$

Succinate:

$$C_4H_6O_4 + 4H_2O \longrightarrow 4CO_2 + 7H_2$$

Fumarate:

$$C_4H_4O_4 + 4H_2O \longrightarrow 4CO_2 + 6H_2$$

L-Malate:

$$C_4H_6O_5 + 3H_2O \longrightarrow 4CO_2 + 6H_2$$

Studies with intact cells have led to the conclusion that these remarkable conversions occur through the reactions of the well-known citric acid cycle coupled with an additional light-dependent process which, either directly or indirectly, effects the oxidation of reduced pyridine nucleotide by liberation of H_2 (13,24). If certain utilizable nitrogen sources such as N_2 or NH_4^+ are added to cells metabolizing in this fashion, the evolution of H_2 is completely inhibited (34) and the quantity of CO_2 produced is greatly diminished (35). These effects are especially clear-cut with N_2, which establishes inhibition very rapidly. In our earliest publications (16,17,36; see also ref. 37) on this phenomenon, we made the obvious suggestion that the inhibition was due to diversion of a reductant, created by light, from the hydrogen-evolving system to reactions of reductive amination of keto acids derived from the organic substrate.

Although a competition of this sort may still be entertained as a first approximation, the effect is evidently more complex since the amount of N_2 actually utilized in short-term experiments is considerably less than the predicted quantity (38). In other words, N_2 appears to inhibit by two mechanisms, one of which is essentially "catalytic." It seems quite possible that the "catalytic" effect of N_2 on H_2 production may be a regulatory cut-off device similar to the so-called "allosteric inhibition" (39) observed in other enzyme systems. Further investigations will be required to unravel this interesting puzzle.

The overall effect of N_2, or ammonia, on the photometabolism of organic substrates by hydrogen-producing cells of *R. rubrum* is to divert carbon from the dissimilatory anaerobic citric acid cycle, and toward assimilatory pathways (5,24). This is hardly unexpected behavior for a cell with an abundant energy supply suddenly faced with a utilizable nitrogen source. The absence of H_2 production during active growth in media containing excess NH_4^+ is partly understandable on the basis of the fact that reduction is required to convert this inorganic nitrogen source to the level of the amino group. There is, however, an additional mechanism which ensures the prevention of hydrogen evolution during growth in ammonium salt media. Substantial evidence has been obtained (33) indicating that NH_4^+, or a metabolic derivative, effectively represses the formation of one or more components of the hydrogen-evolving system. Accordingly, cells harvested from a malate + NH_4^+ medium before the nitrogen source is exhausted are initially incapable of producing hydrogen. After a period of continued illumination in the presence of suitable organic substrates, however, H_2 evolution begins and gradually increases in rate (see Fig. 1).

Experiments (33) with chloramphenicol and other inhibitors indicate that the derepression of hydrogen production involves protein synthesis, which presumably can occur at the expense of amino acids in the endogenous pool. In agreement with expectations, cells harvested several hours after exhaustion of the nitrogen source from cultures grown with limiting amounts of ammonia are immediately capable of photoproducing hydrogen.

This brief review of the carbon and nitrogen metabolism of photosynthetic bacteria should serve, in part, to underline the dynamic and variable aspects of their metabolic behavior. In this respect, the photosynthetic bacteria obviously do not differ materially from other microorganisms. Their metabolic variability is emphasized here because of a tendency to regard photosynthetic bacteria as "laboratory reagents" to be used primarily as a convenient source of photoactive subcellular particles. It is probable that the composition and properties of such particles will differ considerably depending on the organism and its nutritional history, and it is prudent not to underestimate the possibility (5,40,41) that violent disengagement of the photochemical

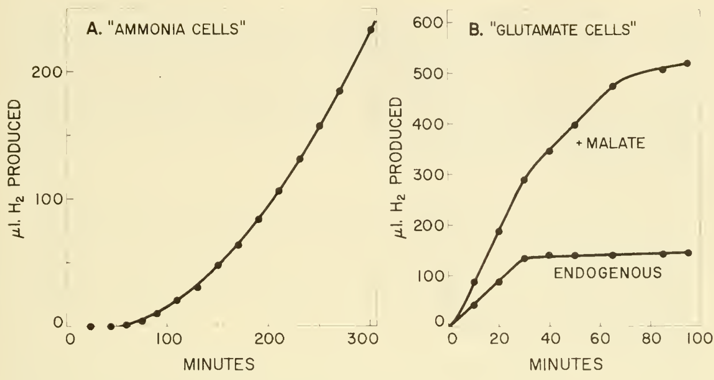

Fig. 1. Kinetics of photoproduction of H_2 by "resting" cells of *R. rubrum* derived from a malate + NH_4^+ (A.) or malate + glutamate (B.) medium. After Ormerod and Gest (13).

In A., the cells were harvested while ammonia was still present in the culture medium. Except for the endogenous control in B., L-malate was added at zero time. For other experimental details, see ref. 13.

apparatus from the complex cellular matrix may result in significant alteration or even total loss of certain biochemical characteristics.

Remarks on the absence of oxygen production in bacterial photosynthesis

Before the discovery of light-induced phosphorylation, the similarities and differences in the autotrophic metabolism of green plants and photosynthetic bacteria could be visualized mainly in terms of formation and subsequent fate of a photoreductant and a photooxidant. According to the concept developed by van Niel (2), the photoreductant would be used for reducing CO_2 in both types of organisms, while disposal of the photooxidant presumably occurs through alternative routes, i.e., conversion to O_2 in green plants and reduction of the potential O_2-precursor by the accessory electron donor in the bacteria.

An appealing experimental system for investigating this hypothesis is furnished by green algae which can switch by "anaerobic adaptation" from typical green plant photosynthesis to a bacterial-type of photoreduction of CO_2 with H_2 (42). The change is reversible in that O_2 formation is resumed if the anaerobic algae are exposed to high light intensities. In a recent extension of studies with such organisms, Bishop (43) has isolated a mutant of *Scenedesmus obliquus* which

apparently has a genetic block in the oxygen-producing mechanism. This organism, consequently, is incapable of producing O_2 when illuminated, but it retains the capacity for photoreducing CO_2 with molecular hydrogen. It seems reasonable that further investigation of algae of the kind under discussion may provide valuable insights into the biochemical evolution of photosynthesis. On the basis of the data at hand, Gaffron (44) has concluded that it is:

> . . . extremely plausible that whatever else had to change to make the release of free oxygen possible—an indispensable part of this last evolutionary step must have been the addition of certain catalysts to an already existing complete mechanism for the fixation of carbon dioxide, for photophosphorylation, and for photoreduction with hydrogen. In place of the reaction leading to molecular oxygen there has been originally a coupling to hydrogenases or dehydrogenases.

According to this point of view, O_2 formation represented the acquisition of a new and singular property. Perhaps there is really no need to ask why the bacteria do not produce O_2; the significant question may be why green plants do.

Gaffron (45) subscribes to the view that the function of the accessory hydrogen donor in autotrophic photosynthesis of the bacterial type is to reduce the potential O_2-precursor (photooxidant) and has marshaled arguments against an alternative hypothesis, *viz.*, that the accessory donor is, in fact, used as a source of hydrogen for CO_2 reduction. In his terms, the alternative stipulates that water is no longer oxidized, but instead NADP is reduced "by a more direct photocatalytic hydrogen (or electron) transport." If we use the formalism of light-dependent "water cleavage," this seems to imply that the only alternative is one in which the "water cleavage" system is entirely eliminated. We can formulate schemes, however, which retain "water cleavage" for the purpose of producing the reductants and oxidants required for cyclic photophosphorylation, or generation of energy-rich precursors of ATP, and postulate that the hydrogen necessary for net reduction is derived from the accessory donor. This type of conception would relegate O_2 production to the category of a unique feature of green plant photosynthesis which is not readily rationalized as a minor comparative biochemical variation.

The notion that the accessory donor serves as the source of hydrogen for CO_2 reduction is an old idea which can be found in some of the earliest discussions (e.g., see ref. 1) on the mechanism of bacterial photosynthesis. Similarly, the concept of a hydrogen transfer, between the added hydrogen donor and CO_2, which is "driven" in some way by light-energy also was proposed in relatively modern terms many years ago (46). The knowledge accumulated during the past fifteen years on electron transfer processes and phosphorylation in a variety

of biological systems makes it possible to reexamine these ideas in a more sophisticated and meaningful way.

Net reducing power in bacterial photosynthesis

There is still no unambiguous supporting evidence for the proposal (see ref. 13) that a photoreductant provides the hydrogen atoms, or electrons, required for *net* formation of reduced pyridine nucleotide or molecular hydrogen. To my mind, it has become increasingly difficult to account for the available biochemical and physiological facts on the basis of this kind of postulated mechanism, which has come to be called "noncyclic electron flow." In this connection, Bose and I (47) have recently examined the experimental basis of the claim (48) that an antimycin-resistant "noncyclic photophosphorylation" (i.e., a phosphorylation presumably dependent on light-stimulated "noncyclic electron flow") system operates in the normal metabolism of purple bacteria. The evidence cited in favor of this conclusion consists essentially of the demonstration, using pigmented particles from *R. rubrum*, of photophosphorylation ostensibly dependent on the presence of both an added reductant (ascorbate + DPIP) and oxidant (NAD). Our studies indicate that the phosphorylation observed in such experiments is in reality cyclic photophosphorylation, which is antimycin-resistant because of the ability of DPIP to effect a by-pass in electron transfer around the antibiotic-sensitive region. The effects of added reductants or oxidants, or both, can be readily explained by the fact that optimal photophosphorylation by isolated particles requires maintenance of a suitable redox potential (49). The particles show very little inherent poise and, consequently, the rate of phosphorylation is quite sensitive to changes in the redox potential caused by electron donors or acceptors which interact with the electron transfer system. It is easy to show, in fact, that in the presence of excess reductant, phosphorylation will not proceed at a significant rate unless a suitable oxidant is added, even though there is no net electron transport (Table 3).

The gas phase in both experiments was 100% H_2. At 30°C. (Exp. I), the hydrogenase in the *R. rubrum* particle preparation is active and when ascorbate + DPIP are also present the particles become "over-reduced," leading to an inhibition of photophosphorylation. It is apparent that the over-reduction effect can be completely prevented, or reversed, by addition of fumarate. At 20°C. (Exp. II), the hydrogenase is practically inactive and inhibition of light-induced phosphorylation by over-reduction is, accordingly, not observed. It is of importance to note that under the conditions employed there was no detectable consumption of H_2 and as much as 8.5 μmoles of Pi could be esterified in the presence of only 0.2 μmole of ascorbate. The results in Table 3 also indicate that DPIP can catalyze an antimycin-resistant bypass in electron transfer associated with photophosphorylation when the overall redox potential is suitably adjusted.

TABLE 3

*Activation of antimycin-resistant cyclic photophosphorylation by DPIP and "redox buffers"**

Experiment	Temperature	Additions	Pi utilized (μmoles)
I	30°C.	None	7.5
		Antimycin A (10 μg)	0.7
		Ascorbate (0.2 μmole) + DPIP (0.2 μmole)	0.7
		Ascorbate + DPIP + fumarate (10 μmoles)	8.5
		Ascorbate + DPIP + fumarate + antimycin A	4.4
II	20°C.	None	4.4
		Antimycin A (10 μg)	0
		Ascorbate (0.2 μmole) + DPIP (0.2 μmole) (0.2 μmole)	5.3
		Ascorbate + DPIP + antimycin A	5.0
		Ascorbate + DPIP + NAD (1 μmole)	7.5
		Ascrobate + DPIP + NAD + antimycin A	5.1

* After Bose and Gest (47). In experiment I, the *R. rubrum* particles contained 0.11 mg bacteriochlorophyll; in experiment II, 0.20 mg. The reaction mixtures contained in a final volume of 3 ml: particles; Tris-HCl pH 8.0, 100 μmoles; ADP, 0.5 μmole; K_2HPO_4, 10.2 μmoles; $MgSO_4$, 5 μmoles; hexokinase, 1 mg; glucose, 30 μmoles. Gas phase, H_2; incubation time, 30 minutes in red light.

As an alternative to "noncyclic electron flow," studies by a number of investigators, notably Chance and his colleagues (50,51), have provided the basis for a completely different, but at least equally plausible, mechanism for light-stimulated electron transfer between added donors and acceptors. These researches, with mammalian mitochondria, indicate that electron flow against an apparent thermochemical gradient can be driven by energy-generating systems. The reaction which has been studied in most detail is the reduction of NAD by succinate, i.e.,

$$\text{succinate} + \text{NAD} \xrightarrow{X \sim \text{ or } X \sim P} \text{fumarate} + \text{NADH}_2$$

Considering the relative redox potentials of the succinate-fumarate $\left(E_0' = +0.031v.\right)$ and $NADH_2$ - NAD $\left(E_0' = -0.32v.\right)$ couples, it can be concluded that an input of energy is necessary to drive the reaction from left to right. This can be provided either by ATP or energy-rich intermediates (precursors of ATP) associated with oxidative phosphorylation. Recent experiments by Griffiths and Chaplain (52) suggest the intriguing possibility that a labile phosphorylated form of NAD may be involved in this and analogous reactions.

An energy-dependent reduction of NAD by succinate has been observed with *R. rubrum* particles, in the sense that a net reaction occurs only when the system is illuminated. The reaction catalyzed by the *Rhodospirillum* system was found by Frenkel (53) to be significantly inhibited when optimal amounts of ADP, Pi, and Mg^{++} were added. This observation suggests, among other possibilities, that there is a competition between the oxidation-reduction reaction and the phosphorylating system for a common intermediate.

The oxidation of succinate to fumarate is a particularly interesting reaction in the metabolism of both mitochondria and photosynthetic bacteria. In both instances, there appears to be a close structural association between succinic dehydrogenase and the phosphorylating electron transfer system. In mitochondria, this physical integration presumably is one of the factors responsible for the unusually high "electron pressure" exerted by succinate on the electron transfer chain. Another property worthy of special attention is the relatively high redox potential of the succinate-fumarate couple. It seems unlikely that the presence of one oxidation-reduction step in the citric acid cycle with this redox character is an accident of nature, and I am inclined to believe that this particular aspect of the succinate-fumarate conversion may be of great importance in the regulation of electron transfer in both aerobic and anaerobic systems.

Purple bacteria such as *R. rubrum* can grow anaerobically in the light with succinate as the primary carbon source and evidently must be able to oxidize succinate to fumarate under these conditions. What, then, is the electron acceptor? There is some evidence (54) from intact cell experiments that the anaerobic oxidation of succinate can be coupled with CO_2 fixation, i.e., the "direct" acceptor in this case is presumably NAD, as in Frenkel's *in vitro* system. There is also evidence (13,24) that a coupling with CO_2 reduction is not obligatory, in that glutamate-grown cells of *R. rubrum* can oxidize succinate with the liberation of H_2; the gas yield (~7 moles H_2/mole succinate) under optimal conditions indicates that the two hydrogen atoms removed in the initial oxidation step must be convertible to molecular hydrogen. To my knowledge, photosynthetic bacteria are the only known organisms which have the apparent potentiality of producing H_2 from the conversion of succinate to fumarate.

Reasoning on the basis of redox potentials, the formation of H_2 from succinate by illuminated cells of purple bacteria would be expected to require an even greater input of energy than the reduction of NAD. Although we could calculate the probable magnitude of the energy requirements for such reactions, or for formation of H_2 from $NADH_2$, it seems wise to heed Mansfield Clark's admonition (55) in this regard. He advises us to: "Beware when the attempt is made to apply such [thermodynamic] data too casually to the dynamic affairs of living cells and to such heterogeneous systems as are living cells." In any event, the contribution of the photochemical system to light-stimulated net electron flow in bacterial photosynthesis can be reasonably visualized in terms of energy-dependent "reverse" electron transfer (see Fig. 2).

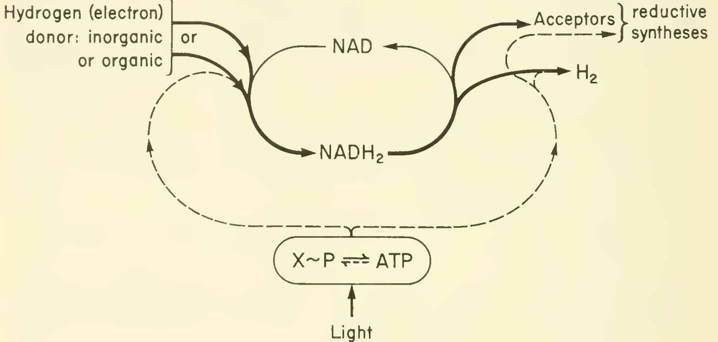

Fig. 2. Scheme for hydrogen (electron) flow from donors to acceptors and photoproduction of H_2 in bacterial photosynthesis. The pyridine nucleotide may be either NAD or NADP; for convenience, only the former is shown.

This hypothesis assumes that electrons (or hydrogen) required for net generation of reduced pyridine nucleotide are derived from an accessory inorganic electron donor or organic compounds. Depending on the redox potential of the donor, or on the steady-state concentrations of the reduced and oxidized forms of donor and pyridine nucleotide, the formation of $NADH_2$ may be promoted by energy-rich intermediates created by the action of light on the photochemical apparatus. Parenthetically, it may be noted that a similar promotion of "reverse" electron transfer by intermediates of oxidative phosphorylation could

explain the unsolved problem (56) of how certain chemosynthetic autotrophs generate reduced pyridine nucleotide from the oxidation of inorganic electron donors of relatively high redox potential.

Reduced pyridine nucleotide would be utilized for a variety of reductive biosyntheses, e.g., conversion of CO_2 to organic compounds, transformation of C_2 and C_3 intermediates to reserve materials (5, 13,27,57), or reductive aminations to provide amino acids for protein synthesis. If, however, the illuminated cell is producing "excess" ATP and $NADH_2$ relative to the demands of the biosynthetic machinery, the reduced electron carrier would be reoxidized, through liberation of molecular hydrogen, by an energy-dependent process. Conditions of this kind apparently obtain during photosynthetic growth with certain amino acids serving as the nitrogen source, and in illuminated resting cell suspensions which are rapidly metabolizing oxidizable compounds in the absence of utilizable nitrogen sources. According to the foregoing conception, H_2 evolution is interpreted as the reflection of a kind of regulatory device which maintains ATP and reduced pyridine nucleotide at levels consistent with the overall rate of biosynthetic activity (13,24,33). Chance and Hollunger (58) have recently suggested that a similar type of control mechanism may operate in mitochondria, *i.e.*, the succinate-dependent reduction of NAD shows great sensitivity to "uncouplers" of oxidative phosphorylation and this could provide a mechanism for delicate regulation of the concentration of $NADH_2$ within the mitochondrion.

The relatively simple scheme shown in Fig. 2 provides a working hypothesis which appears to be compatible with the salient oxidation-reduction features of bacterial photosynthesis. With particular reference to light-dependent H_2 formation, the scheme predicts that this process should be inhibited by compounds which "discharge" or "de-energize" the postulated ATP-precursors. Certain inhibitors of light-induced phosphorylation (such as antimycin A and redox dyes) are, in fact, potent inhibitors of H_2 evolution (24). Addition of such compounds to illuminated intact cells of *R. rubrum* not only abolishes H_2 formation, but usually also causes the cells to resort to an anaerobic fermentation of endogenous reserves (to fatty acids). Such fermentation, which is characteristic of dark anaerobic metabolism, normally does not occur to an appreciable extent during illumination (59). In other words, it appears as if inhibition of the phosphorylating system can have the effect of suppressing overall photometabolism, which in turn results in the appearance of a fermentative pattern frequently seen in heterotrophic anaerobes. This striking phenomenon suggests that photophosphorylation activity inhibits fermentation and, accordingly, the induced transition could be characterized as a "photosynthetic Pasteur effect" (47).

It is encouraging that other types of experiments have given results consistent with the occurrence of energy-linked "reverse" electron

flow in bacterial photosynthesis. Chance and Olson (60) have proposed that a process of this kind can account for the kinetics, obtained by dynamic spectrophotometry, of light-stimulated NAD reduction in intact cells of purple bacteria. Relevant evidence has also been obtained with a model cell-free "photohydrogenase" system in which light stimulates the oxidation of H_2 with fumarate (61). Pigmented particles isolated from *R. rubrum* do not reduce fumarate with H_2 unless a suitable electron carrier is added. When a dye of relatively low redox potential such as brilliant cresyl blue (BCB; $E_0' = +0.047$v.) is supplied, a rapid reaction occurs in darkness as shown in Fig. 3.

Illumination has no effect on the rate of the reaction with BCB serving as the mediator. On the other hand, with DPIP ($E_0' = +0.22$v.) negligible fumarate-reducing activity is observed in the dark and the reaction is now dependent on, or stimulated by, light. Although ferricyanide is rapidly reduced by the particulate hydrogenase, this compound does not catalyze fumarate reduction in dark or light.

According to our interpretation, when DPIP is the mediator, light provides energy for driving hydrogen transfer against an unfavorable thermodynamic gradient, i.e., from $DPIPH_2$ to fumarate. Further evidence for this view has been obtained by Bose (62) using N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD; $E_0' = \sim +0.26$v.) as the catalyst for light-dependent reduction of fumarate by H_2. Table 4 summarizes some of his results showing the effects of various uncouplers of phosphorylation on the light-stimulated oxidation-reduction reaction.

It can be seen that with the first three compounds both the oxidation of H_2 and light-induced phosphorylation were inhibited. The results suggest that of the two processes, photophosphorylation is perhaps somewhat more sensitive to inhibition. With appropriate concentrations of oligomycin, on the other hand, photophosphorylation was inhibited while the oxidation-reduction reaction was consistently stimulated; separate experiments disclosed similar effects with atebrin and gramicidin D. These data show a striking parallelism with the reported (58,63,64) effects of these inhibitors on oxidative phosphorylation and energy-linked "reverse" electron transfer in mitochondria and, consequently, lend further support to the suggested interpretation.

There is good reason to expect that the availability of active cell-free systems and new experimental techniques will lead to an intensification in study of the basic features of light-activated electron transfer pathways in bacterial photosynthesis. Many significant questions remain unanswered, which is not surprising in view of the fact that our understanding of electron transfer in nonphotosynthetic systems is still far from complete in spite of decades of active investigation. One of the prominent unresolved problems in bacterial photosynthesis concerns the nature and extent of cross-connections between the electron carrier systems associated with net electron transfer (i.e., from

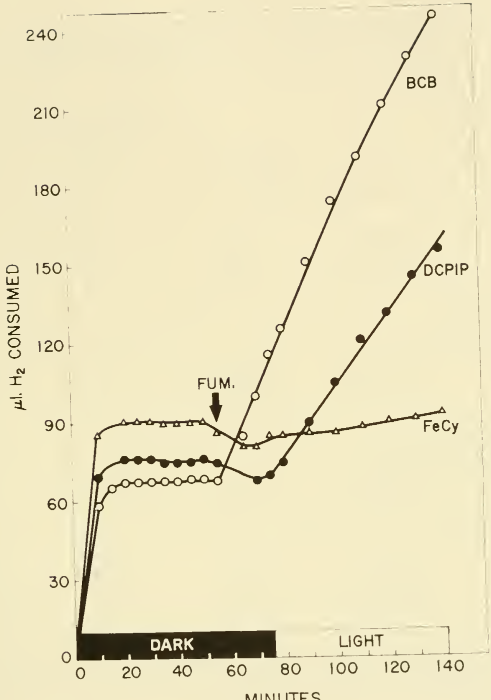

Fig. 3. Oxidation of H_2 with fumarate by *R. rubrum* particles in the presence of redox carriers; light-stimulated catalysis by DPIP. After Bose and Gest (61).

Conditions: Tris-HCl pH 8.0, 150 μmoles; potassium phosphate pH 7.6, 3.5 μmoles; sodium fumarate, 40 μmoles; washed *R. rubrum* particles (from alumina-ground cells) containing 0.98 mg bacteriochlorophyll; water to make 2.0 ml final volume; 0.2 ml of 20% KOH in center well; gas phase, H_2; temperature, 30°C; light intensity, 1000 foot-candles. Redox carriers (approximately 4 μmoles) were added at zero time, and fumarate at 55 minutes. Brilliant cresyl blue, BCB; potassium ferricyanide, FeCy.

TABLE 4

Effects of "uncouplers" on light-stimulated oxidation of H_2 with fumarate and photophosphorylation by R. rubrum *particles**

Inhibitor (*M*)	Particle Conc. (mg bacteriochl./3 ml)	Activity: "Photohydrogenase" (% of control)	Activity: Photophosphorylation (% of control)
Dicumarol:			
5×10^{-4}	0.21	26	3
7×10^{-4}	0.21	16	2.5
1×10^{-3}	0.21	0	0
n-butyl-3,5-diiodo-4-hydroxybenzoate:			
1×10^{-4}	0.18	70	78
5×10^{-4}	0.18	16	4
1×10^{-3}	0.18	0	0
m-chlorocarbonyl cyanide phenylhydrazone:			
5×10^{-5}	0.45	32	0
1×10^{-4}	0.45	0	0
Oligomycin:			
9×10^{-7}	0.56	118	33
3×10^{-6}	0.56	124	14
6×10^{-6}	0.56	124	0
9×10^{-6}	0.56	113	0

* After Bose (62). Particles were prepared as described in ref. 47 and illuminated with red light. Reaction mixtures (in Warburg vessels) contained, in a final volume of 3 ml: particles, as indicated; Tris-HCl pH 7.9, 200 μmoles; TMPD, 2 μmoles; sodium fumarate, 40 μmoles; ADP, 2 μmoles; K_2HPO_4, 40 μmoles; $MgCl_2$, 30 μmoles; hexokinase, 1 mg; mannose, 60 μmoles. Gas phase, H_2 (KOH in center well); temperature, 30°C. The particles were preincubated with inhibitors for approximately 10 minutes and the extent of phosphorylation determined in the usual way after deproteinization of the suspensions used for the manometric assays.

donors to acceptors) and cyclic photophosphorylation. A number of studies (47,49,65-68) indicate that electrons from substrates such as $NADH_2$, succinate, and H_2 can eventually interact with carriers of the photophosphorylating chain. It is possible that such interconnections are merely concerned with maintaining the latter carriers in states of optimal redox balance, but further investigation is required to establish their true import.

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BIOCHEMICAL BASIS FOR THE OBLIGATE PHOTO-AUTOTROPHY OF GREEN BACTERIA OF THE GENUS *CHLOROBIUM*

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Acetate and a variety of other organic compounds can serve as sources of carbon and reducing power for the light-dependent growth under anaerobic conditions of most photosynthetic bacteria. This is not true for certain species of the genus *Chlorobium* (*C. limicola* and *C. thiosulfatophilum*). These green sulfur bacteria grow only anaerobically in light in the presence of exogenous CO_2 and reduced inorganic sulfur compounds (1,2). They may be classified as anaerobic, obligate photoautotrophs. Such restricted requirements for growth substrates are not characteristic of all green bacteria. *Chlorobium chlorochromatii* can grow on peptone and malate (3). *Chloropseudomonas ethylicum* utilizes several carbon sources including ethanol, acetate, and glucose (4).

The inability of *C. limicola* and *C. thiosulfatophilum* to utilize simple organic compounds as the sole carbon source for growth was established by van Niel (1) and Larsen (2) and subsequently confirmed by others (3,5). Although these compounds cannot act as growth substrates in the absence of CO_2, several are photometabolized in the presence of CO_2. Larsen demonstrated a carboxylation of propionate to succinate by cells of *C. thiosulfatophilum*. Sadler and Stanier (5) showed that the growth yield of *C. limicola* was improved by the addition of certain organic supplements. Of these, acetate was the most effective and doubled the cell yield. The gross intracellular distribution of assimilated carbon from acetate was identical with that from CO_2. The incorporation of acetate was strictly dependent on the provision of both CO_2 and sulfide. It was proposed that, whereas other photosynthetic bacteria oxidize acetate to provide the CO_2 and reducing power necessary for the assimilation of other acetate molecules, an enzymic pathway for acetate oxidation is lacking in *C. limicola*. The utilization of acetate for cell growth by this organism would then be dependent upon an exogenous supply of CO_2 and reducing power.

Since the oxidation of acetate in photosynthetic bacteria such as *R. rubrum* can proceed via an anaerobic citric acid cycle (6), extracts of *C. thiosulfatophilum* were examined for citric acid cycle enzymes.

The results obtained support the hypothesis of Sadler and Stanier (5) and provide a possible biochemical basis for the inability of *C. thiosulfatophilum* to utilize acetate in the absence of CO_2.

EXPERIMENTAL PROCEDURE

The green sulfur anaerobe *Chlorobium thiosulfatophilum*, strain L, was cultivated as described by Larsen (7). The growth temperature was 26°C. *Chloropseudomonas ethylicum* was grown at 30°C in a medium containing ethanol and CO_2 as the carbon sources (8). *Chromatium*, strain D, was grown at 35°C in Hendley's medium (9). *R. rubrum* was cultivated as described by Kohlmiller and Gest (10). *Rhodomicrobium vannielii* was grown in a medium containing acetate as the carbon source (11).

Since some of the growth media contained sulfide, an inhibitor of aconitate hydratase (12), the harvested cells were washed several times with 0.05 M Tris buffer, pH 7.8. Cells were broken by passage through a French pressure cell (13). The extracts were centrifuged at 144,000 x g for 60 minutes and the supernatant fluid assayed for enzymic activity. The protein content of the extracts was determined by the biuret reaction (14).

All enzymic measurements were made with a Cary recording spectrophotometer using a slide-wire showing full-scale deflection at an absorbancy of 0.1. The assay temperature was 23°C. Two methods were employed to assay aconitate hydratase. In one, the enzyme was directly assayed by the change in absorbancy at 240 mμ (15). Citrate, cis-aconitate, or isocitrate were used as substrates. In the other, an excess of purified pig heart isocitrate dehydrogenase and TPN were included in the reaction mixture and the reduction of TPN was measured by the absorbancy change at 340 mμ (16). Citrate or cis-aconitate were the substrates. Isocitrate dehydrogenase was measured by the reduction of TPN in the presence of isocitrate (16). Succinic dehydrogenase was assayed by the reduction of cytochrome *c* in the presence of catalytic amounts of *N*-methylphenazonium sulfate (17). The absorbancy change at 240 mμ using malate as substrate was recorded to estimate fumarate hydratase activity (15). Malate dehydrogenase was determined by DPNH oxidation in the presence of oxalacetate (18). Aceto-CoA-kinase was assayed by hydroxamic acid formation (19).

RESULTS

The Conversion of Acetate to Isocitrate

The incorporation of acetate into isocitrate is a prerequisite for any oxidation via the citric acid cycle. Incorporation of acetate into

isocitrate is also required for the assimilation of acetate via the glyoxylate cycle. The condensing enzyme was not directly assayed in extracts of *C. thiosulfatophilum*, but its existence was indicated by experiments with labeled acetate. *Chlorobium* extract, acetate-1-C^{14}, Coenzyme A, $MgCl_2$, and oxalacetate were incubated for various time intervals and the products chromatographed on paper in two dimensions. Autoradiography revealed a radioactive compound which had migrated to the same region of the chromatogram as citrate. This compound cochromatographed with citrate in two different solvent systems. Its appearance was dependent upon the presence of oxalacetate in the reaction mixture. These experiments indicated that *C. thiosulfatophilum* extracts contained condensing enzyme and aceto-CoA-kinase. The presence of the latter enzyme was verified by direct assay using the procedure of Jones and Lipmann (19).

As shown in Table 1, aconitate hydratase activity could not be demonstrated in *C. thiosulfatophilum* extracts. An increase in absorbancy at 240 mμ with either citrate or isocitrate or a decrease with aconitate was not detected. On the other hand, the extracts contained a very active TPN-linked isocitrate dehydrogenase. TPN reduction was not observed in this assay if isocitrate was replaced by either aconitate or citrate even in the presence of an excess of purified pig heart isocitrate dehydrogenase. The same results were obtained with cells grown in the presence of sodium acetate (0.005 M).

In another experiment, a cell-free homogenate of *C. thiosulfatophilum* and the supernatant fluid and sediment from a centrifuged portion of the homogenate (144,000 x g for 60 minutes) were incubated separately with aconitate. After two hours, enzymic assay failed to

TABLE 1

Enzymic assays with extracts of C. thiosulfatophilum

Enzyme	Assay	Substrates	Activity
			μmoles/min/g protein
Aconitate hydratase	Absorbancy change at 240 mμ	Citrate, aconitate or isocitrate	0
Aconitate hydratase	Coupled to isocitrate dehydrogenase	Citrate or aconitate	0
Isocitrate dehydrogenase	TPN reduction	Isocitrate	1940
Isocitrate dehydrogenase	DPN reduction	Isocitrate	0

reveal the presence of isocitrate. Fractions obtained from the supernatant fluid by ammonium sulfate precipitation did not show aconitate hydratase activity. Possible activators such as β-mercaptoethanol, EDTA, and various metal ions were tried without effect. Extracts were also inactive after preincubation with ferrous ions and cysteine under the conditions described for activation of pig heart aconitate hydratase (20).

Aconitate Hydratase in Other Photosynthetic Bacteria

Since aconitate hydratase was not found in *C. thiosulfatophilum*, several other photosynthetic bacteria were examined for this enzyme. The extraction and assay procedures were the same as used for *C. thiosulfatophilum*. The extracts were also assayed for isocitrate dehydrogenase. Both enzymes were present in all the extracts examined (Table 2). For comparison, the aconitate hydratase activity of a pea leaf extract was determined. A value of 21 μmoles aconitate utilized per minute per gram of protein was obtained. The activities of the bacterial extracts are either comparable or higher than this value.

There is no reason to suspect that enzymes of *C. thiosulfatophilum* are easily inactivated during the extraction procedure. In a previous study, *C. thiosulfatophilum* extracts were assayed for thirteen enzymes of the photosynthetic carbon cycle and activity was demonstrated in every case (21). However, the possibility remains that these extracts contain an inhibitor highly specific for aconitate hydratase. Accordingly, the effect of *C. thiosulfatophilum* extracts on the aconitate hydratase

TABLE 2

Aconitate hydratase and isocitrate dehydrogenase activities in extracts of photosynthetic bacteria

Type of bacterium	Source of extract	Aconitate* hydratase	Isocitrate dehydrogenase TPN	Isocitrate dehydrogenase DPN
		µmoles/min/g protein		
Green sulfur	*Chloropseudomonas ethylicum*	25	1080	2.0
Purple sulfur	*Chromatium*	104	76	0.9
Purple nonsulfur	*Rhodospirillum rubrum*	51	460	2.3
Purple nonsulfur	*Rhodomicrobium vannielii*	20	365	5.6

* Assayed by TPN reduction in the presence of aconitate and excess isocitrate dehydrogenase.

TABLE 3

The effect of C. thiosulfatophilum *extract on aconitate hydratase activity*

Except where noted, the enzyme was assayed by following TPN reduction coupled to isocitrate dehydrogenase

Source of enzyme	Protein in assay (μg)	Substrate	*Chlorobium* extract (μg protein)	Absorbancy change in 10 min	Per cent inhibition
Chromatium	59	Aconitate	–	.397	
	59	Aconitate	570	.334	16
	59	Aconitate	550*	.340	14
	59	Citrate	–	.091	
	59	Citrate	570	.070	23
	118	Isocitrate†	–	.133	
	118	Isocitrate	380	.115	14
Pea leaf	280	Aconitate	–	.425	
	280	Aconitate	380	.430	0

* Extract was dialyzed
† Assayed by absorbancy increase at 240 mμ

activities of extracts of other organisms was examined. Variable results were obtained. Some extracts of *C. thiosulfatophilum* had no effect; with other preparations some inhibition resulted. Table 3 shows part of an experiment in which inhibition was obtained. In the same experiment, the aconitate hydratase activities of *Chloropseudomonas ethylicum*, *R. rubrum*, and *Rhodomicrobium vannielii* were decreased from 10 to 30 per cent by the addition of *C. thiosulfatophilum* extract. The inhibition was not removed by dialyzing the extract. Since inhibition was never severe, it appears unlikely that the inability to demon-

TABLE 4

Enzymes involved in succinate oxidation in the green photosynthetic bacteria

Bacterium	Succinic dehydrogenase	Fumarate hydratase	DPN-malate dehydrogenase
	μmoles/min/g chlorophyll	*μmoles/min/g protein*	
Chlorobium thiosulfatophilum	28	285	22
Chloropseudomonas ethylicum	not assayed	233	2170

strate aconitate hydratase in *C. thiosulfatophilum* can be attributed to the existence of inhibitors in the extract.

Other Enzymes of the Citric Acid Cycle

Succinic dehydrogenase, fumarate hydratase, and malate dehydrogenase are present in *C. thiosulfatophilum* (Table 4). Results obtained with another green bacterium, *Chloropseudomonas ethylicum,* are included for comparison. It may be noted that the very active fumarate hydratase activity of *C. thiosulfatophilum* was measured by an assay procedure very similar to one of the assays used for aconitate hydratase. *C. thiosulfatophilum* was not examined for α-ketoglutarate dehydrogenase.

DISCUSSION

The purple photosynthetic bacteria can photometabolize acetate by several alternative pathways. In some, acetate assimilation appears to be predominantly through a glyoxylate cycle (22,23). Others, including *R. rubrum,* lack isocitratase (22) and utilize acetate by an unknown pathway (24). In addition, acetate may be oxidized via an anaerobic citric acid cycle (6), condensed with pyruvate to yield citramalate (25,26) or reduced to a storage compound, poly β-hydroxybutyric acid (27). As pointed out by Gest *et al.* (6), the direction of metabolism "is dictated by the nutritional history of the cells and the conditions of incubation."

Regardless of the initial pathway of incorporation, the eventual utilization of acetate for cellular growth in bacteria which do not contain a glyoxylate cycle is CO_2-dependent (27,28,29), even though growth on acetate may result in a net production of CO_2. This necessitates an enzymic pathway for the oxidation of acetate to CO_2. In *R. rubrum* this can take place via a light-dependent anaerobic citric acid cycle (6,30,31). Among the green bacteria, *Chloropseudomonas ethylicum* can oxidize acetate to CO_2. During growth on acetate there is a net production of CO_2 amounting to 15 to 20 per cent of the acetate utilized. In contrast, *C. limicola* can quantitatively assimilate acetate but only with simultaneous assimilation of CO_2. Experiments with acetate-C^{14} disclosed that only a very small fraction of the acetate taken up by these cells reappeared as labeled CO_2 (5). Since acetate assimilation by *C. limicola* is CO_2 dependent, the mechanism of assimilation may be similar to that in *R. rubrum,* except that whereas in the latter CO_2 and reducing power are produced endogenously from acetate, these must come from an exogenous source in the case of *C. limicola.* Hence while *C. limicola* possesses an enzymic mechanism for acetate assimilation, it does not appear to contain a mechanism for acetate oxidation.

The experiments described in this paper indicate that a closely related bacterium *C. thiosulfatophilum*[1] is deficient in aconitate hydratase. With the exception of α-ketoglutarate dehydrogenase which was not assayed, all the remaining enzymes necessary for the oxidation of acetate via the citric acid cycle are present. A deficiency of aconitate hydratase would, firstly, preclude the possibility of acetate incorporation via a glyoxylate cycle such as occurs in *Chromatium* (23), and secondly, prevent oxidation of acetate via a citric acid cycle. While metabolism via the citric acid cycle cannot result in a net uptake of carbon, it can provide the CO_2 and reducing power necessary for acetate assimilation by a pathway such as that occurring in *R. rubrum* (24). A deficiency of aconitate hydratase could thus explain why acetate alone cannot support the growth of *C. thiosulfatophilum*. Assuming that these cells, like *C. limicola*, can assimilate acetate provided CO_2 and reduced sulfur compounds are present, a pathway for acetate assimilation exists which involves neither the glyoxylate nor citric acid cycles.

In the presence of thiosulfate, the growth of *C. thiosulfatophilum* could theoretically be supported by any substance which is converted by the organism to CO_2. The inability of dicarboxylic acids like succinate and malate to function as growth substrates could be attributed in part to a deficiency of aconitate hydratase. However, in *R. rubrum* these acids can be diverted to form CO_2 and C_3-fragments under conditions where the citric acid cycle is blocked by monofluoroacetate (32). This alternative to metabolism via the citric acid cycle does not appear to operate in *C. thiosulfatophilum*.

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SUMMARY

Experiments were carried out in an attempt to establish a biochemical basis for the obligate photoautotrophy of *Chlorobium thiosulfatophilum*. It is suggested that this organism cannot utilize acetate in place of CO_2 as the growth substrate because it lacks aconitate hydratase. This precludes assimilation of acetate by a glyoxylate cycle as well as oxidation via the citric acid cycle to provide the CO_2

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[1] *C. thiosulfatophilum* is distinguished from *C. limicola* by the inability of the latter to oxidize thiosulfate (7).

and reducing power necessary for the assimilation of acetate by alternative pathways.

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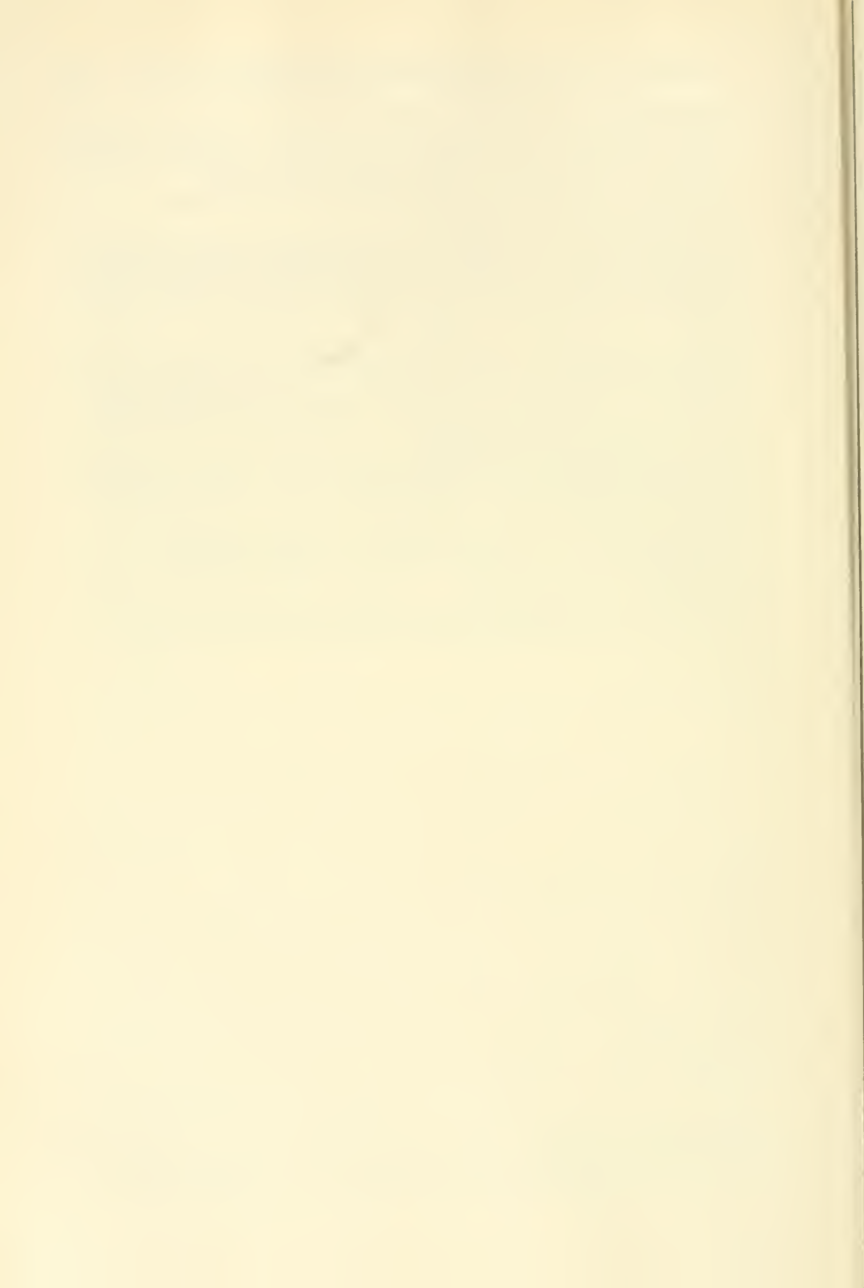

SOME OBSERVATIONS CONCERNING THE PURIFICATION AND PROPERTIES OF THE AEROBIC PHOSPHORYLATION SYSTEM OF *R. RUBRUM* EXTRACTS[1]

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INTRODUCTION

Previous work (1) demonstrated that cell-free extracts of *R. rubrum* carry out an aerobic phosphorylation of adenosine diphosphate in the absence of light. By means of differential centrifugation, the aerobic phosphorylation system of extracts of cells grown aerobically in darkness was found to consist of soluble dehydrogenases and a particulate complex which catalyzed phosphorylation of adenosine diphosphate associated with the oxidation of DPNH.

The following is a report of results of a further analysis of *R. rubrum* extracts by centrifugation in sucrose density gradients. This work indicates that the bulk of the DPNH oxidase activity of crude extracts of cells grown aerobically in darkness is associated with particles which are lighter than those which catalyze aerobic phosphorylation with DPNH. The phosphorylation system appears to be closely associated with succinic dehydrogenase activity. This aerobic phosphorylation system, purified by centrifugation in sucrose density gradients, has been characterized by its response to various inhibitors of electron transport and phosphorylation.

EXPERIMENTAL PROCEDURE

Growth of bacteria and preparation of extracts

Bacteria were grown aerobically in darkness or anaerobically in light, harvested, and washed as previously described (1).

Extracts derived from cells grown aerobically in the dark are designated by the term "dark extracts" and extracts of cells grown anaerobically in the light, by "photosynthetic extracts."

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Extracts were made by disruption of cell suspensions (200-250 mg, wet weight, of washed cells per ml of 0.05 M glycylglycine buffer, pH 8) at 0° in a French Pressure Cell (American Instrument Company, Silver Springs, Maryland) at 15,000 pounds (Carver Press, Fisher Scientific Company).

Intact cells and large debris were removed by centrifugation at 37,000 x g for 30 minutes. The supernatant fluid was centrifuged again for 30 minutes at 37,000 x g. The final supernatant is referred to as the crude extract.

Fractionation of crude extract by centrifugation in sucrose density gradients

All operations were performed at 0-5°C. Following the methods of Britten and Roberts (2), SW39 rotor tubes were loaded with a linear sucrose gradient made by emptying a Bock and Ling mixing device containing 2.3 ml 5% sucrose and 2.3 ml 20% sucrose. All sucrose solutions used also contained 0.05 M glycylglycine pH 8. This was followed by the addition of 0.2 ml of crude extract and 0.2 ml 4% sucrose to the gradient tubes with a smaller mixing device; accordingly, the extract concentration attained its highest value at the minimum sucrose concentration (at the top of the tube). The tubes were centrifuged in the SW39 rotor, following the procedure of Martin and Ames (3). The tubes then were punctured with a hollow needle and nine to ten fractions, each containing 40 drops, collected.

Assay of sucrose gradient fractions

The protein contents of sucrose gradient fractions were estimated routinely by measuring optical density at 280 mμ with a Zeiss spectrophotometer. In other instances, protein was measured by the method of Lowry *et al.* (4). Chlorophyll was estimated on the basis of the optical density of sucrose gradient fractions at 880 mμ.

Enzymatic assays were facilitated by fitting a Leeds and Northrup Speedomax Type G recorder to the spectrophotometer. With special chart paper (No. TCI 1180 Chart paper, Technical Recording Chart Division, Graphic Controls Corporation, Buffalo 10, N. Y.), the full span of the recorder was equivalent to 0.1 optical density unit. About 15 seconds afte. mixing, the time course of enzymatic reactions was followed for one minute. All enzymatic assays were done at room temperature (23°-25°C), unless otherwise stated.

The assay of DPNH oxidase activity consisted of measuring the rate of decrease in optical density at 340 mμ induced by the addition of enzyme preparation to 0.5 ml of 0.1 mM DPNH in 0.1 M K phosphate + 5 mM $MgCl_2$ pH 7.0.

In the succinic dehydrogenase assays, measurements were first made of the decrease in optical density at 600 mμ induced by addition of enzyme to 0.5 ml of 0.1 M K phosphate + 5 mM $MgCl_2$ pH 7.0

containing 7 μg 2,6-dichlorophenolindophenol. The rate observed was deducted from the rate seen upon the subsequent addition of 10 μl of 1 M sodium succinate.

Aerobic phosphorylation was measured by counting the radioactive mannose-6-P produced by incubation of an aliquot of enzyme preparation with radioactive Pi. The standard incubation mixture consisted of the following, together with enzyme, in a total volume of 0.5 ml (pH 7.4): 0.1 μmole DPNH, 0.2 μmole $MgCl_2$, 1.0 μmole K phosphate, 10^7 cpm P^{32}-inorganic phosphate (Nuclear Consultants Corporation, St. Louis 19, Mo.), 0.1 μmole ATP, 10 μmoles mannose, 5.0 μmoles glycylglycine, and 0.25 mg yeast hexokinase (Fraction IV, Sigma Chemical Corporation, St. Louis, Mo.). The reaction was started by addition of enzyme to the mixture contained in a 3 ml tube. After 10 minutes in a 30°C water bath in darkness, the reaction was terminated by adding 10 μl 100% TCA.[2] Zero time controls were prepared by addition of TCA prior to all of the components of the incubation mixture. Mannose-6-P was separated from Pi by electrophoresis. To each acidified incubation sample, 10 μl aliquots of 1 M K_2HPO_4 and 1 M K mannose-6-P were added. A 10 μl aliquot of the mixture was electrophoresed on Whatman 3 MM paper in 0.04 M Na citrate pH 3.6 for 30 minutes at 80 V/cm. With the apparatus used, consisting of lucite tank No. LT-36 fitted with auxiliary steel cooling coils and a 5 K.V. power supply (Savant Instruments, Inc., Hicksville, N. Y.), 4500 volts were applied, with the current ranging from 100 to 160 mA. Thirty samples could be processed in one run. The paper then was thoroughly dried at 50°C for one hour. The spots were visualized by several hours exposure to Kodak No-Screen Medical X-ray Film and by aniline hydrogen phthalate spray (5). In routine assays (*e.g.* inhibition experiments) the X-ray film exposure was omitted. The sugar phosphate spots were cut out (3/4 in. x 1 in. rectangles), placed in vials containing 10 ml POPOP-PPO mixture (4 g PPO and 0.1 g POPOP per liter of toluene), and counted in a liquid scintillation spectrometer (Packard Instrument Co., Inc., La Grange, Illinois). The mannose-6-P count was compared with total phosphate count (obtained by counting a paper rectangle spotted with a 10 μl aliquot of one hundred fold-diluted incubation mixture). In this system, mannose-6-P migrated toward the anode at two-thirds the velocity of Pi.

In studies of the distribution of the aerobic phosphorylation system in sucrose gradient fractions, a correction had to be made for activity which was presumably due to exchange reactions unrelated to DPNH oxidation. This was done by determination of phosphorylation in duplicate tubes containing PMS (0.1 mg/ml). The incorporation of

[2] The abbreviations used are: TCA, trichloroacetic acid; PPO, 2,5-Diphenyloxazole; POPOP, 1,4-bis-2-(5-phenyloxazolyl)-benzene; SDH, succinic dehydrogenase; M-6-P, mannose-6-phosphate; p-F_3COCCP, phenylhydrazone of p-trifluoromethoxyphenyl carbonyl cyanide.

Pi into mannose-6-P in the presence of PMS was subtracted from the corresponding value obtained in the absence of PMS. Previous experiments have established that aerobic phosphorylation is strongly inhibited by PMS (1). The phosphorylation assay thus was an estimation of PMS-sensitive incorporation of Pi into mannose-6-P. The PMS control was not used in studies of the properties of the purified aerobic phosphorylation system (which was completely inhibited by PMS).

Biochemicals were obtained from Sigma Chemical Corporation. PPO and POPOP were obtained from Packard Instrument Company.

RESULTS

Separation of enzymatic activity by sucrose density gradient centrifugation

Initial centrifugation experiments were carried out with crude extracts derived from *R. rubrum* cells grown in darkness under aerobic conditions. Succinic dehydrogenase activity was associated with a collection of particles which sedimented more rapidly than the bulk of the DPNH oxidase (Fig. 1). This was even more evident when the centrifugation time was increased from 40 to 60 minutes (Fig. 2). In both examples, the incorporation of inorganic phosphate into mannose-6-P was associated with succinic dehydrogenase activity.

With crude extracts derived from cells grown anaerobically in the light (Fig. 3), succinic dehydrogenase activity paralleled chlorophyll content. The rate of sedimentation of the dark succinoxidase particles (Fig. 1) appeared to be similar to that of the "chromatophore fraction" (Fig. 3). However, the patterns of DPNH oxidase activity differed strikingly; most of the apparent DPNH oxidase in the photosynthetic extract remained in the top "soluble" fraction (Fig. 3), whereas much of the DPNH oxidase activity of the dark extract was found in particulate form (Fig. 1).

In other experiments it has been found that the specific DPNH oxidase activities of crude "photosynthetic" and "dark" extracts differ considerably, whereas the succinic dehydrogenase activities do not. For example, the specific activity of the DPNH oxidase (at 30°C) of a crude photosynthetic extract was 9.6 mμmoles DPNH oxidized/mg protein/min. This was one-fifth of the value shown by a crude dark extract, 49 mμmoles. On the other hand, the specific activity of succinic dehydrogenase (at 30°C) of the crude photosynthetic extract was 26 mμmoles indophenol dye reduced/mg protein/min, only one and one-half times the value for the crude dark extract, 18 mμmoles.

The aerobic phosphorylation system of the photosynthetic extract, however, appeared to sediment in the same zone as the chromatophore

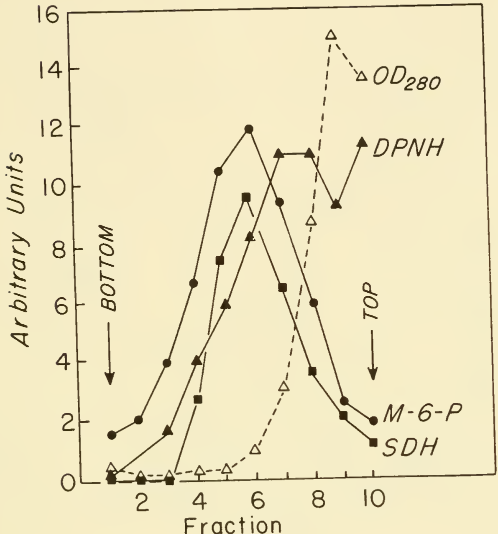

Fig. 1. Activity profile of Crude Dark Extract Centrifuged Forty Minutes at 35,000 rpm—Crude dark extract, 5.9 mg protein, was centrifuged in a sucrose density gradient; fractions were collected and analyzed as described in the text. The first fraction represented the bottom fraction of the tube.

In order to translate arbitrary units into activity stated in terms of each undiluted fraction: one OD_{280} unit (open triangles) represents an optical density of 0.65 at 280 mμ, one succinic dehydrogenase unit (squares) represents 2.2 mμmoles indophenol reduced/ml/min, one DPNH oxidase unit (triangles) represents 6.6 mμmoles DPNH oxidized/ml/min, and one M-6-P unit (circles) represents 2.1 mμmoles M-6-P formed/ml/min.

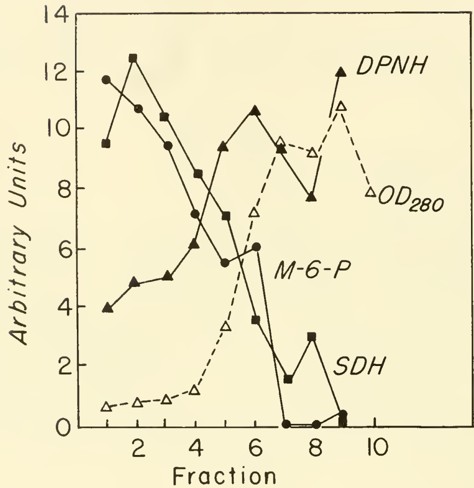

Fig. 2. Activity profile of Crude Dark Extract Centrifuged Sixty Minutes at 35,000 rpm—Crude extract, 8.2 mg protein, was treated in the manner outlined in Fig. 1 (except that the centrifugation time was extended to sixty minutes). The actual values of the arbitrary units were (in terms of undiluted fractions): for OD_{280} (open triangles), one unit represents an optical density of 0.865 at 280 mμ, for succinic dehydrogenase (squares) 2.2 mμmoles indophenol reduced/ml/min, for DPNH oxidase (triangles) 3.7 mμmoles DPNH oxidized/ml/min, and for M-6-P (circles) 1.0 mμmole M-6-P formed/ml/min.

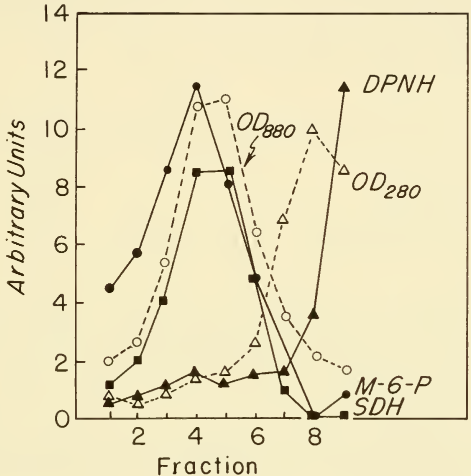

Fig. 3. Activity profile of Crude Photosynthetic Extract—Crude photosynthetic extract, 6.0 mg protein, was centrifuged and analyzed as in Fig. 1. One arbitrary unit represents (in terms of each undiluted fraction): for OD_{280} (open triangles), an optical density of 1.0 at 280 mμ; for OD_{880} (chlorophyll, open circles), an optical density of 0.17; for succinic dehydrogenase (squares), 5.4 mμmoles indophenol reduced/ml/min; for DPNH oxidase (triangles), 4.5 mμmoles DPNH oxidized/ml/min; and for M-6-P (circles), 0.68 mμmoles M-6-P formed/ml/min.

TABLE 1

Substrate Requirement for Aerobic Phosphorylation by a Sucrose Gradient Fraction of Dark Extract

The incubation volume was reduced to 200 μl, with proportionate reduction of all components (see experimental procedure). Each tube contained an aliquot of the sucrose gradient fraction used in Fig. 5 (29 μg protein). The inorganic phosphate content was 0.37 μmoles (10^7 cpm)/200 μl. All values given have been corrected for the zero time control (960 cpm).

Additions	Mannose-6-Phosphate formed (cpm)
1. None	1,600
2. DPNH (0.05 μmole)	163,000
3. DPN (0.05 μmole)	2,200
4. Succinate (0.5 μmole)	21,900
5. DPNH (Extract omitted)	80

fraction. It must be noted that the association of this phosphorylation system with either succinic dehydrogenase or chlorophyll does not appear to be as evident as the parallel distribution of succinic dehydrogenase and chlorophyll seen in Fig. 3, or succinic dehydrogenase and the phosphorylation system of dark extracts (Figs. 1 and 2). The phosphorylation exhibited by the peak fraction in Fig. 3 (fraction 4) has been shown to have the same substrate requirements as the corresponding fraction from sucrose gradient experiments with crude dark extracts (see Table 1).

Preliminary attempts to show a possible interaction between the "nonphosphorylating DPNH oxidase" fractions and the phosphorylation system have met with negative results (Fig. 4). The usual sucrose density gradient experiment (using a crude dark extract) was performed, the assays yielding the usual curves for DPNH oxidase, succinic dehydrogenase, and phosphorylation (curve M-6-P-I). Phosphorylation assays were also made using fraction 5 in combination with each sucrose fraction; the curve obtained (M-6-P-II) is identical within experimental error to the dotted curve calculated by a simple arithmetic addition of activities (expected if there were no interaction of fractions).

Some properties of sucrose gradient fractions having maximum aerobic phosphorylation activity

DPNH was required for aerobic phosphorylation by a sucrose gradient fraction of dark extract (Table 1). Little phosphorylation was seen with succinate. The same substrate requirement has been observed in similar experiments with the corresponding fraction from

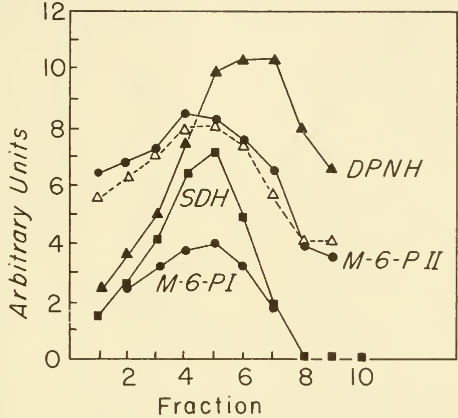

Fig. 4. Effect of Combining Fractions on Aerobic Phosphorylation—Crude dark extract, 8.0 mg protein, was centrifuged in a sucrose density gradient for forty five minutes at 35,000 rpm, fractionated and analyzed as in Fig. 1. Succinic dehydrogenase and DPNH oxidase activities were assayed at 30°C. One arbitrary unit represents: for succinic dehydrogenase (squares), 5.4 mμmoles indophenol reduced/ml/min; for DPNH oxidase (triangles), 7.0 mμmoles DPNH oxidized/ml/min; and for M-6-P (curve M-6-P-I), 4.9 mμmoles M-6-P formed/ml/min. Curve M-6-P-II represents the assay of PMS-sensitive aerobic phosphorylation carried out in the same manner as for M-6-P-I, except that equal volumes of fraction 5 were also added. The dashed curve was calculated by adding the M-6-P value for fraction 5 (in curve M-6-P-I) to the values obtained for each fraction in curve M-6-P-I.

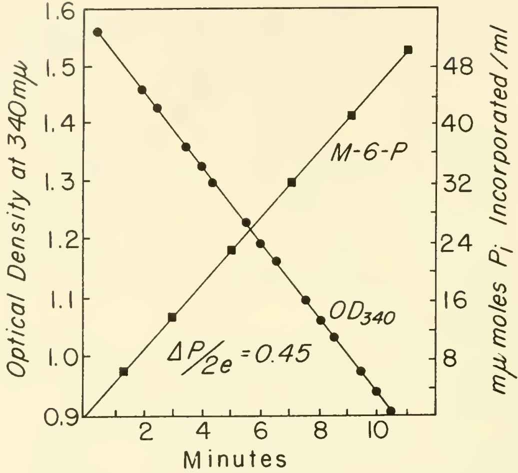

Fig. 5. Kinetics and Efficiency of Aerobic Phosphorylation—An incubation mixture (see experimental procedure) was warmed for five minutes at 30°C (in the absence of enzyme). An aliquot was withdrawn and acidified with TCA (zero time), and the reaction was initiated by adding enzyme (26 μg of a sucrose fraction in a final incubation volume of 734 μl). Optical density at 340 mμ was followed (30°C, thermostated cuvette compartment) and at intervals aliquots were withdrawn and acidified with TCA. The sucrose gradient fraction used contained maximal succinic dehydrogenase activity (in a sucrose density gradient centrifugation of crude dark extract). All values given have been corrected for the zero time blank (0.25 mμmoles/ml). A control tube, lacking DPNH, incubated at 30°C for 12 minutes gave a value of 0.98 mμmoles M-6-P/ml.

TABLE 2

Effects of Antibiotic Inhibitors on Aerobic Phosphorylation

The procedure followed and the enzyme preparation used were the same as in Table 1. Ethanolic solutions of the antibiotics were added to the incubation mixtures (final ethanol concentration, 0.5%).

Inhibitor	*Conc.* μg/ml	*Mannose-6-Phosphate* cpm	*Inhibition* %
Antimycin A	–	162,000	(0)
	5	48,600	70
	0.5	143,000	12
	0.05	147,000	9
Oligomycin	5	13,000	92
	0.5	84,000	48
	0.05	140,000	14
Valinomycin	5	17,800	89
	0.5	117,000	28
	0.05	142,000	12
Dianemycin	5	92,500	43
	0.5	139,000	14
Ethanol control		146,000	10

sucrose density gradient centrifugation of crude photosynthetic extract.

The rates of both oxidation of DPNH and phosphorylation were constant during the incubation period (Fig. 5), with a calculated P/2e ratio of 0.45. This ratio is of the same order as that obtained with washed crude 145,000 x g particles in earlier experiments (1). In separate experiments it has been observed that the rate of DPNH oxidation is independent of the presence of necessary components of phosphorylation, viz., Pi, Mg, and ADP.

In a study of inhibitors of aerobic phosphorylation (Table 2), inhibitory concentrations of the antibiotics antimycin A, Valinomycin, and Dianemycin were one to two orders of magnitude above those effective in sensitive mitochondrial systems (6,7). On the other hand, Oligomycin exerted an effect at a concentration required for inhibition of mitochondrial phosphorylation (6).

It is of interest to note (Table 3) that aerobic phosphorylation was inhibited by low concentrations of a carbonyl cyanide phenylhydrazone derivative (10^{-7}-10^{-6} M), a potent uncoupling agent of mitochondrial oxidative phosphorylation (8); on the other hand, the system was only partially affected by dinitrophenol. In separate experiments, the DPNH

TABLE 3

Effects of Uncoupling Agents and Inhibitors of Electron Transport on Aerobic Phosphorylation

The procedure and enzyme used were as described in Table 1. The solutions of inhibitors were made just before use; p-F_3COCCP and 2,4-DNP were dissolved in excess NaOH.

Inhibitor	Conc. M	Mannose-6-Phosphate cpm	Inhibition %
None		162,000	0
p-F_3COCCP	1×10^{-8}	160,000	1
	1×10^{-7}	131,000	19
	1×10^{-6}	35,000	78
	1×10^{-5}	2,000	99
2,4-DNP	1×10^{-4}	115,000	29
PMS	3×10^{-4}	11,300	93
NaCN	1×10^{-4}	100,500	38
Amytal	1×10^{-3}	56,700	65
NaOH control	1×10^{-3}	141,000	13

oxidase was found to be sensitive to amytal (and the phosphorylation correspondingly so, as shown here). Both DPNH oxidase and phosphorylation were moderately affected by high concentrations of cyanide, and aerobic phosphorylation was inhibited by phenazine methyl sulfate.

DISCUSSION

The fact that the bulk of the DPNH oxidase of crude dark extracts may be separated from the phosphorylation system does not necessarily imply that the two activities were not associated in the cell prior to disruption. Indeed, the nature of the particulates of such extracts is most likely a function of the means used to break the cells. This is exemplified by the experiments of M. Baltscheffsky (9) which demonstrated that the type of abrasive used influenced the amount of "soluble" DPNH oxidase activity obtained in extracts of photosynthetically grown *R. rubrum*. Furthermore, experience with "chromatophore fractions" in *R. rubrum* must be cited; all of the pigment of osmotic lysates of lysozyme-treated *R. rubrum* cells was found to sediment readily in very low centrifugal fields (10).

It must be noted, however, that separation of most of the DPNH oxidase from the phosphorylating system in dark extracts was somewhat unexpected. Previous work with similar extracts (1) repeatedly had shown that DPNH oxidase, succinic dehydrogenase and the phosphorylation system sedimented together under the conditions of the usual differential centrifugation.

Preliminary experiments with crude photosynthetic extracts indicate that the aerobic phosphorylation system sediments at a rate similar to that of the chromatophore fraction. The data are consistent with the concept that the aerobic phosphorylation system is associated with the chromatophore fraction (11). Further proof of association, however, must await results of purification of these particulate systems using other means.

The aerobic phosphorylation system obtained in sucrose density gradient experiments appears to be the same system observed previously in crude washed 145,000 x g particles obtained by differential centrifugation (1). Both the latter particles and the active sucrose fractions required DPNH for activity, giving P/2e ratios below 1; the DPNH oxidase of both was amytal sensitive, but relatively unaffected by antimycin A.

The most interesting observation made in the study of the inhibition of aerobic phosphorylation was that low concentrations of a potent uncoupling agent (F_3COCCP) were strongly inhibitory. This fact and the sensitivity to oligomycin indicate that the mechanisms involved in phosphorylation by this preparation may be similar to those of other sensitive systems (6,8).

SUMMARY

1. Centrifugation in sucrose density gradients of crude extracts of *R. rubrum* cells grown aerobically in darkness has shown that the aerobic phosphorylation system is associated with heavy particles rich in succinic dehydrogenase. The phosphorylation system is separable from the bulk of the DPNH oxidase activity, which is associated with light particles.

2. Similar centrifugation experiments with crude extracts of photosynthetically grown *R. rubrum* indicate that the rate of sedimentation of the aerobic phosphorylation system is similar to that of the chromatophore fraction. Succinic dehydrogenase activity is also closely correlated with the chlorophyll content of the chromatophore fraction.

3. The properties of the aerobic phosphorylation system in sucrose gradient fractions are similar to those of the cruder systems previously studied.

4. The effects of a variety of inhibitors of electron transport and uncoupling agents of oxidative phosphorylation on the aerobic phos-

phorylation system purified by sucrose density gradient centrifugation are described.

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METABOLISM OF PHOTOSYNTHETIC BACTERIA. II. CERTAIN ASPECTS OF CYCLIC AND NONCYCLIC PHOTOPHOSPHORYLATION IN *RHODOSPIRILLUM RUBRUM*

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Investigations with isolated chloroplasts led to the discovery of photosynthetic phosphorylation comprising two photochemical reactions, now called cyclic (Eq. 1) and noncyclic (Eq. 2) photophosphorylation (1-4), which produce the assimilatory power (ATP and reduced pyridine nucleotides) to drive carbon assimilation during photosynthesis.

$$\text{ADP} + \text{Pi} \xrightarrow[\text{chloroplasts}]{h\nu} \text{ATP} \qquad (1)$$

$$2\text{TPN} + 2\text{ADP} + 2\text{Pi} + 4\text{H}_2\text{O} \xrightarrow[\text{chloroplasts}]{h\nu} 2\text{TPNH}_2 + 2\text{ATP} + \text{O}_2 + 2\text{H}_2\text{O} \qquad (2)$$

The terms cyclic and noncyclic photophosphorylation were suggested to denote the difference between a "closed" and an "open" electron transport pathway (coupled with ATP formation) that are envisaged for reactions 1 and 2, respectively (4,5). In cyclic photophosphorylation, the electrons cycle in a "closed" system within the photoreceptor particle, whereas in noncyclic photophosphorylation the photoreceptor particle mediates an "uphill" electron transfer in an "open" system from an external electron donor to an external electron acceptor.

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If the basic photosynthetic mechanisms are the same in green plants and in photosynthetic bacteria, it would be expected that cyclic and noncyclic photophosphorylation of chloroplasts would have appropriate counterparts in photosynthetic bacteria. Such counterparts were indeed found, but only after other investigations revealed fundamental similarities that were at first obscured by differences between the chloroplast and the bacterial systems. Thus, when Frenkel found (6) a light-induced phosphorylation by cell-free preparations of *R. rubrum* and pointed to its similarity to the photosynthetic phosphorylation discovered earlier in spinach chloroplasts (1) he observed that the bacterial particles became substrate (α-ketoglutarate) dependent after washing (6). This was at variance with the unique feature of photosynthetic phosphorylation in chloroplasts, a feature which distinguishes it from oxidative phosphorylation in mitochondria and the anaerobic phosphorylations associated with fermentation: ATP formation in chloroplasts occurs without the contribution of energy by an oxidizable substrate and solely at the expense of the energy contributed by absorbed photons (1,2). However, in later experiments, Frenkel (7) and other investigators (8-10) found that the role of α-ketoglutarate and other organic acids in the bacterial system was catalytic and regulatory and not that of a substrate. Once this fundamental point was clarified, the basic similarity of what we now call cyclic photophosphorylation in chloroplasts and in bacterial chromatophores was no longer in doubt (4,11).

Another basic difficulty surrounded attempts to find a noncyclic photophosphorylation in chromatophores. This difficulty seemed at first insurmountable because photosynthetic bacteria never evolve oxygen (12), whereas oxygen evolution was a part of noncyclic photophosphorylation in chloroplasts (Eq. 2). However, Losada, Whatley and Arnon (13) separated noncyclic photophosphorylation with chloroplasts (Eq. 2) into two component photochemical reactions: (a) a photooxidation of water[3] (OH^-) to yield oxygen (Eq. 3), and (b) the noncyclic photophosphorylation reaction proper (Eq. 4)—a reaction not accompanied by oxygen evolution, but one in which ATP formation is coupled with a light-driven "uphill" electron flow to TPN from an exogenous electron donor other than water (OH^-). The two component reactions have been experimentally separated by using an indophenol dye in its reduced and oxidized forms (A^- and A) as an intermediary electron carrier, in accordance with Eqs. 3 and 4:

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[3] Water and OH^- will be used interchangeably. OH^- represents the hydroxyl ion at neutral pH as in the reaction:

$$4OH^- \left(10^{-7}\ N\right) \longrightarrow O_2 + 2H_2O + 4e^-;\ E'_0 = 0.815\ V\ (pH\ 7)$$

$$4OH^- + 4A \xrightarrow{h\nu} O_2 + 4A^- + 2H_2O \quad (3)$$

$$2TPN + 2ADP = 2Pi + 4A^- + 4H^+ \longrightarrow 2TPNH_2 + 2ATP + 4A \quad (4)$$

Sum:

$$2TPN + 2ADP + 2Pi + 4H^+ + 4OH^- \longrightarrow 2TPNH_2 + 2ADP + O_2 + 2H_2O \quad (2)$$

This subdivision of noncyclic photophosphorylation of chloroplasts into two component reactions revealed that Reaction 4, the noncyclic photophosphorylation proper, was basically independent of oxygen evolution and might, therefore, conceivably occur in photosynthetic bacteria. According to this interpretation, noncyclic photophosphorylation in bacteria would lack Reaction 3, the photooxidation of water, which in chloroplasts reduces an intermediate electron carrier, probably plastoquinone (14,15), corresponding to A in Equation 3. In photosynthetic bacteria, the intermediate donor (A^- in Eq. 4) would not come from a photochemical reaction but would be supplied by the external medium, in accord with the well-known dependence of bacterial photosynthesis on such external hydrogen (or electron) donors as thiosulfate or organic acids (12,16).

The existence in photosynthetic bacteria of a noncyclic electron transport pathway was suggested by the evidence in *Chromatium* cells for a light-driven electron flow from thiosulfate as the electron donor to H^+ and N_2 as the electron acceptors, resulting in the evolution of H_2 and reduction (fixation) of N_2, respectively (17,18). Likewise, the photoreduction of pyridine nucleotide by succinate or the ascorbate-DPIP couple in cell-free preparations of *R. rubrum* (19-21) could also be interpreted as evidence for a light-driven noncyclic electron flow in photosynthetic bacteria. There was no evidence, however, that such noncyclic, light-dependent electron transport in photosynthetic bacteria could be coupled with a simultaneous ATP formation.

Evidence for noncyclic photophosphorylation in chromatophores of *R. rubrum* has recently been presented by Nozaki, Tagawa and Arnon (22). They observed bacterial noncyclic photophosphorylation under conditions when cyclic photophosphorylation was experimentally suppressed, thereby making it possible to distinguish the ATP formed by a noncyclic electron flow mechanism from that formed by a cyclic mechanism. In chromatophores of *R. rubrum*, in which cyclic photophosphorylation was made inoperative, ATP formation was coupled with a light-dependent noncyclic electron flow from an ascorbate-DPIP couple as the external electron donor to DPN as the terminal electron acceptor.

This article reports further work on the nature of bacterial cyclic and noncyclic photophosphorylation in *R. rubrum* chromatophores, and considers particularly the experimental conditions needed to demon-

strate a distinction between these two electron pathways in photophosphorylation. The results confirm and extend the evidence for noncyclic photophosphorylation which we reported earlier (22) and do not support the recent contention of Bose and Gest (23) that the ATP formed concurrently with the photoreduction of DPN by the ascorbate-DPIP couple is the result of cyclic photophosphorylation.

METHODS

R. rubrum was grown as previously described (22), at a temperature of about 27°C in 4.5 1. Pyrex glass-stoppered bottles, completely filled to exclude air. A blower was used to prevent overheating of the culture bottles. An inoculum of approximately 1 per cent was used for each bottle.

The inoculum was grown first for about 30 hours as a stab culture in illuminated 100 ml screw-capped tubes, using nutrient agar containing the components of the nutrient solution (22). At the end of this period some cells were transferred to another stab culture tube. The old stab culture tube was filled with a nutrient solution of the same composition, incubated again for 30 hours, and the liquid portion was used as the inoculum. (This technique was based on a suggestion by Dr. D. M. Geller.) At intervals of several weeks the purity of the cultures was checked by growing the cells in Petri dishes on nutrient agar.

The cells were harvested 40-45 hours after inoculation, the cultures yielding then about 4 g of wet packed cells per liter of nutrient solution. The cells were sedimented by centrifugation for 5 min. at 4000 x *g* at 1°C. The supernatant solution was discarded and the sedimented cells were washed once by suspending them in 0.1 M tris/HCl buffer at pH 7.8. The washed cells were collected by centrifugation.

The method of preparing chromatophores under anaerobic conditions was the same as that previously described (22). Washed chromatophore preparations were suspended in 0.1 M tris buffer, pH 7.8, and either used directly or stored at 4°C for several days under argon prior to use. Bacteriochlorophyll, ATP and $DPNH_2$, and lactate were determined as previously described (22). The experiments were carried out in Thunberg-type cuvettes or in Warburg vessels, which were illuminated by incandescent reflector spot lights. All experiments were carried out under argon unless otherwise specified.

When aged chromatophores were used, hexokinase, d-glucose, and a catalytic amount of ADP were added to the reaction mixture as a trapping system for ATP, to prevent loss of ATP by ATPase activity. (The ATPase activity was negligible in fresh chromatophores but gradually increased upon aging.) The "aged" chromatophores were prepared by storage of fresh preparations at 2°C for 30 days under

argon. The ATP or the glucose-6-phosphate formed was determined by the magnesium ammonium phosphate precipitation method (24) or by the siliconized celite column method (25). Crystalline lactate dehydrogenase (from muscle), crystalline alcohol dehydrogenase, antimycin A, and hexokinase (Type III) were purchased from Sigma Chemical Co. Hydrogenase was partially purified from *Desulfovibrio desulfuricans* on a DEAE-cellulose column.

RESULTS

I. CYCLIC PHOTOPHOSPHORYLATION

Inactivation by pretreatment with salt at pH 5.0

Acidification to pH 5, with acetate buffer, resulted in a coagulation of chromatophores without impairment of phosphorylating activity. However, when the acidification was accompanied by the addition of salt, an appreciable loss of phosphorylation occurred. As shown in Table 1, the loss of phosphorylation is due to the high ionic strength at pH 5, and not to the kind of salt. Ionic strength lower than 0.2 at pH 5 or high ionic strength at pH 7.8 had little effect. The inhibitory effect of high salt concentration at pH 5 was observed in the endogenous system as well as in the presence of phenazine methosulfate (Table 1). Attempts to reactivate phosphorylation in the salt-treated chromatophores were unsuccessful.

TABLE 1

Inactivation of cyclic photophosphorylation by pretreatment with salt

Pretreatment with salt	ionic strength	pH	μmoles ATP formed/mg Bchl/hr endogenous	PMS
None	0.05	7.8	140	400
NaAc	0.28	5.0	110	395
NaAc + NH_4Cl	0.63	7.8	-	386
NaAc + NH_4Cl	0.53	5.0	-	22
NaAc + KCl	0.78	5.0	25	36
NaAc + KNO_3	0.53	5.0	-	18

Prior to the photophosphorylation reaction the chromatophores were exposed for 5 min at 0°C to the indicated salt mixture, collected by centrifugation (5 min) and resuspended in 0.1 M tris buffer, pH 7.8. The reaction mixture for photophosphorylation included in a final volume of 3 ml, chromatophores (containing 100 μg bacteriochlorophyll), 1 mg hexokinase and the following in μmoles: tris buffer, pH 7.8, 100; $MgCl_2$, 5; ADP, 0.5; $K_2HP^{32}O_4$, 10; D-glucose, 30; and where indicated, phenazine methosulfate (PMS), 0.3. The reaction was run at 20°C for 15 min in the light (20,000 lux).

Effect of aging and heat treatments

As previously reported (see Table 1 in ref. 5), chromatophores from *R. rubrum,* when freshly prepared under anaerobic conditions, have a complete system for cyclic photophosphorylation. Cyclic photophosphorylation in chromatophores (unlike chloroplasts) proceeds without the addition of exogenous cofactors or water-soluble extracts. When chromatophores were prepared under argon (22), even washing them three times in succession had little effect on phosphorylating activity (cf. 26).

The full phosphorylating activity of chromatophores stored anaerobically (under argon) at ca. 4°C was readily restored to original levels. As shown in Table 2, full phosphorylating activity was restored to chromatophores stored for 30 days by the addition of ascorbate, $DPNH_2$ or phenazine methosulfate. In both fresh and aged chromatophores, phosphorylation, in the presence of ascorbate or $DPNH_2$ but in the absence of phenazine methosulfate, was completely inhibited by antimycin A.

Table 3 shows that chromatophores heated in air for 10 min. at 50°C lost about 60 per cent of their endogenous phosphorylating activity. This loss of activity was restored by the addition of phenazine methosulfate or ascorbate. Heating at 50°C under argon caused no impairment of phosphorylating activity.

It appears that the loss of phosphorylating activity on aging or heating in air is probably the result of the oxidation of one or more electron carriers. Either this oxidation is reversed by the addition of a reductant (ascorbate or $DPNH_2$), or the oxidized site may be bypassed by the addition of phenazine methosulfate.

Effect of redox agents

Horio and Kamen (26) have shown that a high phosphorylation activity of chromatophores depended on the presence of an appropriate amount of ascorbate to provide a redox potential of about 0 volt. At lower concentrations of ascorbate the system was "overoxidized" and

TABLE 2

Restoration of Cyclic Photophosphorylation in Aged Chromatophores

Additions	μmoles ATP/formed/mg Bchl/hr fresh	aged
None	135	41
5×10^{-3} M ascorbate	200	135
3×10^{-3} M $DPNH_2$	137	145
10^{-4} M phenazine methosulfate	352	304

Illumination and components of the phosphorylation reaction mixture were the same as given in Table 1.

TABLE 3

Effect of Heat Treatment on Cyclic Photophosphorylation

Additions	μmoles ATP formed/mg Bchl/hr Heated in air	Heated in argon	Control
none	77	201	192
ascorbate	176	204	203
phenazine methosulfate	307	328	307

Heat pretreatment was for 10 min at 50°C. The final concentration of ascorbate was 5 x 10^{-3} M. The conditions and components of the phosphorylation reaction mixture were the same as given in Table 1.

at higher concentrations the system became "overreduced" for optimum photophosphorylation. The effect of a particular ascorbate concentration was influenced by the gaseous atmosphere. Thus, an overreducing effect of a high concentration of ascorbate under anaerobic conditions was mitigated by the admission of air (cf. also 23).

As shown in Table 4, we have confirmed the effect of ascorbate concentration in argon and in air on cyclic photophosphorylation. ATP formation in air or argon was strongly inhibited by antimycin A. No appreciable antimycin A-resistant photophosphorylation occurred with the addition of ascorbate alone at any of the ascorbate concentrations tested (10^{-5} to 10^{-2} M).

TABLE 4

Effect of Ascorbate and Antimycin A on Cyclic Phosphorylation under Argon and Air

Ascorbate concentration	μmoles of ATP formed/mg Bchl/hr Argon	Argon, antimycin A present	Air	Air, antimycin A present
none	182	0.4	37	0.7
10^{-5} M	248	0.4	57	0.6
10^{-4} M	332	1.3	86	0.4
10^{-3} M	373	2.0	160	1.0
10^{-2} M	178	2.2	224	1.2

50 μg bacteriochlorophyll was used in all cases. Other components of the photophosphorylation reaction mixture were the same as given in Table 1. Where indicated, 10 μg of antimycin A was added.

Table 5 shows that the addition of $DPNH_2$ fully restored the phosphorylating activity of aged chromatophores. Although $DPNH_2$ is a stronger reducing agent than ascorbate, it appears that the addition of $DPNH_2$ alone, even at a concentration as high as 1.7×10^{-2} M, cannot result in a pronounced overreduction of the phosphorylating system.

TABLE 5

Effect of $DPNH_2$ on Cyclic Photophosphorylation by Fresh and Aged Chromatophores

Chromatophores	$DPNH_2$ concentration	μmoles ATP formed/mg Bchl/hr
Fresh	none	135
"	3.3×10^{-3} M	137
Aged	none	69
"	3.3×10^{-6} M	107
"	3.3×10^{-5} M	138
"	3.3×10^{-4} M	167
"	3.3×10^{-3} M	162
"	1.7×10^{-2} M	140

The reaction mixture for fresh chromatophores lacked the hexokinase system but contained instead 10 μmoles ADP. Other conditions and components of the phosphorylation reaction mixture were the same as given in Table 1.

Overreduction with $DPNH_2$, at the relatively low concentration of 3.3×10^{-3} M, was observed, however, in the presence of methyl viologen (Table 6). Similar results were obtained in the presence of benzyl viologen, but DPIP was not effective. Table 6 also shows that the inhibitory effect on photophosphorylation by overreduction with ascorbate was markedly enhanced by the presence of DPIP or phenazine methosulfate. Overreduction by H_2 plus hydrogenase was greatly enhanced by the addition of methyl (or benzyl) viologen (Table 6); in this system, as with $DPNH_2$, DPIP was much less effective. That these dyes have indeed enhanced overreduction is indicated by the reversibility of their effects on admission of air to the system (Experiments B and C in Table 6).

These results suggest that these dyes have a strong affinity for the phosphorylating system of chromatophores into which they promote an electron flow not only from a moderately reactive reductant such as $DPNH_2$ but also from the more reactive ascorbate.

The affinity of phenazine methosulfate and DPIP for the phosphorylating system of chromatophores is so strong that, under certain conditions, they catalyze cyclic photophosphorylation in the presence of

TABLE 6

Inhibition of Cyclic Photophosphorylation by Dyes under Reducing Conditions

Experiment	Treatment	μmoles ATP formed/mg Bchl/hr
A	3.3 x 10^{-4} M $DPNH_2$	137
	10^{-4} M methyl viologen	154
	$DPNH_2$ + methyl viologen	7
B	6.7 x 10^{-3} M ascorbate	239
	6.7 x 10^{-5} M 2,6-dichlorophenol indophenol (DPIP) + 6.7 x 10^{-3} M ascorbate	9
	ascorbate, DPIP, air	240
C	10^{-4} M phenazine methosulfate (PMS)	314
	10^{-4} M PMS + 6.7 x 10^{-3} M ascorbate	9
	PMS, ascorbate, air	240
D	H_2	146
	H_2 + hydrogenase	55
	H_2 + hydrogenase + 10^{-4} M methyl viologen	2

A 16-day old chromatophore preparation was used in Experiments B and C. Other conditions and components of the phosphorylation mixture were the same as given in Table 1. Anaerobic conditions were employed except as otherwise indicated.

antimycin A (10,23). It seems likely that DPIP, like phenazine methosulfate (10), acts as a bypass agent around an antimycin A-sensitive site in cyclic photophosphorylation (cf. 23).

The ability of these dyes to act as bypass agents for the antimycin A-sensitive site of cyclic photophosphorylation is influenced by the redox status of the system. As shown in Table 7, phenazine methosulfate did not bypass antimycin A inhibition in the presence of 5 x 10^{-3} M ascorbate. Likewise, DPIP failed to catalyze an antimycin A-insensitive phosphorylation in the presence of 10^{-3} M ascorbate.

The influence of ascorbate concentration on the bypass effect of DPIP is of special relevance to our subsequent discussion. As shown in Table 7, DPIP catalyzed an appreciable antimycin A-insensitive cyclic photophosphorylation in the presence of 10^{-4} M ascorbate. However, this cyclic photophosphorylation was abolished at concentrations of ascorbate of 10^{-3} M or higher. Attention is called to these experimental conditions, since, as will be shown later, an antimycin A-insensitive noncyclic photophosphorylation was measured in the presence of DPIP but at concentrations of ascorbate at which cyclic photophosphorylation is excluded (Table 7).

TABLE 7

Reactivation of Antimycin A-Inhibited Cyclic Photophosphorylation by Dyes

Experiment	Treatment	Per cent of phosphorylating activity
A	Control	6
	Phenazine methosulfate (PMS)	96
	PMS + 5 x 10^{-3} M ascorbate	3
B	Methyl viologen (MV)	1
	MV + DPIP + 6.7 x 10^{-3} M ascorbate	145
C	DPIP	2
	DPIP + 10^{-5} M ascorbate	1
	DPIP + 10^{-4} M ascorbate	22
	DPIP + 10^{-3} M ascorbate	2

The phosphorylating activity of a similar system without antimycin A was designated as 100 per cent. 10 μg of antimycin A was included with all the treatments shown above. Where the dyes were added their respective concentrations were: phenazine methosulfate, 6.7 x 10^{-5} M; methyl viologen, 10^{-4} M; 2,6-dichlorophenol indophenol (DPIP), 6.7 x 10^{-5} M. Fresh chromatophores were used in Exper. B, 4-day old chromatophores in Exper. A and 30-day old chromatophores in Exper. C. Other conditions and components of the phosphorylation mixture were the same as given in Table 1.

II. PYRIDINE NUCLEOTIDE REDUCING SYSTEM IN CHROMATOPHORES

Frenkel (19) and Vernon and Ash (20,21) have shown that chromatophores of *R. rubrum* can photoreduce DPN by succinate, reduced FMN or the ascorbate-DPIP couple. We have confirmed the photoreduction of DPN, using succinate or the ascorbate-DPIP couple as the electron donor (22). The reduction of DPN by chromatophores is a photochemical reaction; our attempts to replace light with ATP were unsuccessful. $DPNH_2$ is more reducing than the electron donors used, and photons absorbed by the photosynthetic pigments supply the additional energy needed to drive the electron transfer against the thermodynamic gradient, by what appears to be a light-induced noncyclic electron flow.

Washed chromatophores were found to be highly specific in their ability to photoreduce DPN; TPN was not reduced. The addition of ferredoxin from *R. rubrum*, *Chromatium*, or spinach (27) did not change the rate or specificity of DPN photoreduction. However, we found that washed chromatophores were able to photoreduce TPN as rapidly as DPN when the reaction mixture included a water-soluble extract of *R. rubrum* cells (the supernatant solution from the chromatophore preparation).

The TPN-reducing factor in the water-soluble extract was found to be heat stable and was identified as DPN by paper chromatography after isolation with the aid of a Duolite A-2 resin (28). Fig. 1 shows that chromatophores photoreduced TPN in the presence of either catalytic amounts of DPN or the boiled water-soluble extract of *R. rubrum* cells.

The fact that TPN reduction was mediated by catalytic amounts of DPN indicated that the chromatophores contained a transhydrogenase. This conclusion is supported by the results shown in Fig. 2. Transhydrogenase activity in washed chromatophores was determined by measuring the TPN reduced *in the dark* (as a change in optical density at 340 mμ) in the presence of an added alcohol dehydrogenase system and catalytic amounts of DFN. TPN was not reduced without DPN. DPN was added either as the pure chemical or as a cell extract, i.e., the boiled and Duolite A 2-treated supernatant solution from the chromatophore preparation.

III. NONCYCLIC PHOTOPHOSPHORYLATION

As already mentioned, we have interpreted the photoreduction of DPN as evidence for a noncyclic electron flow in *R. rubrum* chromatophores. To demonstrate noncyclic photophosphorylation in chromatophores, it was necessary to establish that the photoreduction of DPN was accompanied by ATP formation under conditions such that ATP formation by cyclic photophosphorylation was excluded.

Effect of antimycin A

Photoreduction of DPN by succinate is inhibited by antimycin A (22). However, antimycin A does not inhibit the photoreduction of DPN by the ascorbate-DPIP couple (22). It should be noted that the photoreduction of DPN by the ascorbate-DPIP couple in the presence of antimycin A, which we reported previously (see Fig. 2 in ref. 22), occurred at a concentration of ascorbate (6.7×10^{-3} M) at which cyclic photophosphorylation catalyzed by DPIP is suppressed (Table 7). Thus, the ATP formation (shown again in Table 8) which could not have occurred via cyclic photophosphorylation because of the high ascorbate concentration, could only have resulted from noncyclic photophosphorylation.

Requirement for electron donor and acceptor

Table 8 shows ATP formation by chromatophores in the presence of antimycin A, DPIP, and 6.7×10^{-3} M ascorbate. Appreciable photophosphorylation occurred only in a complete noncyclic electron transport system, i.e., in the presence of both an electron donor (ascorbate + DPIP) and an electron acceptor (DPN). Little photophosphorylation occurred when either the electron donor or the electron acceptor, or both, were omitted. Table 8 also shows that little photophosphorylation

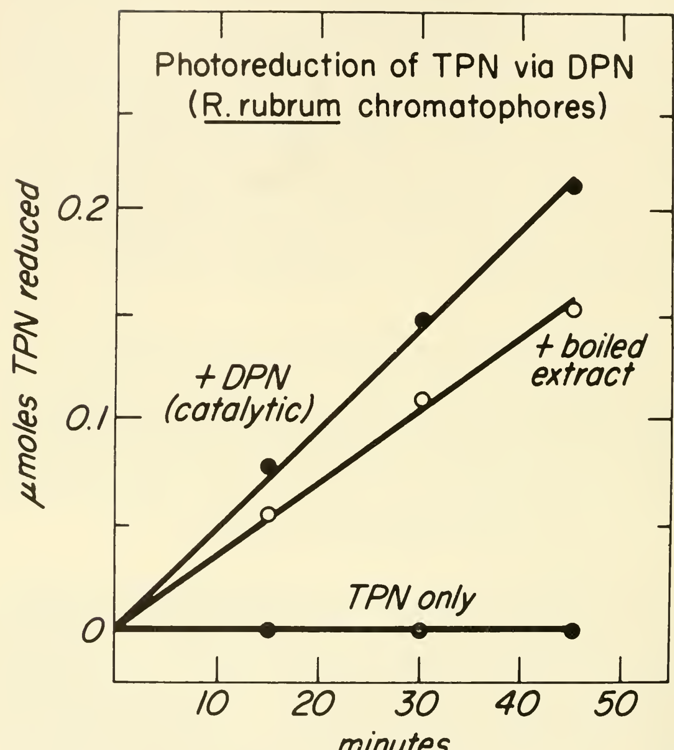

Fig. 1. Photoreduction of TPN via DPN by *R. rubrum* chromatophores. The reaction mixture included, in a final volume of 3 ml, chromatophores (containing 30 μg bacteriochlorophyll) and the following (in μmoles): tris buffer, pH 7.9, 100; magnesium chloride, 5; sodium ascorbate, 20; 2,6-dichlorophenolindophenol, 0.2; TPN, 2. Also added was 0.05 μmole DPN or boiled extract (see text) as indicated. The reaction was carried out in Thunberg type cuvettes at 20°C. TPN reduction was measured at 15 min intervals as the increase in optical density at 340 mμ. Illumination, 10,000 lux.

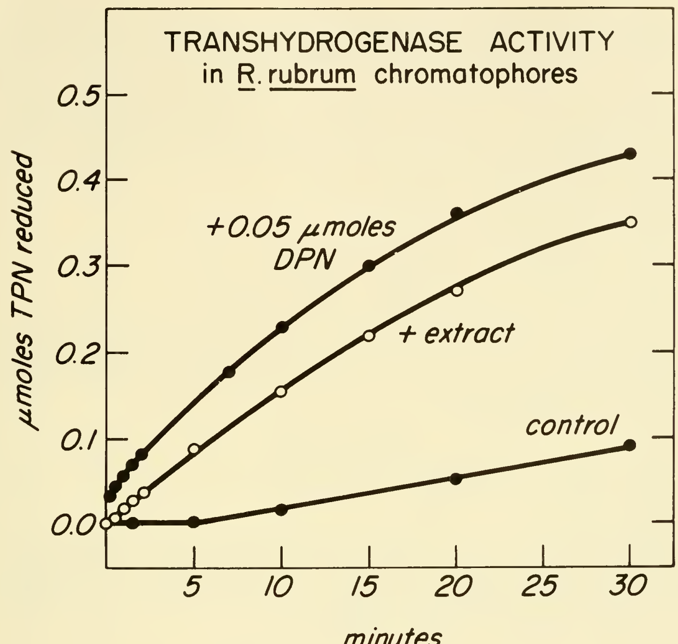

Fig. 2. Transhydrogenase activity in *R. rubrum* chromatophores. The reaction mixture contained, in a final volume of 3 ml, alcohol dehydrogenase, chromatophores (containing 32 μg bacteriochlorophyll) and the following (in μmoles): tris buffer, pH 7.9, 100; magnesium chloride, 5; ethanol, 170; TPN, 2. Also present was 0.05 μmoles DPN or extract (see text) as indicated. The reaction was carried out in Thunberg type cuvettes at room temperature in the *dark*.

occurred in a complete system in which the photoreduction of DPN was inhibited by the addition of the inhibitor phenyl mercuric acetate (22).

Stoichiometry

The stoichiometry of the ATP formed and the DPN reduced in the course of noncyclic photophosphorylation is shown in Table 9. The theoretical ratio of $ATP/DPNH_2 = 1$ was obtained when the $DPNH_2$ formed was trapped by an added lactate dehydrogenase system. Without the lactate dehydrogenase system, the ratio $ATP/DPNH_2$ was

TABLE 8

Noncyclic Photophosphorylation

	Additions	μmoles ATP formed/mg Bchl/hr
Complete system	Ascorbate, DPIP,DPN	44.8
Electron acceptor omitted	Ascorbate, DPIP	5.4
Electron donor omitted	DPN	7.6
Electron donor and acceptor omitted	none	5.0
Complete system, PMA inhibited	Ascorbate, DPIP,DPN, PMA	6.2

All vessels contained 10 μg antimycin A. The final concentrations of the added components were: ascorbate, 6.7 x 10^{-3} M; 2,6-dichlorophenol indophenol (DPIP), 6.7 x 10^{-5} M; DPN, 6.7 x 10^{-4} M; and phenyl mercuric acetate (PMA), 10^{-4} M. Other conditions and components of the phosphorylating mixture were the same as given in Table 1.

greater than one, suggesting that the chromatophores might have contained a $DPNH_2$ reoxidizing system.

DPN dependence

Since the concept of noncyclic photophosphorylation (22) envisages that the ATP formed by this pathway is obligatorily coupled with an electron flow from the ascorbate-DPIP couple to DPN, ATP formation

TABLE 9

Stoichiometry of ATP and $DPNH_2$ Formed in Noncyclic Photophosphorylation

	In the presence of DPNH trapping system		In the absence of DPNH trapping system	
Time (min)	ATP formed (μmoles)	$DPNH_2$ formed (μmoles)	ATP formed (μmoles)	$DPNH_2$ formed (μmoles)
10	0.38	0.35	—	0.16
20	0.60	0.57	0.55	0.29
30	0.81	0.73	—	0.43
40	0.97	0.93	0.95	0.53

The reaction mixture included, in a final volume of 3 ml, chromatophores containing 40 μg of bacteriochlorophyll, and the following in μmoles: tris buffer, pH 7.8, 100; $MgCl_2$, 5; ADP, 5; $K_2HP^{32}O_4$, 5; ascorbate, 20; 2-6 dichlorophenol indophenol, 0.2; DPN, 5. Where the DPN trapping system was used, DPN was reduced to 0.2 μmoles, and 10 μmoles of pyruvate and 25 μg of lactate dehydrogenase were added. All vessels contained 10 μg of antimycin A. The reaction was run in Thunberg type cuvettes at 20°C in the light (10,000 lux).

should stop when DPN is completely reduced. But such a DPN-dependent formation would not be expected if the ATP formed in the experiments represented by Tables 8 and 9 is the result of a DPIP-catalyzed cyclic photophosphorylation.

Fig. 3 shows a dependence of ATP formation on DPN availability that is consistent with the mechanism of noncyclic photophosphorylation. A limited amount of DPN (0.3 μmoles) was used as the electron

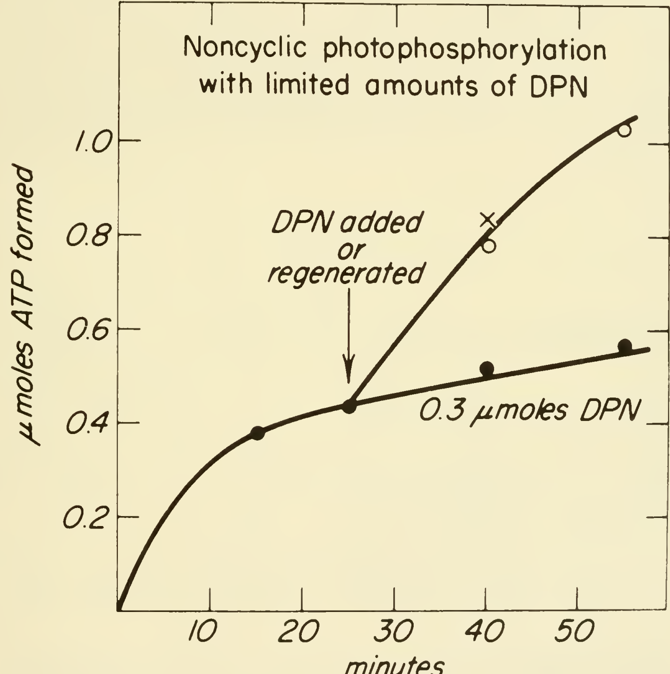

Fig. 3. Dependence of noncyclic photophosphorylation on DPN. The reaction mixture contained, in a final volume of 3 ml, 1 mg hexokinase, 10 μg antimycin A, chromatophores (containing 45 μg bacteriochlorophyll) and the following (in μmoles): tris buffer, pH 7.9, 100; magnesium chloride, 5; ADP, 0.5; $K_2HP^{32}O_4$, 10; sodium ascorbate, 20; 2,6-dichlorophenol indophenol, 0.2; DPN, 0.3. Additional DPN (2 μmoles) or a DPN regenerating system (lactate dehydrogenase plus 10 μmoles sodium pyruvate) was added at the time indicated by arrow.

acceptor for noncyclic photophosphorylation. After this quantity of DPN was completely reduced, the rate of ATP formation greatly decreased. However, when substrate amounts of DPN or a DPN-regenerating system was then added to the reaction mixture, the rate of ATP formation became greatly accelerated.

Effect of aging

Another line of evidence which supports the existence of both cyclic and noncyclic photophosphorylation in chromatophores is the differential stability of these two systems to aging. As was already shown in Table 5 and is again demonstrated in Table 10, chromatophores retained a capacity for cyclic photophosphorylation after at least 30 days of storage; such decrease in activity as occurred during this period was fully restored by the addition of ascorbate. By contrast, Table 10 shows that chromatophores stored for 30 days lost completely the capacity for noncyclic photophosphorylation and that this loss was irreversible.

TABLE 10

Loss of Noncyclic Photophosphorylation in Aged Chromatophores

Photophosphorylating system	Additions	μmoles of ATP formed/mg Bchl/hr: fresh chromatophores	μmoles of ATP formed/mg Bchl/hr: aged chromatophores
Noncyclic	ascorbate, DPIP, DPN, antimycin A	43	2
Cyclic	none	133	41
Cyclic	ascorbate (5×10^{-3} M)	–	135
Inhibited cyclic	antimycin A (10 μg)	9	5

Noncyclic photophosphorylation was carried out under the same conditions as given in Table 8. Experimental conditions and reaction mixture for cyclic photophosphorylation are given in Table 1.

We have suggested elsewhere (4,5) that cyclic and noncyclic photophosphorylation in chloroplasts and, by extension, in chromatophores, share a common site for ATP formation. If this hypothesis is correct, it would follow that the loss of noncyclic photophosphorylation on storage is the result of the inactivation of the DPN-reducing system without which ATP formation could not occur by the noncyclic pathway, but which would not affect ATP formation by the cyclic pathway. This interpretation is consistent with the experimental findings. Chromatophores kept at ca 4°C for a week lost more than 90 percent of their

capacity for DPN reduction but, as mentioned previously, they retained their capacity for cyclic photophosphorylation even after storage for 30 days.

DISCUSSION

The results of this investigation confirm and extend the previous finding of noncyclic photophosphorylation in chromatophores of *R. rubrum* (22). The capacity of *R. rubrum* chromatophores to catalyze a noncyclic electron flow, i.e., a light-driven, "uphill," unidirectional electron transfer against the thermodynamic gradient, has already been seen in the photoreduction of DPN by the ascorbate-DPIP couple (19-21). Our experimental findings provide evidence that this noncyclic electron flow in chromatophores is coupled with ATP formation under experimental conditions which exclude ATP formation by cyclic photophosphorylation.

Noncyclic photophosphorylation is distinguished from cyclic photophosphorylation in chromatophores by its joint dependence on an external electron donor system (ascorbate-DPIP couple) and an external electron acceptor (DPN). Losada et al. showed (13) that a similar "bacterial type" of noncyclic photophosphorylation, in which the ascorbate-DPIP couple is the electron donor and TPN is the electron acceptor, can be carried out by spinach chloroplasts once the use of the natural electron donor for chloroplasts, water (OH^-), is experimentally suppressed.

Bose and Gest have recently argued (23) that the noncyclic photophosphorylation which we have previously found in *R. rubrum* chromatophores is, in fact, a cyclic photophosphorylation catalyzed by the dye DPIP, which acts as a bypass for the antimycin A-sensitive site. They explain the joint requirement for an added reductant (ascorbate + DPIP) and oxidant (DPN) as resulting from "their action in establishing a redox environment which permits efficient operation of cyclic LIP [photophosphorylation]" (23).

The experiment reported by Bose and Gest (Exp. II, Table 6 in ref. 23) which comes closest to ours was carried out under hydrogen gas, in the presence of 1 μmole DPN, 0.2 μmoles DPIP and 0.2 μmoles of ascorbate (in 3 ml), i.e., under conditions where the system was not overreduced. The relevance of this experiment and the accompanying arguments to our previous experiments and to those reported now is not apparent. Our experiments (for example, Table 2 in ref. 22) were carried out under argon gas, in the presence of 2 μmoles DPN, 0.2 μmoles DPIP, and 20 μmoles ascorbate (in 3 ml), i.e., in the presence of 100 times more ascorbate than used by Bose and Gest, thereby bringing about a degree of "overreduction" which suppresses the cyclic photophosphorylation that might otherwise have been promoted by

DPIP. In our experiments ascorbate was present in such great excess that it served not only as an inhibitor of cyclic photophosphorylation but also as an electron donor for noncyclic photophosphorylation.

As discussed previously (18), photosynthesis in plants and bacteria is now seen as having in common two photochemical processes, cyclic and noncyclic photophosphorylation. However, the bacterial noncyclic photophosphorylation never produces oxygen—a consequence of the inability of the bacterial system to use water as the electron donor.

It seems reasonable to consider that photosynthesis in plants and bacteria depends to a different degree on cyclic and noncyclic photophosphorylation. In conventional plant photosynthesis, noncyclic photophosphorylation would appear to be the dominant photochemical process since, apart from its contribution of ATP, it is the exclusive mechanism for bringing about a hydrogen transfer (via TPN) from water to CO_2. The role of cyclic photophosphorylation in plants would thus appear to be that of supplementing the ATP needs for carbon assimilation which are not fully met by noncyclic photophosphorylation.

In bacterial photosynthesis, on the other hand, cyclic photophosphorylation seems to be the dominant photochemical process, because it provides a most effective anaerobic mechanism for generating ATP for biosynthetic purposes. Photosynthetic bacteria, unlike plants, have no exclusive dependence on a photochemical reaction for the generation of reduced pyridine nucleotide. With some bacterial electron or hydrogen donors, as for example, with hydrogen gas or malate, the reduction of pyridine nucleotide requires no input of light energy; it can proceed with the aid of appropriate enzyme systems in the dark (5,11). However, with certain other electron donors, such as thiosulfate or succinate, an input of light energy becomes necessary for the reduction of pyridine nucleotide, and in such cases a noncyclic electron flow with an accompanying ATP formation would become a component of bacterial photosynthesis.

SUMMARY

Features of cyclic and noncyclic photophosphorylation in chromatophore preparations of *R. rubrum* were investigated with special reference to the experimental conditions needed to demonstrate a distinction between these two electron pathways in bacterial photophosphorylations.

Noncyclic photophosphorylation was demonstrated in a system in which the ascorbate-dichlorophenolindophenol couple served as the electron donor and DPN as the electron acceptor. Cyclic photophosphorylation under these conditions was suppressed by the presence of antimycin A and an excess of ascorbate.

Chromatophores retained a capacity for cyclic photophosphorylation even after extended storage but lost irreversibly the capacity for noncyclic photophosphorylation after storage for a few days. This loss was associated with an inactivation of the DPN reducing system.

Chromatophores, unlike chloroplasts, photoreduce DPN but not TPN. However, chromatophores contain a $DPNH_2$-TPN transhydrogenase and photoreduce TPN in the presence of catalytic amounts of DPN.

The effect of ascorbate concentration was investigated in relation to the use of dichlorophenolindophenol, methyl viologen and phenazine methosulfate as "bypass agents" for cyclic photophosphorylation when it was inhibited by antimycin A.

Ascorbate, reduced DPN and H_2 plus hydrogenase were compared, in the presence and absence of dyes, as regulators of the oxidation-reduction state during cyclic photophosphorylation.

Cyclic photophosphorylation was found to be irreversibly inactivated by a salt treatment at pH 5.

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PHOTOPHOSPHORYLATION IN *RHODOSPIRILLUM RUBRUM*. ABOUT THE ELECTRON TRANSPORT CHAIN AND THE PHOSPHORYLATION REACTIONS

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INTRODUCTION

It has long been recognized that the phosphorylation reactions in the light-induced production of ATP in photosynthesis are closely linked to electron transport. The various results in the field of electron transport and phosphorylation which led to a realization of this circumstance caused what may be regarded as an intrusion into the area of the classical union between photochemistry and carbohydrate metabolism in photosynthesis. Today many agree that the following light-induced sequence of events may occur in all photosynthetic organisms:

1. The photochemical reactions.
2. The electron transport-linked photophosphorylation.
3. The assimilation of carbon dioxide.

In this presentation some aspects of the electron transport and phosphorylation reactions of photophosphorylation in isolated chromatophores from the photosynthetic bacterium *Rhodospirillum rubrum* will be discussed in general and, where it appears to be justified, in specific terms.

ABOUT THE ELECTRON TRANSPORT CHAIN

High rates of cyclic photophosphorylation can be obtained in isolated bacterial chromatophores without any added electron carriers. In contrast to isolated chloroplasts from green plants, where so far the cyclic process has been found to occur only in the presence of such added compounds, the bacterial system is thus particularly suitable for studies of the physiological electron carriers and carrier sequences which participate in the cyclic photophosphorylation process.

Frenkel's (1) discovery in 1954 of light-induced phosphorylation in extracts from *R. rubrum* led to the immediate question: is the process linked to electron transport? Experimental support for this possibility was first reported by Smith and M. Baltscheffsky (2,3), who demonstrated that low concentrations of 2-*n*-heptyl-4-hydroxyquinoline-N-oxide (HOQNO), which were known to specifically inhibit mitochondrial electron transport, strongly inhibited photophosphorylation, thereby causing an oxidation of cytochrome c_2 and a probable reduction of a *b*-type cytochrome. These findings indicated the following similarity between electron transport reactions in mitochondrial oxidative phosphorylation and bacterial photophosphorylation:

$$\rightarrow b\text{-type cytochrome} \xrightarrow{\text{HOQNO}} c\text{-type cytochrome} \rightarrow$$

Additional evidence for the participation of cytochrome c_2 in bacterial photophosphorylation was recently obtained by Horio and Kamen (4), and of both cytochrome *b* and cytochrome c_2 by Nishimura (5).

Another similarity became apparent, when one of us (H. B.) obtained results indicating that a flavoprotein functions as electron carrier in the cyclic photophosphorylation system of *R. rubrum* (6). In brief, the evidence was: 1) stimulation of photophosphorylation by rather high concentrations of FAD, 2) inhibition by rather low concentrations of atebrin and 3) reversal of the atebrin-induced inhibition by rather high concentrations of FAD. It was also shown that both the basal rate of photophosphorylation and the stimulation caused by added FAD were strongly inhibited by HOQNO and antimycin A (Table 1 in reference 6). This eliminated the possibility that a nonphysiological bypass had occurred from the added FAD to cytochrome c_2 (Horio and Kamen (7) have recently emphasized that such bypass reactions may occur at high concentrations of FAD). Our data thus suggested resemblance between bacterial photophosphorylation and mitochondrial oxidative phosphorylation at the flavin level, giving experimental support for the occurrence of the following electron transport sequence in both systems:

$$\rightarrow \text{flavoprotein} \rightarrow b\text{-type cytochrome} \xrightarrow{\text{HOQNO}} c\text{-type cytochrome} \rightarrow$$

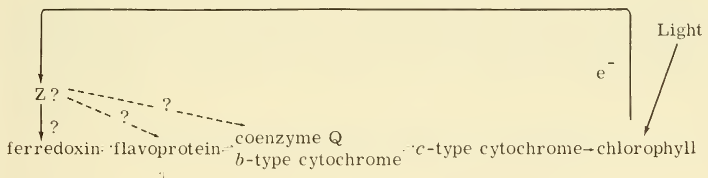
The first direct evidence for a photochemical reaction sequence

$$c\text{-type cytochrome} \longrightarrow \text{chlorophyll}$$

was obtained with the photosynthetic bacterium *Chromatium* by Chance and Nishimura (8), who demonstrated that this reaction is temperature-independent from 80° to 298°K. Recently, Clayton (9) reported that

coenzyme Q can be photochemically reduced and thus may be a primary electron acceptor in isolated chromatophores of photosynthetic bacteria. The presence of the electron carrier ferredoxin (the plant enzyme has long been known as photosynthetic pyridine nucleotide reductase or methemoglobin reducing factor) in photosynthetic bacteria has been reported by Tagawa and Arnon (10). To further compare bacterial photophosphorylation with mitochondrial oxidative phosphorylation, one may endeavour to state that the site of cytochrome *c* oxidase in oxidative phosphorylation is occupied by chlorophyll in bacterial photophosphorylation, where the pigment in the light may serve as a "cytochrome c_2 photooxidase." While the general picture on the oxidizing side of the chain today appears to be quite similar to its mitochondrial counterpart, the present knowledge about the electron transport sequence at the reducing side is still rather limited. For example: is ferredoxin or coenzyme Q or another compound the primary acceptor of electrons from chlorophyll, or may more than one agent act in this capacity?

The present uncertainty in this region is reflected in the multiple choices of our very tentative scheme for the electron transport reactions in cyclic photophosphorylation of chromatophores from *R. rubrum:*



Recently one of us (H. B.) has tried to outline what similarities and differences one may find between the above and various other electron transport pathways (11). That outline and the above discussion may be taken as an expression of our conviction that the variability observed between different systems for biological electron transport is, in the final analysis, due to nothing but variations on a general and basically common theme.

ABOUT THE PHOSPHORYLATION REACTIONS

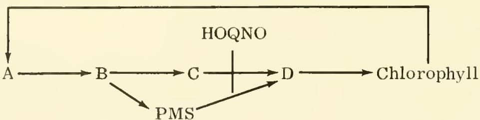
The fact that one may inhibit the "physiological" cyclic electron transport chain in chromatophores from *R. rubrum* with site-specific

agents such as HOQNO (2) and antimycin A (12) and obtain a bypass around the site of inhibition by adding phenazine methosulfate (12,13) (PMS, methyl-phenazonium sulfate) has made it possible to investigate the question of whether one or several phosphorylation sites are linked to the electron transport. Our evidence for the presence of two sites in the "physiological" chain and one in the "PMS" chain has been given (14-17). The discussion of the phosphorylation reactions will be limited to some possible implications of the results which we obtained with the uncoupling agent valinomycin, which is known to uncouple the phosphorylation at all three phosphorylation sites in the electron transport system in animal mitochondria (18), but which appears to uncouple only one of two existing phosphorylation sites in cyclic photophosphorylation.

Fig. 1 shows our general interpretation of the effects obtained with valinomycin on bacterial photophosphorylation (14-16). If this interpretation is correct, there is a valinomycin-sensitive phosphorylation site in a region of the cyclic electron transport chain containing only dark electron transport reactions and a valinomycin-insensitive phosphorylation site in a region which contains the photochemical reactions and possibly one or several dark electron transport steps.

It may well be more than a coincidence that, when sensitivity to valinomycin is used as an indicator, the bacterial photophosphorylation reactions are similar to those in oxidative phosphorylation in the region where only dark reactions occur, but different in the region where the photochemical reactions are localized. Obviously, a possible site

(The arrows indicate the direction of the electron transport. A, B, C and D indicate physiological electron carriers.)



Valinomycin-sensitive phosphorylation linked to the region:

B ⟶ C ⟶ D

Valinomycin-insensitive phosphorylation linked to the region:

D ⟶ Chlorophyll ⟶ A ⟶ B

Fig. 1. Interpretation of the effects obtained with valinomycin on bacterial photophosphorylation.

for this difference in a photosynthetic system is in the region of its unique component, the chlorophyll. The fact that a valinomycin-insensitive phosphorylation appears to be localized in the same part of the cyclic electron transport pathway as the chlorophyll brings into focus a recent suggestion by Calvin (19) that chlorophyll might be a site for an ATP-producing reaction pattern. He considered the possibility that light energy would allow the 9-10 enol in chlorophyll to react with orthophosphate and that a subsequent dehydration reaction would give the necessary energy-rich configuration

$$\overset{|}{\underset{|}{C}} = \overset{|}{C} - O - \overset{\overset{O}{\|}}{\underset{\underset{O^-}{|}}{P}} - OH$$

to allow a phosphorylation of ADP to ATP.

It is tempting to speculate that if such a reaction pattern does exist the rate of phosphate addition to the enol group and thus also the rate of ATP-formation at the chlorophyll level could be determined by the rate of electron transport at another part of the chlorophyll molecule (for example, at the δ-bridge carbon atom (20)) over the conjugated double-bond system. Phosphorylation would here not be "coupled" to electron transport in the classical sense, but only "linked" to it to give a rate-dependency. The phosphorylation reactions at such a site may well have properties which are quite different from an ordinary electron transport-coupled phosphorylation, as, for example, insensitivity to valinomycin.

CONCLUDING REMARKS

Our knowledge today about the electron transport and phosphorylation reactions in bacterial photophosphorylation is far from complete. If indeed, as our evidence indicates, there are two phosphorylation sites in the cyclic electron transport chain of *R. rubrum*, and if a difference in response to valinomycin means a basically different reaction pattern in an energy-transfer step, then it would seem to be important that the material under investigation should be as active and intact as possible in order to minimize erroneous results due to partial and perhaps selective inactivation.

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LIGHT-INDUCED AND DARK STEPS OF BACTERIAL PHOTOPHOSPHORYLATION

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INTRODUCTION

The analysis of light-induced and oxygen-activated absorption changes in purple bacteria (1-9) and studies of heme proteins isolated from these organisms (10-17) have indicated that several electron transfer catalysts are functioning in light- and oxygen-activated oxidation-reduction systems. If photophosphorylation is coupled with the oxidation-reduction reactions between these electron carriers, there should exist at least two distinct phases in the process of photophosphorylation, namely, a light-induced primary step and light-independent dark processes (electron transfer and ATP synthesis).

The flashing light technique has been effective in studying the kinetics of photophosphorylation and distinguishing between the primary light-induced step and the dark processes. By this technique, it was observed that photophosphorylation took place in the dark after short, flashing illumination. This "delayed" process of photophosphorylation was affected by certain reagents and temperature. Analysis of the delayed photophosphorylation, combined with spectroscopic studies of the cytochrome system, revealed the presence of two steps in the delayed photophosphorylation; viz., electron transfer and coupled phosphorylation.

When the dark periods between flashes are sufficiently long, the amount of delayed photophosphorylation per flash is indicative of the amount of substance reacting during the flash. The maximal amount of the delayed process per flash is unaffected by temperature or chemical reagents. From the maximal amount of delayed photophosphorylation observed, a value of two was suggested as the tentative number of ATP molecules formed per electron transfer in the oxidation-reduction chain. The comparison of relative quantum efficiencies in the presence of inhibitors and an activator (MPM) is also suggestive of two phosphorylating sites. Some of the data in this paper have been published (18-20).

MATERIAL AND METHODS

Chromatophores of the purple bacterium *Rhodospirillum rubrum* were isolated as reported previously (18). The rate of photophosphorylation was measured under near-infrared illumination with a recording pH meter (21), or by phosphate analysis in the reaction medium (18).

The light source used was a direct-current incandescent lamp used in conjunction with an infrared filter (Wratten 88A) and a water layer (5 cm thick). This filter combination passed near-infrared illumination longer than 720 mμ in wavelengths. The light intensities used were all above the level of saturation under continuous illumination, unless otherwise stated. Flashing illumination was furnished by a rotating sector (18).

For the experiments on the maximum amount of ATP formation by a single flash, a xenon flash tube (flash duration 0.5 msec) was triggered at intervals of 60 sec. The light from the flash tube was filtered through two Wratten 88A infrared filters.

RESULTS

Presence of delayed photophosphorylation after flash

In the first type of experiments, the duration of light period was kept constant at 1.45 msec, and lengths of dark periods were changed. When the rates of phosphorylation were expressed in terms of phosphate esterified per illuminated time, we observed a remarkable rise in the rate of phosphorylation with increasing dark period, indicating the presence of delayed photophosphorylation after the flash. From the curves (rate per illuminated time vs. dark period), we calculated by differentiation the rate of phosphorylation after the flash, as shown in Fig. 1. It was found that the decay of delayed photophosphorylation was dependent on light intensity. The half-life of decay was shorter with low intensity flashes and was longer with strong flashes.

Light-induced phase of photophosphorylation

The rapid light-induced reaction which takes place in the short flash was studied in the second type of experiment, where sufficiently long dark periods were inserted between short flashes (181-1449 μsec), and the duration of a light-dark cycle was kept constant at 8.70 msec. The amount of phosphate esterified per minute per bacteriochlorophyll was plotted against the flash duration (Fig. 2). When the length of flashes was sufficiently short, the rate of phosphorylation was proportional to the flash length. The rate was greater under flashes of high light intensity, but under continuous illumination the rate was identical with these three light intensities (indicated in Fig. 2 by 8.70 msec light

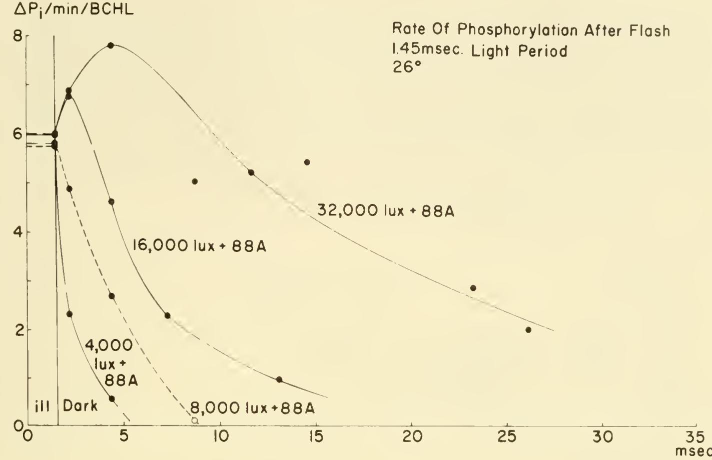

Fig. 1. Rate of phosphorylation after flash (Pi esterified in moles/min/mole bacteriochlorophyll). Type A illumination, 26°C.

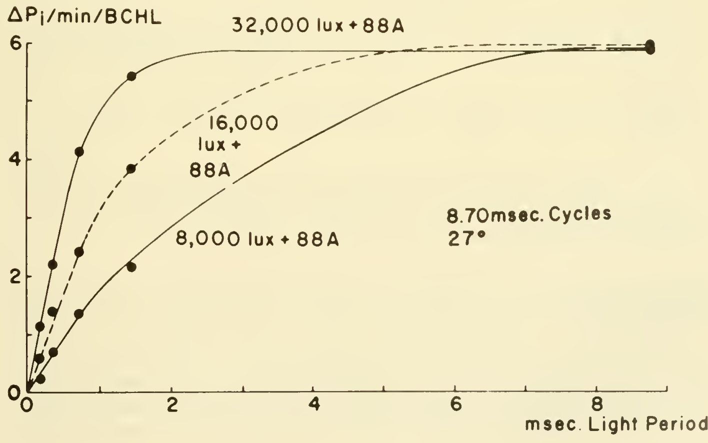

Fig. 2. Rate of photophosphorylation (Pi esterified in moles/min/mole bacteriochlorophyll) and length of light period. Type B illumination, 27°C.

period). The initial tangents of the curves (ATP formation rate vs. flash duration) were proportional to light intensity. From these experiments it is indicated that the extent of the primary photochemical reaction is proportional to the amount of energy in the short flashes, but utilization of the first chemical product(s) is a dark process and requires a longer time than its production.

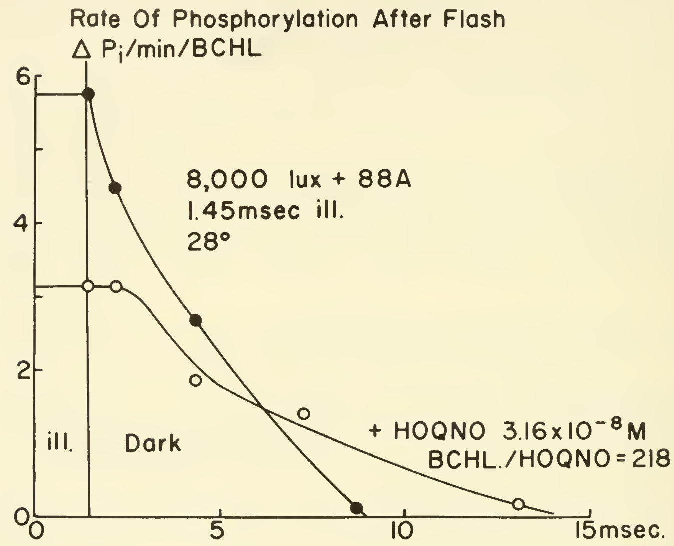

Fig. 3. Effect of HOQNO on rate of phosphorylation after flash (Pi esterified in moles/min/mole bacteriochlorophyll). ●—●, no HOQNO; ○--○, 3.16×10^{-8} M HOQNO. Type A illumination, 8000 lux + 88A filter, 28°C.

Effect of HOQNO,[1] *MPM and temperatures on the delayed photophosphorylation*

The rates of delayed phosphorylation in the presence (3.16×10^{-8} M) and absence of HOQNO are compared in Fig. 3. It is apparent that the rate of decay of delayed photophosphorylation is markedly lowered by HOQNO. The lower level of phosphorylation under continuous illumination, as well as the slower decay of delayed photophosphorylation

[1] Abbreviations: HOQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; MPM, methylphenazonium methosulfate ("phenazine methosulfate").

in the presence of HOQNO, is probably due to inhibition of electron transfer between cytochromes *b* and *c* (8).

The rate of photophosphorylation is markedly increased by MPM under sufficient illumination (22,23). The rates of phosphorylation after the flashes were calculated from a flashing light experiment. A remarkable acceleration of decay of delayed phosphorylation by the added MPM was noticed. The effect of MPM on the kinetics of delayed photophosphorylation was like a reversal of the effect caused by HOQNO.

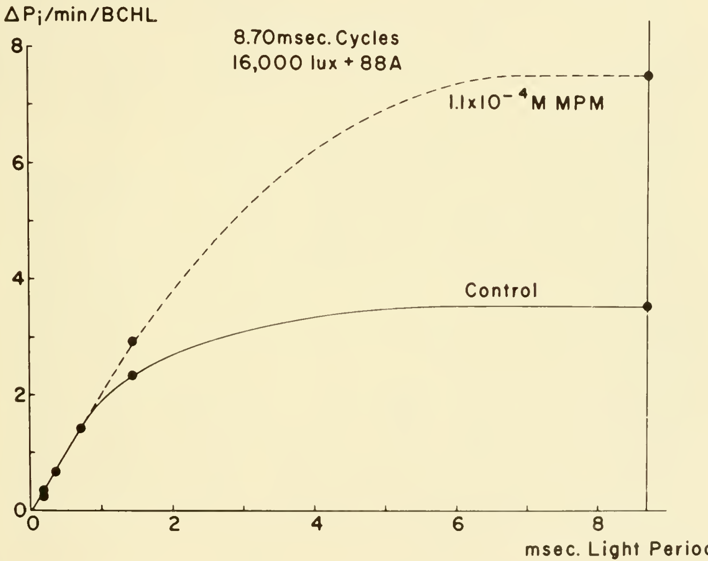

Fig. 4. Effect of MPM on photophosphorylation in Type B illumination. Ordinate: Pi esterified in moles/min/mole bacteriochlorophyll. Abscissa: length of light period. ——, no MPM; - - -, 1.1 x 10^{-4} M MPM. 16,000 lux + 88A filter, 28°C.

In the second set of experiments, the effect of MPM on the light-induced phase of photophosphorylation was investigated. The rate of phosphorylation was proportional to the length of the flash when the flash duration was sufficiently short. The tangents of these two curves were identical in the absence and presence of MPM (Fig. 4).

These data clearly indicate that MPM does not affect the primary light-induced phase of photophosphorylation.

The kinetics of photophosphorylation under intermittent illumination at two different temperatures (15° and 26°C) were also studied. The half-life of delayed photophosphorylation was lengthened at the lower temperature. It is concluded that a low rate of photophosphorylation at a low temperature under continuous illumination results from a slower dark process.

Amount of ATP synthesis caused by a single flash

The area covered by the curve of delayed photophosphorylation corresponds to the number of ATP molecules formed per chlorophyll molecule after a single flash. When the amounts of total delayed photophosphorylation at different temperatures were compared, it was found that the two values were approximately the same, though the rate of photophosphorylation under continuous illumination was lower at lower temperatures. The results are shown in Table 1.

The presence of reagents such as HOQNO or MPM caused a marked change in the rate of photophosphorylation, but the amounts of total delayed photophosphorylation after a single flash in the presence and absence of these reagents were approximately the same (Table 1). These results indicate that the substances accumulating during the short flash are consumed through dark processes, and phosphorylation takes place accompanying the dark processes. Different temperatures, HOQNO, and MPM do not affect the rapid photochemical process which takes place during the flash. These factors influence the rate of dark reactions of photophosphorylation. When the dark period is sufficiently long, the total amount of delayed process is determined by the amount of first product formed by a photochemical process during flash, hence changes in rate of the dark process would have little effect on the amount of total delayed photophosphorylation. This concept agrees well with other data that indicated the presence of three steps for photophosphorylation; i.e., a rapid photochemical process, a second slower process of electron transfer, and a third process of phosphorylation coupled with the second process.

The maximum amount of ATP synthesis per flash was determined by combining the methods employing the sensitive recording pH meter and the xenon flash tube illumination (20). An example of pH recordings is shown in Fig. 5. The single flash yield of photophosphorylation at the saturation level and the rate of phosphorylation under continuous illumination are tabulated in Table 2. Single flash yields are expressed as molar ratios by comparison with the bacteriochlorophyll and cytochrome concentrations.

TABLE 1

Amount of delayed photophosphorylation per single flash

Exp. no.	Temp. (degrees C.)	Addition	Light intensity	Delayed photo-phosphorylation (ATP in moles/flash/mole BChl)	Rate of photo-phosphorylation under continuous illumination (ATP in moles/min/mole BChl)
1*	26		8000 lux + 88A	3.00×10^{-4}	5.74
	15		8000 lux + 88A	2.75×10^{-4}	2.61
2*	28		8000 lux + 88A	2.83×10^{-4}	5.74
	28	HOQNO 3.16×10^{-8} M	8000 lux + 88A	2.67×10^{-4}	3.13
3*	28		16000 lux + 88A	3.14×10^{-4}	3.01
	28	MPM 1.1×10^{-4} M	16000 lux + 88A	3.34×10^{-4}	8.51
4**	25		8000 lux + 88A		6.38
	25		3.58×10^{-2} joule/cm^2/flash (+88A)***	4.7×10^{-2}	

* Experiments 1-3 were carried out under repeating flashes with a rotating sector. The rate of phosphorylation was determined by inorganic phosphate analysis. ** In Experiment 4, the amount of phosphorylation per flash was determined with a recording pH meter under irradiation by an electronic flash. *** Stored energy in the capacitor.

TABLE 2

Single flash yield of photophosphorylation and rate of photophosphorylation under continuous illumination (both under saturating light intensities)

Exp. no.	Single flash yield		Rate under continuous illumination ΔATP/min/BChl (mole/min/mole)
	ΔATP/BChl (mole/mole)	ΔATP/cytochrome (mole/mole)	
1	0.048	0.92	6.05
2	0.037	0.71	4.90
3	0.056	1.07	8.18
Mean	0.047	0.90	6.38

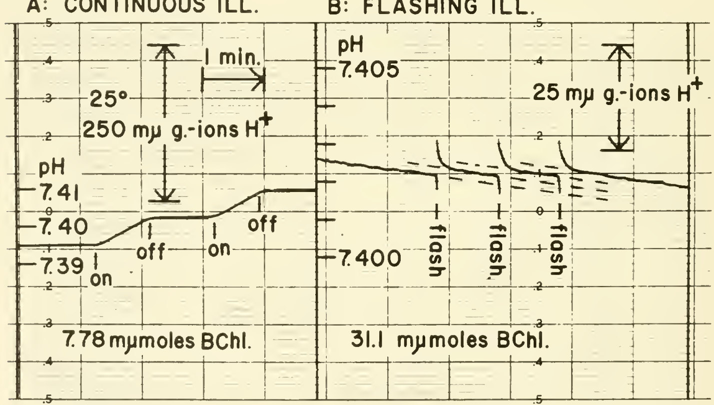

Fig. 5. Recordings of pH change by illumination of *R. rubrum* chromatophores. 25°C. A: continuous illumination, 8,000 lux + 88A filter, 0.1 pH unit full scale, rate of photophosphorylation = 6.05 ATP/min/bacteriochlorophyll. B: flashing illumination, 3.58×10^{-2} joule/cm^2/flash + 88A filter, 0.01 pH unit full scale.

Relative efficiency of light-energy utilization in different systems of phosphorylation

The comparison of photophosphorylation rates for different phosphorylating pathways under low intensity continuous illumination indicates the relative quantum efficiency of such systems. In this series of experiments the following four systems were studied under conditions of limiting light intensity: (a) untreated chromatophores, (b)

+ MPM, (c) + MPM + antimycin A, (d) + MPM + HOQNO. The concentrations of HOQNO and antimycin A were chosen to give both 100 per cent inhibition of photophosphorylation in the absence of MPM, and at the same time to give almost full recovery of phosphorylation when the inhibitor was added with MPM (under high light intensities).

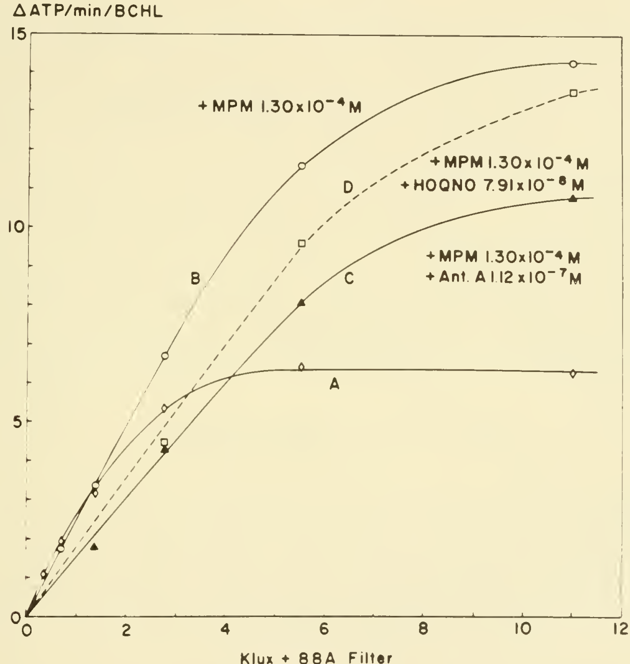

Fig. 6. Rates of photophosphorylation in different systems. Ordinate: Pi esterified in moles/min/mole bacteriochlorophyll. Abscissa: light intensity. A: untreated chromatophores, B: + 1.30 x 10^{-4} M MPM, C: + 1.30 x 10^{-4} M MPM + 1.12 x 10^{-7} M antimycin A. D: + 1.30 x 10^{-4} M MPM + 7.91 x 10^{-8} M HOQNO. Temperature 24°C.

The rates of photophosphorylation in these four systems in the low light intensity range are shown in Fig. 6. Curves A, B, C, and D correspond to systems (a), (b), (c), and (d) described above. The comparison of tangents of these curves at zero light intensity shows the relative quantum efficiencies for the different systems. It is seen from

the figure that the efficiency is slightly lowered in the presence of MPM as compared to untreated chromatophores. In the systems (c) and (d), where reactions of cytochromes of *b* and *c* types are inhibited

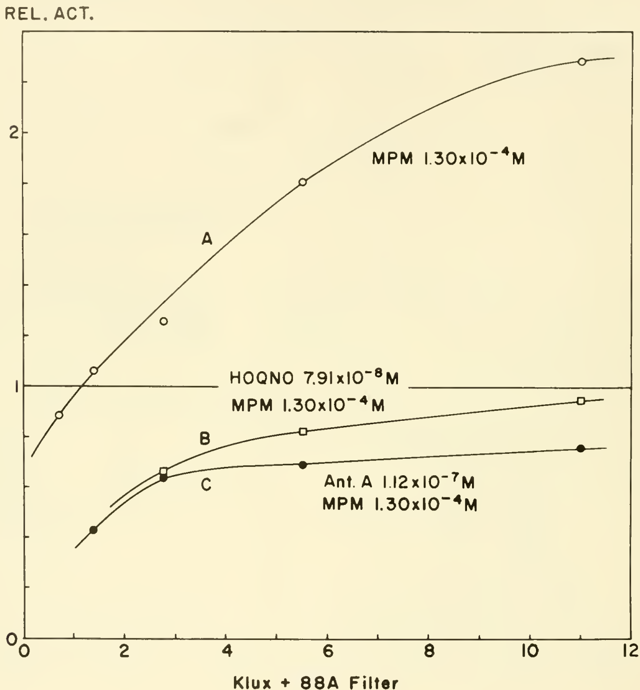

Fig. 7. Comparison of phosphorylative systems at different light intensities. Curves indicate ratios of phosphorylation rates. A: MPM-catalyzed phosphorylation/normal phosphorylation. B: phosphorylation of MPM-HOQNO system/MPM-phosphorylation. C: phosphorylation of MPM-antimycin A-system/MPM-phosphorylation. Concentrations of reagents are the same as in Fig. 6. Temperature 24°C.

and the flow of electrons is bypassed by MPM, the efficiencies are about half of the original value. This means that one of the phosphorylating sites is located close to the oxidation-reduction site of cytochromes *b* and *c*, and bypassing this site with MPM reduces the

quantum efficiency to one half. Knowing the maximum amount of delayed photophosphorylation from a single flash, we postulated two sites of phosphorylation on the electron transport chain in *R. rubrum* chromatophores as a tentative value (see Discussion). The absolute quantum efficiency measurement of photophosphorylation (24) and valiomycin experiments (25) by Baltscheffsky and others are generally in good agreement with our present studies.

The operation of the different phosphorylating sites is a function of light intensity. For example, the MPM activation of photophosphorylation is more marked under high light intensities. Likewise, the recovery of antimycin A or HOQNO inhibition by MPM is greater under higher light intensities. These results are summarized in Fig. 7. Curve A is the ratio of MPM-catalyzed phosphorylation to normal phosphorylation, Curves B and C are ratios of HOQNO- (or antimycin A-) MPM phosphorylation/MPM phosphorylation, respectively. The concentrations of MPM, HOQNO, and antimycin A were identical to those in Fig. 6. These facts suggest that in low light intensity experiments the untreated chromatophores are more efficient in the energy utilization than other systems. However, MPM-bypassed electron transfer becomes greater under higher intensities, and the overall rate of phosphorylation can be high in the presence of MPM even if the normal electron transfer is blocked and one of the phosphorylating sites is lost.

DISCUSSION

It is concluded that the photochemical and dark processes in photophosphorylation can be separated by the technique of flashing illumination. The first photochemical step is rapid and occurs only during illumination. The rate of steady state phosphorylation is limited by the rate of dark process, possibly by the rate of electron transfer. The amount of total delayed photophosphorylation is proportional to the amount of light absorbed when flashes are sufficiently short.

The first step can be the formation and accumulation of oxidized cytochrome (which is rapid and temperature independent) (1-9) and some unknown reduced substance. The reduction of pyridine nucleotide is less rapid than cytochrome oxidation (26). The second process (light-independent phase of photophosphorylation) would include the transfer of electrons from the reduced low-redox potential system to the oxidized high-redox potential system (oxidized cytochrome) and the associated reactions which lead to phosphorylation of ADP. MPM and higher temperatures accelerate the decay of delayed photophosphorylation, whereas HOQNO retards this decay. Under continuous illumination, higher temperatures and MPM raise the level of steady state photophosphorylation; HOQNO lowers the level. These data can

be explained in terms of an increase or decrease of electron flux at a bottleneck point. This point is most likely the HOQNO- and antimycin-sensitive site (between cytochromes *b* and *c*?). Evidence supporting this conclusion is as follows: (a) the reaction between cytochromes *b* and *c* is inhibited by HOQNO or antimycin A in light- and oxygen-activated oxidation-reduction reactions (8), (b) photophosphorylation is inhibited strongly by HOQNO or antimycin A (22,23), (c) inhibition of photophosphorylation by HOQNO or antimycin A is largely diminished in the presence of MPM (22,23), (d) the rate of photophosphorylation increases in the presence of MPM under sufficiently high light intensities, and (e) the decay of delayed photophosphorylation is accelerated by MPM and is decelerated by HOQNO.

The mean value for the maximum single flash yield of ATP synthesis, 0.047 ATP/bacteriochlorophyll, is much higher than the amount of total delayed photophosphorylation by a single flash of light intensity of 32,000 lux (+ infrared filter) in the repeating flash experiments. For the maximal amount of delayed photophosphorylation, the light intensities used in the repeating flash work were apparently not sufficient. Since ATP synthesis during the flash is negligible as compared to the total delayed photophosphorylation when the flash is short and the light intensity is high, the maximum single flash yield obtained by the xenon flash can be regarded as equal to the amount of delayed photophosphorylation. The use of the infrared flashes for the activation of chromatophores excluded the possibility of participation of carotenoids as the primary light-absorbing pigments. Therefore, the number of light quanta absorbed by chromatophores is limited by the number of bacteriochlorophyll molecules (except for nonspecific absorption by chromatophore materials). If we assume that the maximal yield of ATP/bacteriochlorophyll is attained when all the bacteriochlorophyll molecules are excited by the infrared flash, the *minimum* quantum yield for the delayed photophosphorylation will take the same value as the maximal value of single flash yield per bacteriochlorophyll, i.e., $ATP/h\nu$ = ATP/bacteriochlorophyll = 0.047.

There have been many discussions concerning the primary photochemical reaction in photosynthesis (6,26-31). Except for activated electronic states of assimilatory pigments, the first chemical process which takes place in bacterial photosynthesis is probably the light-induced oxidation of cytochrome. The rapidity and the temperature independence of the process suggest that the oxidation of cytochrome takes place during the short illumination and the rest of the photosynthetic reactions proceeds in the dark. As the mechanism of photophosphorylation in photosynthetic bacteria, the following scheme seems most feasible.

$$\text{BChl} + \text{Cytochrome } c\left(\text{Fe}^{2+}\right) \xrightarrow{h\nu} \text{BChl}^{-} + \text{Cytochrome } c\left(\text{Fe}^{3+}\right) \quad (1)$$

$$BChl^{-} + Y + H^{+} \longrightarrow BChl + HY \qquad (2)$$

$$\text{Cytochrome } c\,(Fe^{3+}) + HY \longrightarrow \text{Cytochrome } c\,(Fe^{2+}) + Y + H^{+} \qquad (3)$$

$$m\left(ADP + Pi + nH^{+}\right) \longrightarrow m\left(ATP + H_2O\right) \qquad (4)$$

In this scheme, Eq. 1 indicates the photochemical oxidation of cytochrome *c*, and Eq. 2 shows the formation of the primary reduced substance HY. Eqs. 3 and 4 indicate electron transfer between the oxidized cytochrome *c* and the reduced substance in the oxidation-reduction chain and the coupled phosphorylation.

The maximal yield of delayed photophosphorylation found in this work is 0.90 ATP/cytochrome (Table 1). This figure is calculated on the basis of total cytochrome concentration in the chromatophores. If we assume the light-induced oxidation of *c*-type cytochrome only, the yield (ATP/photochemically oxidized cytochrome) becomes higher. It is calculated to be around 2 on the basis of relative concentrations of different heme protein species in *R. rubrum* cells (9), if the saturating yield is obtained when the cytochrome *c* is fully oxidized by the strong flash. This value (ATP/photochemically oxidized cytochrome = ~2) corresponds to the yield of ATP formation per electron transferred in the oxidation-reduction chain of chromatophores (factor m in Eq. 4). This suggests that the number of phosphorylating sites in the redox chain (Eq. 3) is probably two if the two sites are located in series on the chain. Yet this is a rather tentative value for the number of ATP molecules synthesized per electron transferred by the redox chain, and it remains to be scrutinized further. The increased rate of electron transfer at the rate-determining site resulting from the addition of MPM would lead to an increase in the rate of overall electron transport, followed by an increased rate of photophosphorylation. It must be noted, however, that in the presence of MPM (and HOQNO or antimycin A) the probable loss of one of the phosphorylating sites is expected. The lowering of the quantum efficiency of photophosphorylation by the addition of these reagents (Baltscheffsky, Baltscheffsky and Olson, 24, and our present data) suggests the loss of a phosphorylating site.

The amount of total delayed photophosphorylation per flash was not appreciably affected by temperatures, MPM, or HOQNO when the dark periods were sufficiently long. Other data indicate that these factors affect only the dark steps of photophosphorylation. When the dark period is sufficiently long, the amount of total delayed process is determined by the amount of the primary product formed by the photochemical process during flash. Therefore, it is understood that the changes of the rate of dark processes (consumption of the primary

products and coupled phosphorylation) cause little effect on the amount of total delayed photophosphorylation, though the time required for the completion of the dark reactions changes markedly.

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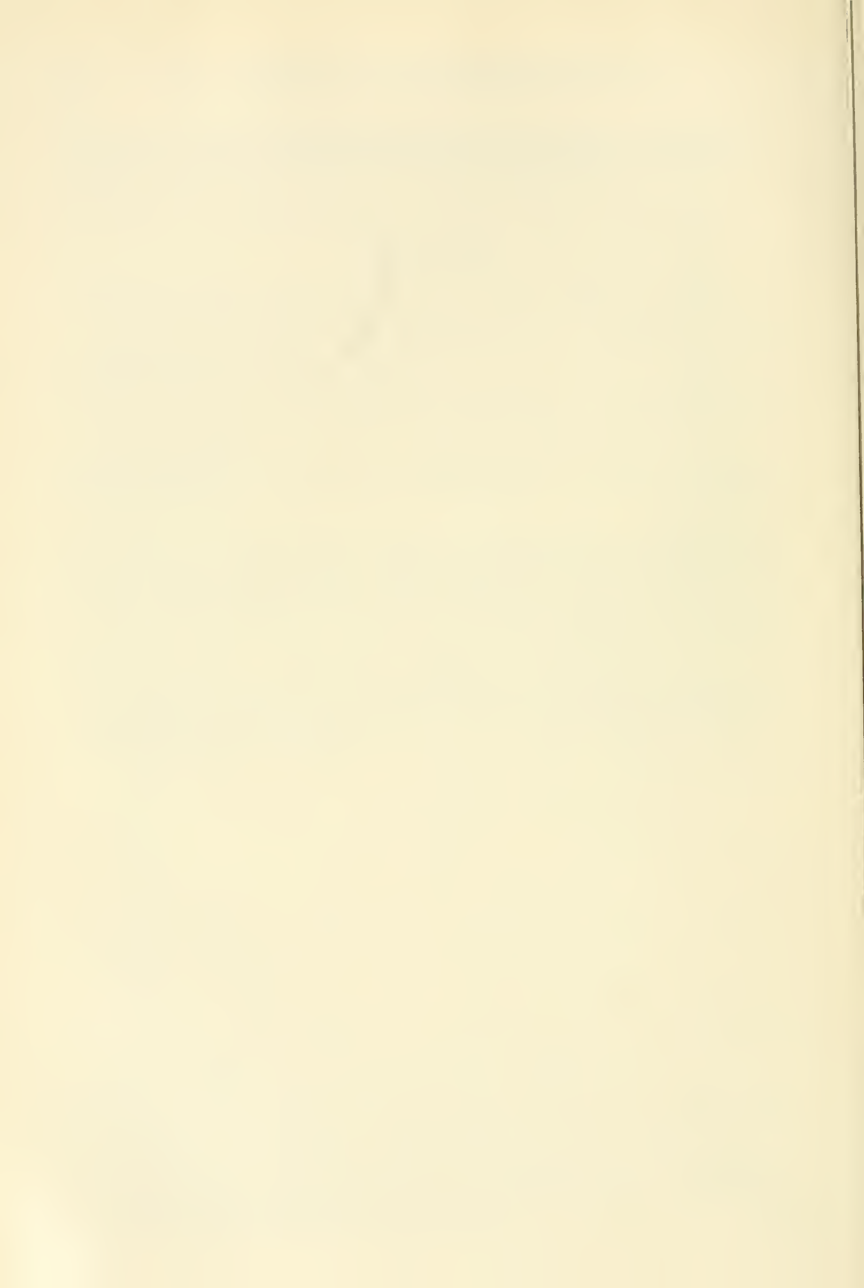

THE EFFECT OF UBIQUINONE$_2$ ON PHOTOPHOSPHORYLATION IN PARTICLES OBTAINED FROM *RHODOSPIRILLUM RUBRUM* GROWN IN MEDIA CONTAINING DIPHENYLAMINE[1]

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It has previously been shown that cells of *R. rubrum* grown in the presence of diphenylamine contain greatly reduced amounts of ubiquinone$_{10}$ (coenzyme Q_{10})[2] (1,2). Chromatophore particle preparations from these cells (DPA particles) carry on photophosphorylation at a lower rate than particles from normally grown cells. The DPA particles require a catalytic amount of a reductant in order to maintain cyclic photophosphorylation. It was observed (2) that photophosphorylation in the presence of 1 mM succinate was greatly stimulated by the addition of UQ_2, however with lower concentrations of succinate (0.05 mM) addition of UQ_{10} completely inhibited phosphorylation. The stimulation of photophosphorylation by UQ_2 was completely abolished by antimycin.

Since reporting these results many experiments have been performed to elucidate the site of these various effects. The results were often puzzling in that it was soon found that depending on conditions one could obtain either stimulation or inhibition of light-induced photophosphorylation (LIP), and there was a great variation between preparations. It gradually became clear that what we were witnessing was the extreme sensitivity of the DPA chromatophore system to changes in redox balance brought about by the various agents added, i.e., there appeared to be very little poising capacity of the redox systems in these preparations. These experiments will be fully reported elsewhere but a few typical experiments shown in the following tables will suffice to show that UQ_2 can overcome the inhibitory effect of antimycin merely by changes in the redox balance of the

[1] This investigation was supported in part by a Public Health Service Research Career Program Award (GM-K-3-993-C3).

[2] The following abbreviations are used: UQ_{10} = ubiquinone$_{10}$, UQ_2 = ubiquinone$_2$, suffix indicating number of isoprenoid units in side chain; LIP = light-induced phosphorylation; DPA particles = chromatophore particles from cells grown in diphenylamine; PMS = phenazine methyl sulfate.

system. These results are in agreement with those reported by Kamen and collaborators (3,4), Vernon and Ash (5), and Geller and Lipmann (6) over the past few years. In particular, these results support the recent finding of Bose and Gest (7) showing that dyes such as DPIP may, under certain conditions, overcome an antimycin inhibition of light-induced, cyclic electron transfer.

As shown in Table 1, when DPA particles are incubated with succinate, one always observes a stimulation of LIP by the addition of catalytic amounts of UQ_2. In this particular preparation PMS also greatly stimulated LIP. When succinate and PMS are both present one observes an inhibition which could be considered to result from the over-reduction of some carrier or of the dye. Similarly, in the case where UQ_2 stimulated LIP, an oxidation of some component occurred. In agreement with this concept is the fact that UQ_2 will relieve some of the inhibition due to over-reduction by succinate (compare vessels 3 and 6, Table 1). Further corroboration appears from a comparison of vessels 5 and 6, Table 1. Here again it is reasonable to consider that succinate is acting as an inhibitor by over-reducing a component of the system.

In the presence of antimycin, LIP stimulation by succinate is completely blocked, whether UQ_2 is present or not. PMS-stimulated LIP

TABLE 1

The Effect of Succinate, PMS, UQ_2 and Antimycin on LIP in DPA Particles

Vessel No.	Additions	No Antimycin	With Antimycin
1	Succinate	4.1	0
2	PMS	15.0	9.2
3	Succinate + PMS	4.7	2.3
4	Succinate + UQ_2	13.0	0
5	PMS + UQ_2	23.2	16.5
6	Succinate + PMS + UQ_2	12.8	10.6

Chromatophore particle preparations were prepared from *Rhodospirillum rubrum* grown in diphenylamine as previously described (2). Experiments were carried out as follows in Warburg manometer vessels. The main compartment contained 20 μmoles $MgCl_2$, 35 μmoles KH_2PO_4, pH 7.0, 100 μmoles glycylglycine buffer, pH 7.4, 40 μmoles of glucose, 0.5 mg of hexokinase (Sigma type III), 0.8 mg of chromatophore particle protein and additions as indicated above. 10 μmoles of ADP were in the side arm. Total volume, 2.5 ml. The cups were filled in the dark and gassed with pre-purified nitrogen for 5 minutes. Then the ADP was tipped and the light (1200 ft-candles on each vessel) was turned on. At the end of the incubation period (usually 45 minutes or 1 hour) the reaction was stopped and phosphate uptake determined as previously described (2). The figures in the table represent micromoles of phosphate esterified in 1 hour. The additions consisted of 2 μmoles succinate, 0.03 mg PMS, 3 μg antimycin, 0.08 μmoles of UQ_2 in 5 microliters alcohol.

is, however, inhibited about 38 per cent. This result is in agreement with the commonly observed bypass of antimycin inhibition by PMS (6). When succinate and PMS are added together one observes again an appreciable inhibition in the presence of antimycin which is bypassed in the presence of UQ_2 (compare vessels 3 and 6, Table 1). Thus it would appear that UQ_2 by affecting the redox balance of the system even in the presence of antimycin can stimulate or inhibit LIP. This concept is further borne out by a comparison of vessels 2 and 5 incubated in the presence of antimycin. Here again one could reason that UQ_2 has affected the redox balance of the system being acted on by the light-reduced PMS, in the pathway bypassing antimycin, so that stimulation of LIP is obtained. If one now compares vessels 5 and 6 incubated in the presence of antimycin, one must conclude that succinate has affected the redox balance towards over-reduction so that inhibition of LIP occurs.

Similar results to the foregoing can be obtained from experiments using the ascorbate-DPIP couple which has been used in studies on photooxidation and photoreduction in chromatophore particles (3,5,8). Although the LIP of DPA particles is completely inhibited when high concentrations of ascorbate (4 mM) are used, it is maintained when the concentration is low (0.02 mM), as shown in Table 2. Addition of UQ_2 or DPIP leads to complete inhibition of phosphorylation, again presumably due to alteration in the redox balance of the system. Addition of UQ_2 to the ascorbate-DPIP couple leads again to the establishment of a new redox balance which is favorable for increased LIP. Similarly, addition of DPIP to the ascorbate-UQ_2 couple leads to the same effect. In the presence of antimycin the stimulating effect of ascorbate alone is completely abolished. In this sense the DPA particles behave differently from normal particles where in some cases ascorbate-induced LIP appears to bypass antimycin inhibition (7,8). Upon the

TABLE 2

The Effect of Ascorbate, DPIP and UQ_2 on LIP in DPA Particles

Vessel No.	Additions	No Antimycin	With Antimycin
1	Ascorbate	5.2	0
2	Ascorbate + UQ_2	0	0
3	Ascorbate + DPIP	0	1.4
4	Ascorbate + DPIP + UQ_2	9.2	3.8

Conditions same as for Table 1. Figures represent micromoles of phosphate taken up in 1 hour, using 0.6 mg of chromatophore particle protein. The additions, where indicated, consisted of 0.5 μmoles of ascorbate, 0.2 μmoles of DPIP and 0.08 μmoles of UQ_2.

addition of DPIP it can be observed that the inhibitory effect of antimycin with ascorbate alone is partially relieved, and furthermore the inhibitory effect of UQ_2 is bypassed, just as in the case without antimycin (compare vessels 2 and 4, Table 2). On the other hand, a comparison of vessels 3 and 4 also shows that UQ_2 acts to bypass an antimycin inhibition of LIP with the ascorbate-DPIP couple.

The results outlined in Tables 1 and 2 indicate that, even in the presence of antimycin, agents affecting redox balance can produce stimulation or inhibition of LIP, depending on the particular redox balance established in the system. These findings extend the observations of Bose and Gest (7) on the effect of DPIP in bypassing an inhibition of electron transport by antimycin. The stimulatory effect of UQ_2 on LIP in the presence of succinate appears to be due to an effect on the redox balance of an electron carrier in the particles. In this connection, it is of interest that succinate readily reduces UQ_{10} in the dark in chromatophore particles (10). In the case of the stimulation by UQ_2 in the presence of PMS the effect may also possibly be directly related to the new redox balance established as a result of interaction of UQ_2 with the light-reduced dye. It seems reasonable to speculate that the effects observed with UQ_2 and PMS in the presence of antimycin are due to this dye interaction and may not necessarily involve direct reaction with a second electron carrier site different from that where UQ_2 and succinate interact.

The quinone normally present in *R. rubrum* is UQ_{10}. Since the E_0' of UQ_2 would not be expected to vary greatly from the +0.100 ±0.01 volts calculated for UQ_{10} (9), it could be assumed that the redox balance at the UQ_{10} site in the cyclic electron transport scheme would be directly affected by the addition of UQ_2.[3] On the basis of E_0' values UQ_{10} would fit in somewhere between *Rhodospirillum* heme protein (+0.01 volts) and cytochrome c_2 (+0.310 volts). Since the DPA particles have greatly reduced amounts of UQ_{10} the addition of UQ_2 would certainly influence the redox balance to a much greater extent than in the case of the normal particles, where little effect of UQ_2 can be observed. UQ_{10} is insoluble in aqueous media, which may explain why the addition of this substance has a negligible effect on LIP. As in the case of PMS, it is difficult to distinguish whether the effects of UQ_2 on the interaction of the ascorbate-DPIP couple in LIP are due to direct reaction with these reagents or to some action on an electron transport carrier in the particles. Nonetheless, a most important point resulting from the foregoing experiments, and one which requires emphasis, is that evidence for the existence of noncyclic photophosphorylation in bacterial systems (8) based on the inhibitory effect of antimycin must

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[3] Moret *et al.* (11) have measured the redox potential of a series of UQ analogues with varying isoprenoid side chains. They found that the length of the isoprenoid chain did not influence the final value of +0.098 volts at pH 7.4.

be carefully examined, to exclude the possibility that various reducing and oxidizing agents have not bypassed the antimycin sensitive site by changes in the redox balance of the system. Such changes could lead to the re-establishment of cyclic photophosphorylation.

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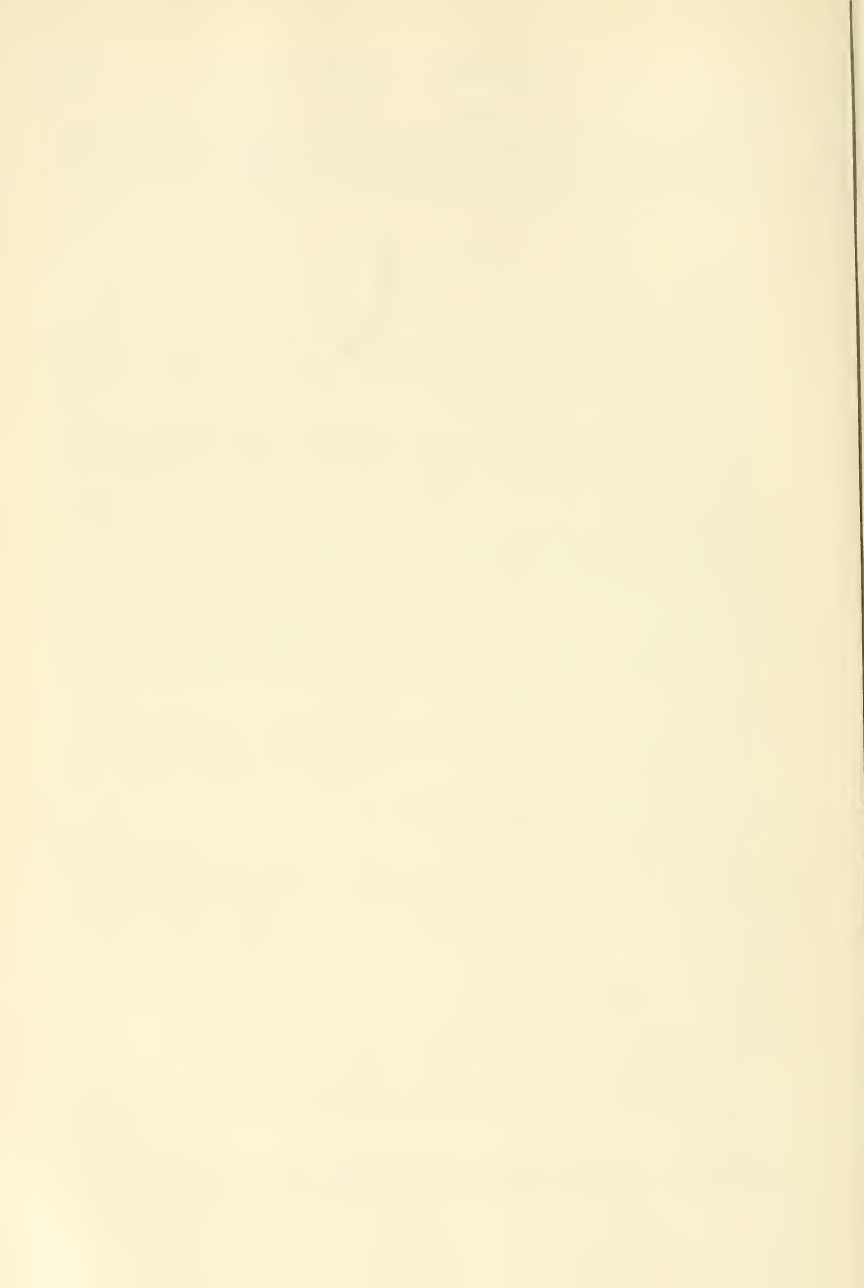

PHOTOSYNTHETIC PHOSPHORYLATION WITH BACTERIAL CHROMATOPHORES: CATALYSIS BY A NATURALLY OCCURRING FACTOR (PHOSPHODOXIN)[1]

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A light-induced phosphorylation of ADP with cell-free preparations from the photosynthetic bacterium *Rhodospirillum rubrum* was first observed by Frenkel (1-3). This light-induced process, which has been termed photosynthetic phosphorylation, has subsequently been observed with chromatophores from *Chromatium* (4-6) and *Chlorobium* (4). Photosynthetic phosphorylation by bacterial chromatophores occurs in the absence of an exogenous electron acceptor at a rate which is compatible with the growth rate of whole cells. This is in sharp contrast to the low endogenous rate of photosynthetic phosphorylation with spinach chloroplasts (7,8), which is only one to two per cent of the photosynthetic capacity of intact spinach leaves. Whereas the endogenous rates observed with chromatophores and chloroplasts are markedly different, both may be increased in the presence of suitable cofactors. For example, photosynthetic phosphorylation by chromatophores and by chloroplasts is stimulated about 10-fold (5,9) and as much as 2,000-fold (10), respectively, in the presence of the dye methyl phenazonium methosulfate. It seemed reasonable, therefore, that the intact organism might contain some component(s) which isolated chromatophores and chloroplasts either lacked or contained in low concentration. Thus, a study of the natural factors involved in photosynthetic phosphorylation was initiated.

A water-soluble, heat-stable factor which stimulates the rate of photosynthetic phosphorylation by spinach chloroplasts as much as 200-fold (8) has been isolated from both spinach leaves and chloroplasts. Further study revealed the presence of a similar factor[2] in many photosynthetic organisms, including bacteria (8). In this report,

[1] Contribution No. 116 of the Charles F. Kettering Research Laboratory. Supported in part by a research grant (GM 10129-01) from The National Institutes of Health, U. S. Public Health Service.

[2] It is not known whether the factor isolated from different organisms is the same or different.

additional data will be presented concerning this factor, which we propose to call *phosphodoxin*, e.g., spinach phosphodoxin or *Rhodospirillum rubrum* phosphodoxin.

METHODS

Experiments on light-induced formation of ATP^{32} were conducted with a 1 ml reaction mixture containing the following components in μmoles: Tris-HCl buffer, pH 7.8, 48; $MgCl_2$, 2; ADP, 1; Pi + Pi^{32} (containing from 0.5 to 1 μcurie), 1; bacteriochlorophyll or chlorophyll, less than 30 μgrams. ATP^{32} was assayed as previously described (8). In some experiments ATP was determined spectrophotometrically with glucose, hexokinase, NADP, and Zwischenferment. The methods used for preparation of chromatophores and chloroplast fragments and for chlorophyll determination were those previously reported (8). A method of isolating the naturally occurring factor has been described elsewhere (8). The reaction mixtures were illuminated laterally in 1-cm cuvettes at 2,500 foot-candles. Light intensity was varied by varying the distance of the reaction mixtures from the light source (11).

RESULTS

Photosynthetic phosphorylation with *R. rubrum* chromatophores was markedly stimulated by the addition of the factor isolated from whole cells of the same organism (Fig. 1). The formation of ATP was more linear with time and fell off slower in the presence of the factor than in its absence.

One characteristic of the factor(s) is its ability to stimulate photosynthetic phosphorylation by chromatophores, regardless of the organism from which it is isolated, i.e., an algae, a higher plant, a flagellate, or a bacterium (8). Further demonstration of these cross-reactions between organisms is given in Table 1 and Figs. 2 and 3. The rates of endogenous photosynthetic phosphorylation of both spinach chloroplasts and *R. rubrum* chromatophores were stimulated by the addition of the factor(s) isolated from spinach, *Chromatium*, or *R. rubrum* (Table 1). Photosynthetic phosphorylation by spinach chloroplasts was strikingly stimulated by the factor isolated from *Chromatium* (Fig. 2), and likewise *Chromatium* chromatophores were stimulated by the factor isolated from spinach (Fig. 3).

In the presence of the factor, anaerobic conditions did not affect the rates of photosynthetic phosphorylation observed with *R. rubrum* chromatophores during a two-minute illumination (Fig. 4, upper two curves). With longer illumination periods, a slight stimulation occurred

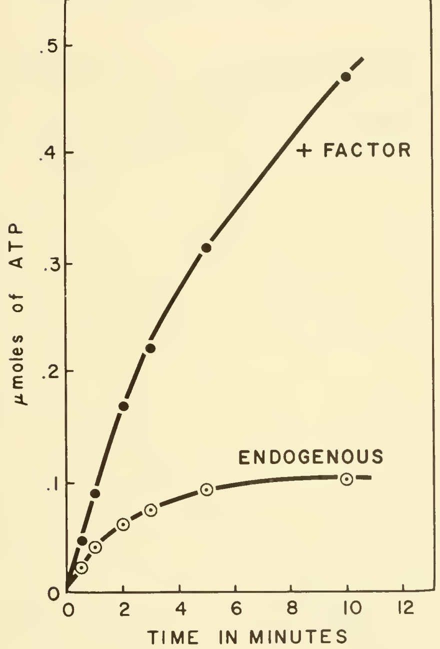

Fig. 1. Time course of the effect of the factor isolated from *R. rubrum* on photophosphorylation with *R. rubrum* chromatophores.

TABLE 1

Activity of chromatophores and chloroplasts with factor from other organisms. Activity is expressed as μmoles of ATP per mg bacteriochlorophyll or chlorophyll per hour

Source of factor	*Rhodospirillum rubrum* chromatophores		Spinach chloroplasts	
	Endogenous	Plus factor	Endogenous	Plus factor
Spinach	121	194	1.5	196
Chromatium strain D	47	125	1.5	115
Rhodospirillum rubrum	68	206	1.0	54

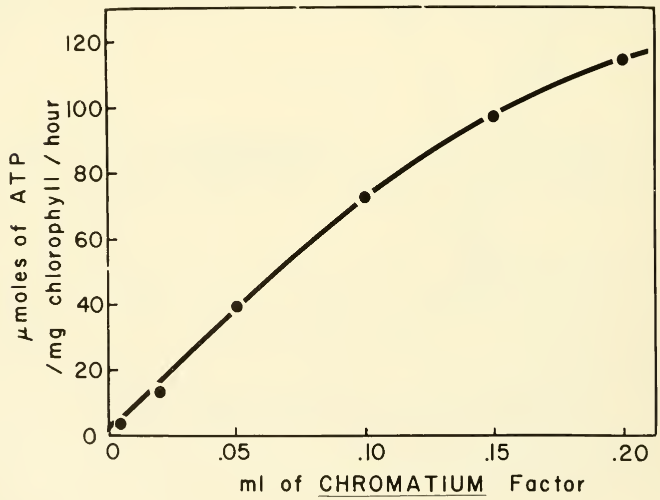

Fig. 2. Effect of concentration of the factor isolated from *Chromatium* on photophosphorylation with spinach chloroplasts.

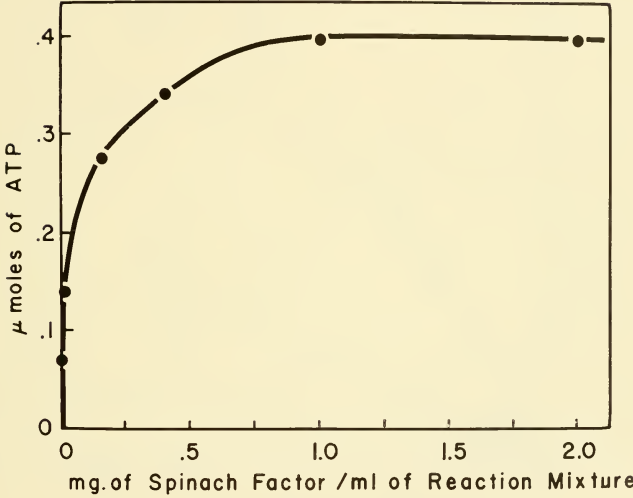

Fig. 3. Effect of concentration of the factor isolated from spinach on photophosphorylation with *Chromatium* chromatophores.

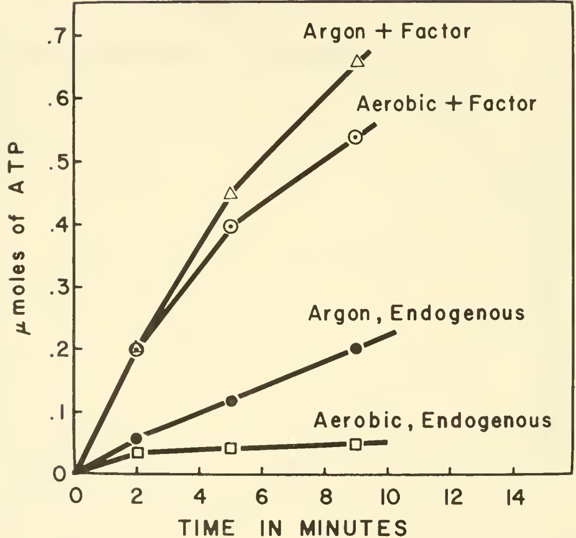

Fig. 4. Effect of anaerobic conditions and time on photophosphorylation with *R. rubrum* chromatophores in the absence and presence of the factor isolated from *R. rubrum*.

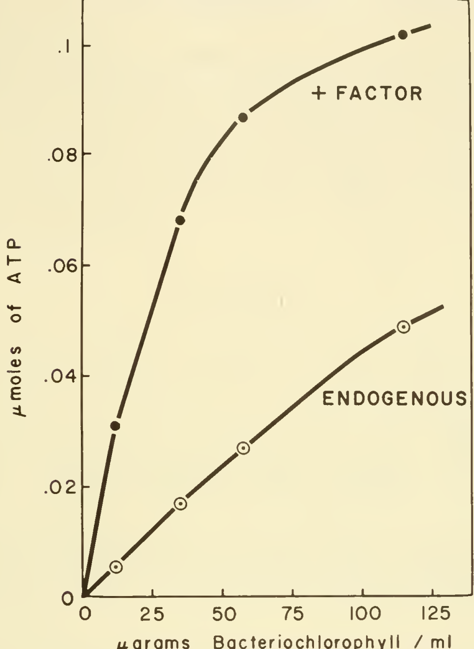

Fig. 5. Effect of bacteriochlorophyll concentration on photophosphorylation by *R. rubrum* chromatophores in the absence and presence of the factor isolated from *R. rubrum*.

under argon, probably resulting from a stimulation of the endogenous ATP production under argon (Fig. 4, lower two curves).

Linearity of ATP production by *R. rubrum* chromatophores with increasing bacteriochlorophyll concentration (up to 30 μgrams) in the presence of the factor is indicated in Fig. 5.

The usual response of photosynthetic phosphorylation with bacteria to increasing light intensity was observed (Fig. 6) with *R. rubrum* chromatophores in the presence of the factor isolated from *R. rubrum*. Saturation was reached at 500 foot-candles in both the absence (unreported data) and presence of the factor. This is about double the saturation intensity previously reported for *R. rubrum* (3) and for

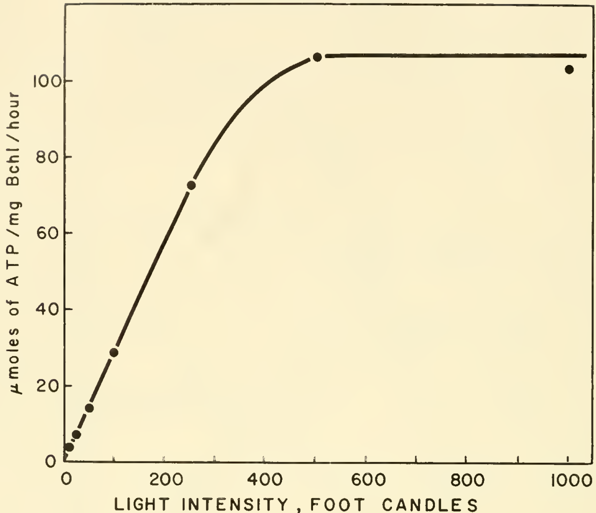

Fig. 6. Effect of light intensity on photophosphorylation by *R. rubrum* chromatophores in the presence of the factor isolated from *R. rubrum*.

Chromatium (5). It is pertinent to point out that photosynthetic phosphorylation by spinach chloroplasts does not show proportionality with light intensity below about 50 foot-candles (11). A broad pH optimum between 7.4 and 8.4 has also been observed with chromatophores in the presence of the factor.

Horio and Kamen (12) have reported that the low photosynthetic phosphorylation capacity of "washed chromatophores" could be restored to a maximal rate by the addition of "chromatophore washings." One component of the "chromatophore washings" was identified as cytochrome c_2. Heating, aerating, or freezing the "chromatophore washings" resulted in a loss of photosynthetic phosphorylation-activation capacity. The factor reported in this paper does not appear to be the same as those studied by Horio and Kamen, since it is not destroyed by these treatments and does not contain cytochrome c_2.

CONCLUSIONS

Photosynthetic bacteria contain a water-soluble, heat-stable factor (phosphodoxin) which stimulates photosynthetic phosphorylation by both chromatophores and spinach chloroplasts. Spinach chloroplasts contain a similar factor which stimulates photosynthetic phosphorylation by bacterial chromatophores. The stimulated reaction by bacterial chromatophores is linear with time, bacteriochlorophyll, and light intensity. Anaerobic conditions do not affect the stimulated reaction during short illumination periods.

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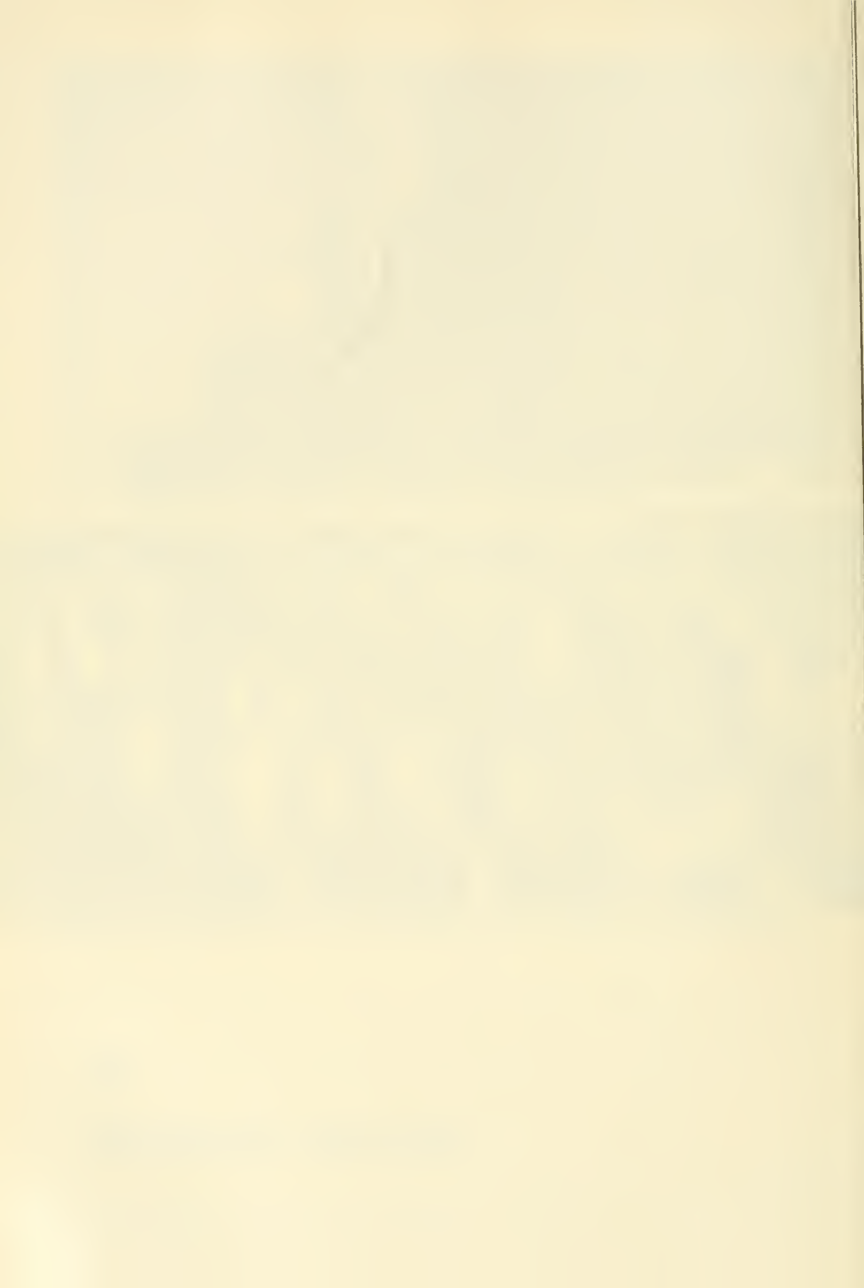
Top: B. Chance; *Bottom:* a section of the audience.

III

ELECTRON TRANSPORT



PHOTOOXIDATION AND PHOTOREDUCTION REACTIONS CATALYZED BY CHROMATOPHORES OF PURPLE PHOTOSYNTHETIC BACTERIA[1,2]

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Photooxidation Reactions:

Oxidation and reduction reactions are the essence of both plant and bacterial photosynthesis. The photosynthesizing cell utilizes the energy obtained from light to effect oxidation and reduction reactions directed primarily toward control of the oxidation level of carbon compounds and an associated production of ATP. It was early recognized that under the influence of light, cells of the photosynthetic bacteria had the ability to oxidize molecules of either inorganic sulphur or organic compounds and reduce carbon dioxide in a coupled reaction (1). French demonstrated a photooxidation of ascorbic acid by bacterial extracts of *Rhodovibrio* (2), and later Vernon and Kamen reported a photooxidation of cytochrome *c* and $DPIPH_2$[3] by chromatophores of *Rhodospirillum rubrum* in the presence of air (3). Since these reactions were performed aerobically, they were subject to the criticism that the photooxidation could represent a nonbiological oxidation catalyzed by the chlorophyll itself. Subsequently, however, it was demonstrated that the reduced forms of DPIP, cytochrome *c*, methylene blue, and indigo carmine were photooxidized by chromatophores of *R. rubrum* in the absence of oxygen if alternate electron acceptors were added, such as fumarate (4).

Lindstrom has extended the studies with $DPIPH_2$ and has shown a coupling of $DPIPH_2$ photooxidation with the photoreduction of sulfate

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[1] Contribution No. 107 from the Charles F. Kettering Research Laboratory.

[2] The term "chromatophore" is used in this presentation to mean the photosynthetically active particle or fragment which is obtained upon rupture of the intact cell by sonic oscillation. There is some question concerning the origin of these particles, but whether they exist as separate entities in the cell or are part of the cell membrane does not significantly affect the conclusions drawn from these experiments.

[3] In addition to the standard abbreviations, the following are used: TMPD, N,N,N',N'-tetramethyl-*p*-phenylenediamine; MB, methylene blue; MBH_2, reduced form of MB; PMA, phenylmercuric acetate; HQNO, 2-heptyl-4-hydroxyquinoline-N-oxide; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid).

(5). He has also studied the stability of the photooxidase system in *R. rubrum* chromatophores (6).

In a series of investigations, which were designed primarily for studying the photoreduction system of chromatophores, Frenkel demonstrated that a photoreduction of NAD could be coupled to a photooxidation of reduced FMN (7). Succinate also supported the photoreduction of NAD, and a photooxidation of this compound was implied. Vernon and Ash also studied the photoreduction of NAD, which was coupled to a photooxidation of succinate (8).

Ample evidence has accumulated during the past several years showing that whole cells of the photosynthetic bacteria are capable of catalyzing a photooxidation of intracellular cytochrome components. Duysens (9) and Chance (10) observed an oxidation of cytochrome upon illuminating cells of *R. rubrum* under anaerobic conditions. This observation was later confirmed by the experiments of Chance and Smith (11). The light-induced oxidation of intracellular cytochrome has been extensively investigated for the bacterium *Chromatium* (12,13,14). Since this photooxidation proceeds at temperatures as low as 80°K, this reaction is probably one of the primary photoreactions taking place after absorption of a light quantum by the chromatophore (12).

Photoreduction Reactions:

In the intact cell, the reducing phase of photosynthesis is evidenced in terms of carbon dioxide reduction. At the chromatophore level, the earlier experiments of French (2) and Vernon and Kamen (3) showed a photoreduction of oxygen. A photoreduction of NADP by chromatophores of *R. rubrum* was reported by Vernon (15). The experiments of Frenkel (7) showed that NAD is photoreduced by *R. rubrum* chromatophores coupled with a photooxidation of added $FMNH_2$. Subsequent experiments (8) showed that NAD photoreduction could be coupled with succinate, and that NAD was the nicotinamide nucleotide of choice in these reactions. Nozaki et al. (16) demonstrated that NAD photoreduction could also be coupled to the oxidation of $DPIPH_2$ in the presence of ascorbate. Other photoreductions observed with *R. rubrum* chromatophores have involved methyl red and tetrazolium blue (17), the disulfide DTNB (18) and sulfate ion (5). A summary of these photoreactions and the rates which have been observed to date is shown below in Table 6.

The photoreduction of intracellular NAD by *R. rubrum* cells was shown in the investigation of Duysens and Sweep (19). A similar photoreduction was observed with *Chromatium* cells by Olson (20), and Amesz (21) has recently completed an extensive investigation on NAD photoreduction with *R. rubrum* cells. Evidence has been presented for a photochemical reduction of ubiquinone contained within the chromatophores of *Chromatium* and *Rhodopseudomonas spheroides* (22).

Also, Nishimura has reported a photoreduction of cytochrome *b* in cells of *R. rubrum* which have been poisoned with antimycin A or HQNO (23,24). In this case, the cyclic electron transport system is apparently blocked, allowing a direct demonstration of cytochrome *b* photoreduction.

PHOTOOXIDATION REACTIONS CATALYZED BY *R. RUBRUM* CHROMATOPHORES

It is apparent that a number of photochemical oxidation and reduction reactions are now available for use in investigation of the electron transport system contained in chromatophores of the photosynthetic bacteria. I would like to present some detailed information on one of these reactions, namely the photooxidation of $DPIPH_2$. A preliminary report of some of these data has already appeared, and the experimental methods used to obtain the data reported here are essentially those which were described in this previous communication (25). The experiments were performed under anaerobic conditions with chromatophores prepared by sonic oscillation followed with two washings by centrifugation of the particles sedimenting in the centrifugal range of 20,000 to 100,000 x g.

The use of a modified Spectronic 505 recording spectrophotometer (25) permitted the photooxidation of $DPIPH_2$ to be followed in detail. Fig. 1 presents the results obtained with and without an added oxidant present. With only chromatophores present in the reaction system, a fast initial reaction was observed which saturated after two to three seconds of reaction time. The presence of either NAD or fumarate in the reaction system allowed a secondary slower reaction to take place following the initial fast reaction. In all cases a dark back-reaction was observed when the light was turned off. In the reaction system containing NAD, the NADH formed in the reaction was immediately converted back to NAD by means of an enzyme system consisting of lactic dehydrogenase and pyruvate. In the absence of this trapping system the secondary slow reaction was not observed, since there is an active NADH-DPIP diaphorase present in the chromatophores. The secondary slow reactions which are coupled to NAD and fumarate are no different from the coupled photoreactions previously observed (4, 8,16), both in mechanism and rate, as shown below.

The most interesting aspect of Fig. 1 is the initial fast photooxidation of $DPIPH_2$. The rapid and definite saturation of this reaction indicates that the oxidation of the $DPIPH_2$ is coupled to the reduction of components contained within the chromatophore. Fig. 2 shows the relationship of this initial fast reaction to the concentration of chromatophores contained within the reaction system. Not only is the rate of the initial fast reaction proportional to the chromatophore concentra-

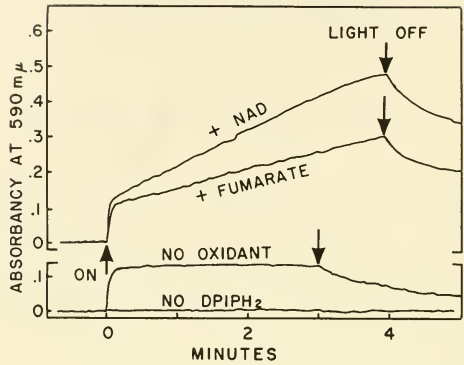

Fig. 1. Photooxidation of $DPIPH_2$ in the absence and presence of added oxidants. The basic reaction system contained 33 mM Tris buffer, 66 μM $DPIPH_2$ (reduced with ascorbic acid until a faint blue color remained) and *R. rubrum* chromatophores equal to the concentration of 0.22 mg BChl in a final volume of 8.0 ml (except for the case where no $DPIPH_2$ was present, in which experiment 0.48 mg BChl was present). When fumarate was present the pH was 8.0 and the final concentration of fumarate was 0.75 mM. For the experiment with NAD, the pH employed was 8.5 and the system also contained 0.5 mM DPN, 1.3 mg of lactic dehydrogenase, and 3.1 mM sodium pyruvate. Anaerobic conditions were obtained by three evacuations with alternate flushing with argon gas. Experiments were carried out using Thunberg tubes which were modified by joining the main tube through a T-joint with 2 one-cm Pyrex absorption cells. These cells were spaced to fit into the cell holder of a Spectronic 505 recording spectrophotometer which had been modified to allow one arm of the reaction vessel to be illuminated by a tungsten lamp mounted outside the housing of the spectrophotometer. The light intensity reaching the reaction vessel was 1.05 x 10^6 ergs per second per square cm. The experiments were run at 30°C for the fumarate system, and 20°C for the NAD system. For further details concerning the chromatophore preparation procedure and other techniques employed see reference 25.

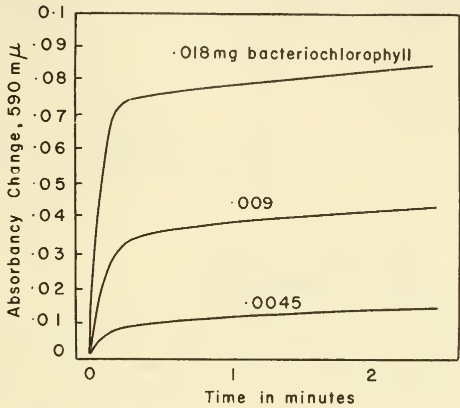

Fig. 2. Influence of *R. rubrum* chromatophore concentration upon the initial fast photooxidation of $DPIPH_2$. Experimental conditions as for Fig. 1.

tion, but the extent of the reaction is also directly proportional to the chromatophore concentration, supporting the idea that the photooxidation of the $DPIPH_2$ observed in the fast reaction is coupled to the photoreduction of components contained within the chromatophore. The stoichiometry of the reaction, as shown below, is also consistent with this hypothesis.

The data presented in Fig. 1 are traces made of the actual recording and show the noise inherent in the system. In subsequent figures a smooth curve has been drawn over the original tracing, so that the noise is not apparent.

Another electron donor which reacts in a similar manner in this system is N,N,N',N'-tetramethyl-*p*-phenylenediamine, abbreviated TMPD, whose response is shown in Fig. 3. It resembles $DPIPH_2$ in all respects, including the initial fast reaction and the saturation in the absence of an added oxidant. It differs only in that it appears to react faster than does $DPIPH_2$. This compound is an excellent donor of electrons, and has been shown to react with chloranil (26), and with cytochrome *c* contained in mammalian mitochondrial systems (27).

The ability of *R. rubrum* chromatophores to photoxidize the reduced forms of methylene blue and cytochrome *c* was reported earlier (4). The kinetics of such oxidations obtained under the present conditions are shown in Fig. 4. For these experiments the methylene blue was reduced enzymatically by succinate in the presence of the chromatophores prior to illumination, and fumarate was then added to poise

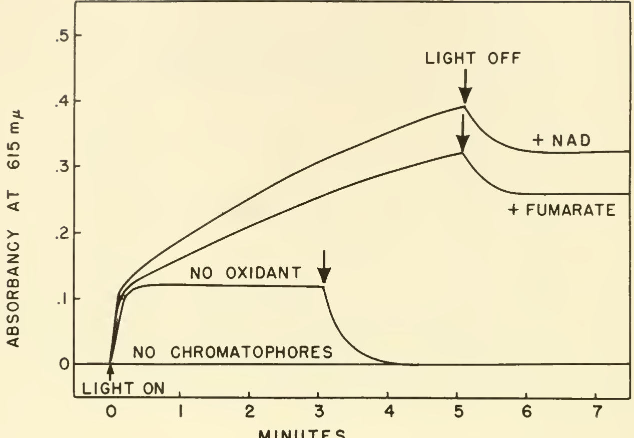

Fig. 3. Photooxidation of TMPD by *R. rubrum* chromatophores. The experimental conditions were as given for Fig. 1, except that 0.1 mM TMPD was substituted for $DPIPH_2$. The BChl concentration was 0.196 mg in the 8 ml reaction system.

the system. Illumination in this case resulted in a fast reaction followed by a secondary slower reaction which was coupled to the photoreduction of the added fumarate. When the light was turned off, a biphasic reaction was observed. The initial fast back-reaction can be correlated with reduction of methylene blue by the reduced components in the chromatophore, while the secondary back-reaction is due to the enzymatic reduction of methylene blue by the succinate present in the system.

In the case of ferrocytochrome *c*, a reaction was observed in the absence of added oxygen, but the initial reaction was slow and the extent of the reaction was less than that observed with $DPIPH_2$. In this case also, a coupled photooxidation could be obtained when fumarate was added to the system. These reactions observed are in agreement with the data on photooxidations previously reported (4), but the present experiments show there are two phases for the photooxidation of both these compounds.

The response of ferrocyanide in the *R. rubrum* chromatophore system was examined, and the results of this experiment are shown in Fig. 5. Although some absorbancy change was noted when the system was illuminated, this was largely due to absorbancy changes which take

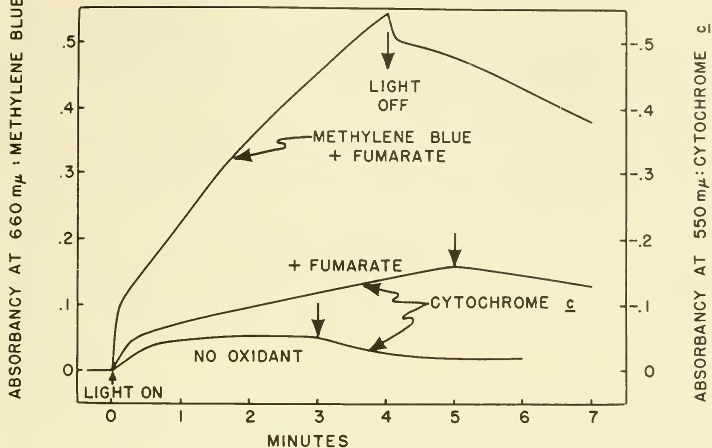

Fig. 4. Photooxidation of reduced methylene blue and ferrocytochrome *c* by *R. rubrum* chromatophores. The experimental conditions were those given for Fig. 1, except that $DPIPH_2$ was replaced with the oxidants listed. Ferrocytochrome *c* was prepared by reduction with borohydride. When present, it was 5 mg per 8 ml and the BChl concentration was 0.190 mg. For the experiment involving MB, 3.1 mM succinate was present initially to reduce the MB via the succinic dehydrogenase contained in the chromatophore particles. Following the enzymatic reduction of MB, sufficient fumarate was added to make the solution 0.75 mM in fumarate. For this experiment the BChl concentration was 0.128 mg.

place within the chromatophores themselves at this wave length. There was no coupled reaction with either fumarate or NAD present in the system. The inactivity of ferrocyanide in the present case is somewhat surprising, since ferricyanide has been shown to interreact with bacterial chromatophores in two ways. Goedheer has shown that ferricyanide will cause a bleaching of bacteriochlorophyll in *R. rubrum*, which corresponds to an oxidation of the pigment (28). Calvin and Androes have also reported that mixtures of ferro- and ferricyanide of different redox potentials influence the photoinduced ESR signal observed with chromatophores of *R. rubrum* (29). One possibility for the inactivity of ferrocyanide in the present reaction is that it may be reduced as rapidly as it is oxidized by the bacterial chromatophores. If the ferrocyanide must react through the cytochrome c_2, whose standard redox potential is below that of the ferrocyanide system, the resulting slow

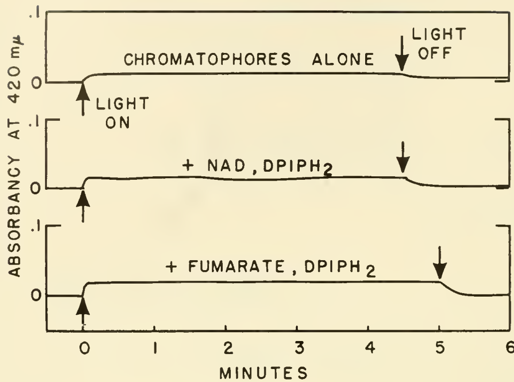

Fig. 5. Inability of *R. rubrum* chromatophores to photooxidize ferrocyanide. The experimental conditions were the same as those given for Fig. 1, except that 1 mM potassium ferrocyanide was substituted for $DPIPH_2$. The BChl concentration was 0.212 mg per 8 ml reaction system.

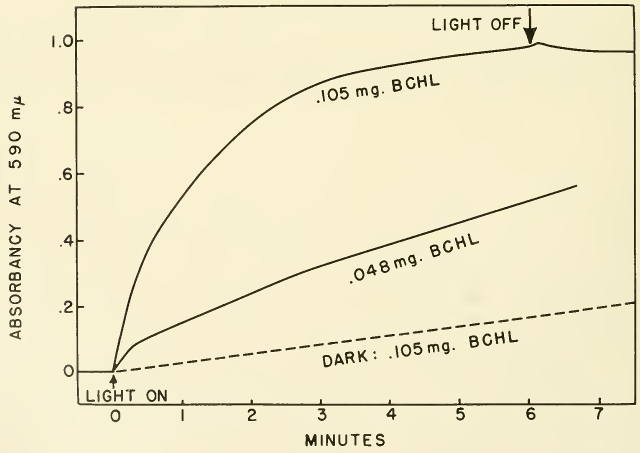

Fig. 6. Photooxidation of $DPIPH_2$ by *R. rubrum* chromatophores under aerobic conditions. The experimental procedures given for Fig. 1 were employed with air present. No oxidant was added.

rate of photooxidation could easily be balanced by reduction reactions from the photoreduced chromatophore components.

One of the early reactions observed with *R. rubrum* chromatophores was the photooxidation of ascorbate in the presence of DPIP and molecular oxygen (3). The ability of chromatophores to photooxidize reduced dye in the presence of oxygen is shown in Fig. 6. The oxidation of the dye in the presence of oxygen is a very stable reaction and is not appreciably influenced by heating the chromatophores to 60°C. The stability of this system has been studied by Lindstrom (6). By decreasing the chromatophore content in the reaction system, it was possible to observe the usual biphasic reaction shown for the other photooxidations. It is interesting, however, that the initial photooxidation rate in the presence of oxygen was significantly lower than that observed under anaerobic conditions. The reason for this is not immediately apparent.

PHOTOOXIDATION REACTIONS CATALYZED BY *CHROMATIUM* AND *RHODOPSEUDOMONAS SPHEROIDES* CHROMATOPHORES

Other photosynthetic bacteria were investigated to see if their photosynthetically active particles could also photooxidize $DPIPH_2$ in a manner similar to that observed with *R. rubrum*. Fig. 7 presents the

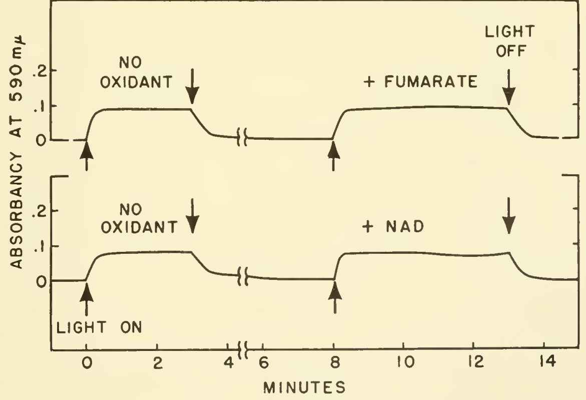

Fig. 7. Photooxidation of $DPIPH_2$ by *Chromatium* chromatophores. The experimental conditions given for Fig. 1 were employed with *Chromatium* chromatophores being used at a concentration equal to 0.264 mg BChl.

data obtained with *Chromatium* chromatophores. In the absence of added oxidants, an initial fast reaction was observed. Although the rate of the reaction was less than that observed with *R. rubrum*, the back-reaction was faster than that observed with *R. rubrum*. The slower initial photooxidation rates may merely reflect the fact that a faster back-reaction obtains with these bacterial particles.

The most significant difference observed between *R. rubrum* and *Chromatium* lies in the fact that the latter particles are unable to couple the photooxidation of the reduced dye with either fumarate or NAD reduction, as shown in Fig. 7. To further check on this problem, corollary experiments were done in which NAD reduction was attempted in the presence of either succinate or ascorbate-DPIP, which systems were designed for detection of NADH accumulation. All experiments of this nature were negative.

Chromatium chromatophores were tested for their ability to photoreduce methyl red, which is active in the photoreduction system of *R. rubrum* chromatophores (17). Fig. 8 shows that *Chromatium* chromatophores were able to photoreduce this dye, although the observed rate was less than that obtained with *R. rubrum*. All indications point to a relatively simple system being involved in the photoreduction of methyl red and tetrazolium blue in the presence of the ascorbate-DPIP couple. As shown below, this activity in the case of *R. rubrum* chromatophores is more stable than is the NAD or fumarate reducing systems.

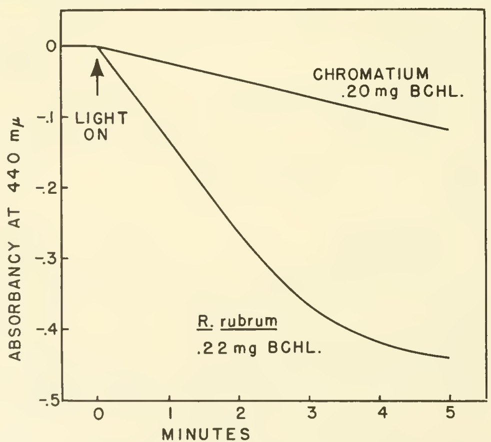

Fig. 8. Photoreduction of methyl red by *R. rubrum* and *Chromatium* chromatophores. The experimental conditions given by Ash et. al. (17) were employed. The BChl concentrations were 0.20 for *R. rubrum* and 0.20 for *Chromatium* for the 8 ml reaction system.

The reason for the failure to show NAD reduction with *Chromatium* chromatophores is not immediately apparent. Arnon reported that he was able to obtain a photoreduction of NAD in the presence of ascorbate and DPIP (30). In our laboratory, however, we have not been able to obtain a photoreduction of NAD with *Chromatium* chromatophores under any circumstance. This is surprising, since this bacterium would be expected to have an active system for NAD photoreduction, because it must reduce carbon dioxide via the photosynthetic route for all of its carbon compounds.

Another photosynthetic bacterium, *Rhodopseudomonas spheroides*, was examined in the usual photooxidation systems with the results given in Fig. 9. Again, this organism showed the capacity to photooxidize $DPIPH_2$ in a fast reaction which soon saturated in the usual manner. Like *Chromatium*, this bacterium did not have the ability to sustain the secondary coupled reactions with either NAD or fumarate, although there was a hint of a slow coupled reaction when fumarate was added to the system. In this case also, it was not possible to demonstrate directly a photoreduction of NAD when DPIP and an excess of ascorbate were present as the electron-donating system (31). The reason for inactivity in NAD reduction in the case of both *Chromatium* and *Rhodopseudomonas spheroides* is not known. One should probably look at the method of chromatophore preparation to see if inactivation of enzymes or other factors is involved in this situation.

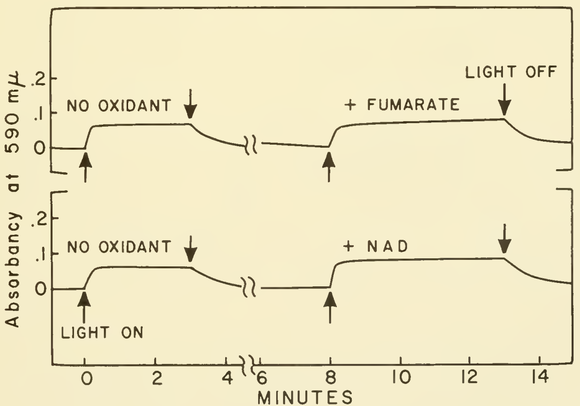

Fig. 9. Photooxidation of $DPIPH_2$ by *Rps. spheroides* chromatophores. The experimental conditions given for Fig. 1 were employed, except that *Rps. spheroides* chromatophores equal to 0.110 μmoles of BChl per 4 ml were employed.

CHARACTERISTICS OF THE $DPIPH_2$ PHOTOOXIDATION SYSTEM OF *R. RUBRUM* CHROMATOPHORES

From the extent of the fast reaction observed with both *R. rubrum* and *Chromatium*, it is possible to calculate the amount of $DPIPH_2$ converted in the photooxidation reactions. Table 1 presents the results of such calculations, showing that for *R. rubrum* there is one mole of $DPIPH_2$ oxidized for every 6 moles of BChl contained in the chromatophore. The value for *Chromatium* is approximately one mole of $DPIPH_2$ oxidized for each 7.5 moles of BChl. A logical compound to consider as the intrachromatophoral oxidant for $DPIPH_2$ is ubiquinone.

TABLE 1.

Ratio of BChl molecules to $DPIPH_2$ photooxidized by chromatophores. The experimental conditions for R. rubrum *were those given for Fig. 1, with only slight variation in BChl content among the different experiments. The experimental conditions for Chromatium were the same as those given for Fig. 7.*

	$BChl/DPIPH_2$
R. rubrum	
Average of 57 samples	5.9
Chromatium	
Average of 5 samples	7.5

This compound is present in chromatophores of both *Chromatium* and *R. rubrum* in high concentrations. Fuller et al. have reported a ratio of about five for chlorophyll to ubiquinone in *Chromatium* (32). Lester and Crane (33) have reported a value of 4.3 μmoles ubiquinone per gram dry weight for *R. rubrum*, which compares with 2.9 for *Chromatium*. Nishimura (34) has recorded a value of 19 for the ratio of chlorophyll to cytochrome in *R. rubrum*. When this ratio is coupled to the information presented by Geller (35) on the heme protein and ubiquinone content of *R. rubrum* chromatophores, a ratio of about three chlorophylls to one ubiquinone can be calculated. Thus, there is sufficient ubiquinone present in the chromatophores to account for the observed photooxidation of $DPIPH_2$ and TMPD in the absence of added external oxidants. The succeeding paper presents definite evidence that ubiquinone is reduced as a function of added $DPIPH_2$ with *R. rubrum* chromatophores. Clayton (22) has previously presented evidence that a photoreduction of ubiquinone is a primary event following light ab-

sorption by *Chromatium* chromatophores. When all these data are considered, it appears likely that the photooxidation of $DPIPH_2$ and TMPD is linked to the photoreduction of ubiquinone contained within the chromatophore itself.

The response of the *R. rubrum* chromatophores to varying concentrations of NAD and fumarate have allowed the calculation of a Michaelis constant for these secondary slow reactions. In the case of NAD, a Km of 2.5×10^{-5} was calculated, while the corresponding Km for fumarate was 1×10^{-4} molar. These values are in the range expected of ordinary enzymatic reactions, and agree fairly well with the

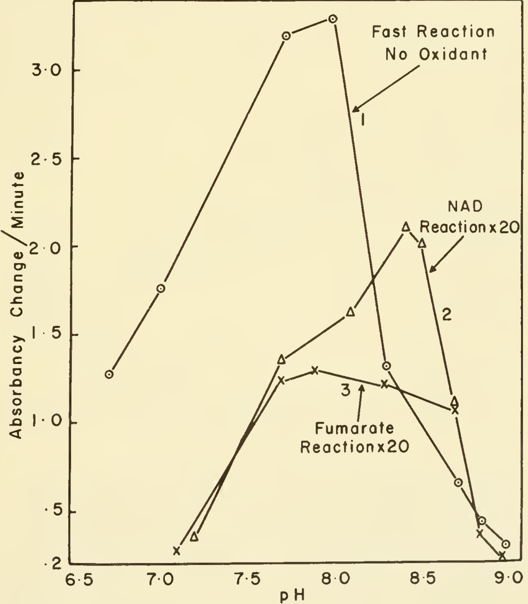

Fig. 10. pH optima for the three reactions involving $DPIPH_2$ photooxidation with *R. rubrum* chromatophores. The experimental conditions given for Fig. 1 were employed utilizing 0.20 mg of BChl in the reaction system. Tris buffer was employed for the pH range from 7.0 to 9.0 and phosphate buffer was employed for the lower pH values. The rates for the slow reactions were multiplied by 20 to place them on the same scale as the fast reactions.

response to NAD and NADH concentrations observed by Horio and Kamen in the case of NADH-RHP reductase and the NAD photoreduction supported by succinate with *R. rubrum* chromatophores (36,37).

The pH optima of these reactions are shown in Fig. 10. The effect of temperature on the photooxidation of TMPD was examined by following the reaction at room temperature and at the temperature of liquid nitrogen. These experiments, shown in Fig. 11, were carried out in collaboration with and through the courtesy of Dr. Britton Chance. The photooxidation of TMPD did not proceed at the temperature of liquid nitrogen, indicating an ordinary chemical reaction was involved in this photooxidation. This distinguishes it from the physical process which results in cytochrome oxidation in *Chromatium* (12).

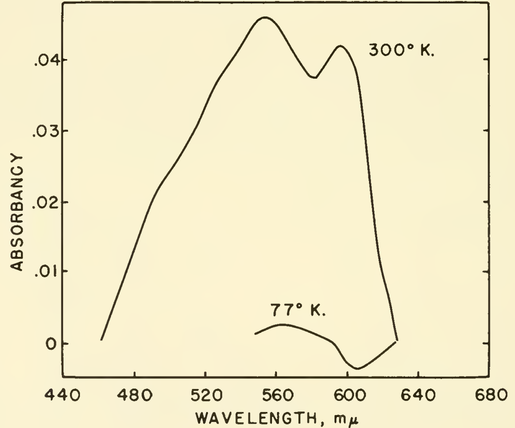

Fig. 11. Lack of TMPD photooxidation at 77°K. Experimental conditions given for Fig. 3 were used, except that the reactions were run aerobically and contained 0.20 mg of bacterial chlorophyll. These experiments were performed by Dr. Britton Chance using the spectrophotometer described previously (12). The curve corresponding to 300°K was taken directly from the recording following 30 seconds illumination time with red light. The curve corresponding to 77°K was plotted by taking the difference between the tracing obtained before illumination and following 70-second illumination. The minimum observed at 600 mμ is characteristic of light absorption changes which occur with chromatophores alone.

The effect of flavins upon the photooxidation of $DPIPH_2$ was examined. Fig. 12 shows the effect of adding FMN to the *R. rubrum* system. At a level of 3×10^{-7} M, FMN exerted an appreciable stimulation with the NAD slow reaction but had no effect upon the coupled fumarate reaction. FAD gave about one-third the stimulation in the NAD system and had no effect upon the fumarate system. Riboflavin was inactive in both systems. One striking feature of the FMN stimulation was the low concentration at which FMN was effective, with half maximal stimulation at a concentration of about 10^{-8} M FMN. The response in the present system to FMN should be compared with the stimulating effect this nucleotide has upon the DPNH-cytochrome c_2 reductase system in the purified fractions obtained by Horio and Kamen (36).

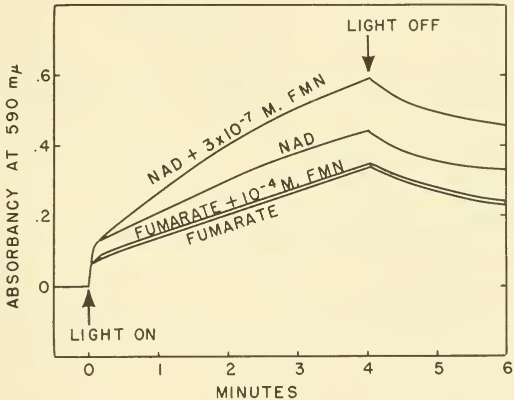

Fig. 12. The effect of FMN on $DPIPH_2$ photooxidation by *R. rubrum* chromatophores. The experimental conditions for Fig. 1 were used with 0.21 mg of BChl present.

This dark enzymatic reaction is stimulated half-maximally at about 10^{-7} M FMN. FAD is less active than FMN, but riboflavin resembles FMN in its activity. It is possible that the same flavoprotein is involved in the present photooxidation reactions and in the dark enzymatic reactions studied by Horio and Kamen.

Addition of quinacrine to the various oxidation systems produced the interesting results shown in Fig. 13. A clear separation of the coupled photooxidations with fumarate and NAD was observed, the NAD-coupled photooxidation being completely inhibited while the fumarate-coupled oxidation was markedly stimulated. This shows clearly that

two systems are involved, one for NAD and one for fumarate. Quinacrine has been shown to have potent effects upon electron transfer systems in photosynthetic bacteria. Baltscheffsky has shown that this compound is an inhibitor of the photophosphorylation process (38). Furthermore, the inhibition observed in his experiments was partially relieved by the addition of FAD, while FMN was less active. It should be noted, however, that extremely high concentrations of FAD and FMN were required for these reactivations, and at such high concentrations the flavin nucleotides are active in nonenzymatic reactions.

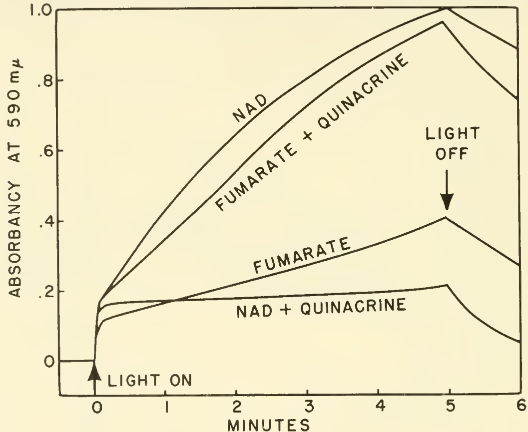

Fig. 13. The effect of 2×10^{-4} M quinacrine on $DPIPH_2$ photooxidation by *R. rubrum* chromatophores. The experimental conditions employed for Fig. 1 were used with 0.21 mg of BChl present.

The experiments of Ash et al. (17) show that quinacrine has a striking effect upon the photoreduction of methyl red and tetrazolium blue by *R. rubrum* chromatophores. The stimulating effect of quinacrine on a very similar system has been found by Bose and Gest (39). In their experiments, *R. rubrum* chromatophores catalyzed the photoreduction of fumarate by hydrogen gas. This photoreduction required the presence of DPIP, which was reduced enzymatically by the hydrogen gas. Therefore, the two systems are essentially the same, consisting of $DPIPH_2$ and fumarate. In both cases $DPIPH_2$ is photooxidized and fumarate is reduced, and in both cases quinacrine markedly stimulates the reactions.

The effect of respiratory inhibitors upon both the fast and coupled slow reactions is shown in Table 2. Phenylmercuric acetate (PMA), a compound which inhibits by combination with sulfhydryl groups, inhibited both the NAD and fumarate slow reactions. It was less effective on the fast reaction, and had little effect on the aerobic photooxidation. As shown below, the fast reaction and the aerobic photooxidation reactions are stable activities which are resistant to heating. This indicates that the fast reactions and the aerobic reaction involves only a portion of the electron transport chain, and this portion is relatively inert to various treatments and outside agents.

The inhibitions caused by antimycin A and HQNO also reveal that the coupled slow reactions are most sensitive. However, in general the

TABLE 2.

Effect of inhibitors upon the photoreactions of R. rubrum *chromatophores. The reaction conditions were as given for Fig. 1. The concentration of BChl was 0.20 mg.*

	Per Cent Inhibition				
	NAD		Fumarate		Aerobic
	Fast RX	Slow RX	Fast RX	Slow RX	
PMA, 10^{-4} M	25	87	13	70	6
" 10^{-5} M	12	47	4	63	0
Antimycin A, 1.1×10^{-4} M	18	56	0	24	0
" " 2.3×10^{-5} M	5	16	0	0	-
HQNO, 1.4×10^{-4} M	9	46	10	19	0
" 2.9×10^{-5} M	5	21	—	—	-

reactions reported here are less sensitive to both antimycin A and HQNO than either the photophosphorylation process or the photoreduction of NAD coupled to succinate. 10^{-5} M antimycin A inhibits over 90 per cent of the activity in the NAD-succinate system (16) and in the photophosphorylation process (16,40). HQNO is also over 90 per cent effective in these reactions at a concentration of 10^{-5} M (16,40). It would appear, therefore, that the antimycin A and HQNO inhibitions observed in the present case are of a different nature from the inhibitions observed in the photophosphorylation process. This has significance when considering the mechanism of the reduction of fumarate and NAD by $DPIPH_2$. As discussed below, the inhibition pattern is not consistent with the idea that the reduction of these compounds is due to a reversed electron transfer coupled to ATP utilization, since at the level

of inhibitors used (10^{-5} M) ATP formation is inhibited over 90 per cent and the reported photooxidation reactions are only slightly decreased.

The effect of ADP and Pi on the various reactions is shown in Table 3. Again a distinction is apparent between the NAD-coupled and the fumarate-coupled slow reactions. Whereas the NAD reaction was inhibited by the addition of these components, the fumarate reaction was stimulated. If it is true that stimulation of a reaction by ADP and Pi indicates a phosphorylation in that reaction sequence, this would indicate that the photoreduction of NAD accompanying $DPIPH_2$ oxidation does not involve a phosphorylation, whereas the photoreduction of fumarate is accompanied by ATP formation. These conclusions should be considered as only tentative, however, since ADP can have effects other than that of stimulating a reaction coupled to ATP formation.

TABLE 3.

Effect of ADP + Pi on the photoreactions of R. rubrum *chromatophores. The reaction conditions were those given for Fig. 1 with a BChl concentration of 0.21 mg.*

Concentration	Per Cent Stimulation			
	NAD		Fumarate	
	Fast RX	Slow RX	Fast RX	Slow RX
10^{-3} M. ADP 10^{-3} M. Pi	22	-69	12	32
"	25	-40	22	38

However, it should be noted that the concentrations used in these experiments are below those reported by Horio to give inhibition of NAD reduction associated with succinate oxidation (37).

The activity of heated chromatophores is shown in Fig. 14. For these experiments aliquots of chromatophores were heated at the indicated temperatures for five minutes, following which the reaction was run in the usual fashion. The rates of the slow reactions were multiplied by ten in order to place them on the same scale as the fast reactions. From this information it is apparent that heating to 40°C was sufficient to destroy the reaction coupled to NAD, and heating to 50°C inactivated the system involving fumarate. Heating to 60°C had very little effect upon the fast reaction observed. Indeed, to inactivate the fast reaction with *R. rubrum* chromatophores, a temperature of 80°C for five minutes must be employed. This emphasizes the stability of the fast reaction, and again implicates a very stable and perhaps frac-

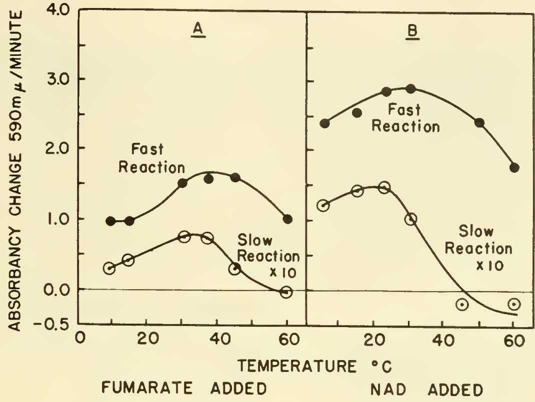

Fig. 14. Heat stability of $DPIPH_2$ photooxidation reactions with *R. rubrum* chromatophores. The experimental conditions outlined for Fig. 1 were employed with 0.20 mg of BChl present. The chromatophores were heated at the indicated temperature for 5 minutes before addition to the reaction system and commencement of the illumination period. The photooxidation reactions were run at the usual temperatures. The rates for the slow reactions were multiplied by 10 in order to place them on the same scale as the fast reactions.

tional part of the entire electron transport system for the observed fast reaction. The stability of the fast reaction reported here agrees with the previously reported stability of the aerobic photooxidation of $DPIPH_2$ as reported by Lindstrom (6).

The rates of various related dark enzymatic reactions for chromatophores of *R. rubrum* and *Chromatium* are given in Table 4. These are the various dark enzymatic reactions which might be expected to influence the photoreactions under investigation. The *R. rubrum* chromatophores have a very potent NADH-DPIP diaphorase, which is most likely the enzyme which has been studied in some detail by Horio and Kamen (36). *R. rubrum* has very weak $DPIPH_2$ oxidase activity and a moderate NADH oxidase activity. *Chromatium* also has a potent NADH-DPIP diaphorase activity, and has a very active $DPIPH_2$ oxidase activity. This is surprising because this organism lives under anaerobic conditions and does not practice a respiration involving oxygen, where a terminal oxidase would be expected to function.

TABLE 4.

Rates of dark enzymatic reactions of chromatophores. The reaction mixtures (4 ml total volume) contained chromatophores equivalent to 0.1 and 0.165 mg of BChl for R. rubrum *and* Chromatium *respectively, with the buffer concentrations listed in Figs. 1 and 7. The experimental system for NADH-DPIP diaphorase also contained 1.5 μmoles of NADH and 0.27 μmoles of DPIP, that for $DPIPH_2$ oxidase contained 0.27 μmoles of $DPIPH_2$ and that for NADH oxidase contained 0.6 μmoles of NADH. The diaphorase assay was performed under anaerobic conditions at 25°C.*

	μmoles/hr/mg Chlorophyll	
	R. rubrum	*Chromatium*
NADH-DPIP Diaphorase	18	17.5
$DPIPH_2$ Oxidase	1	15
NADH Oxidase	7.7	2.4

CORRELATION OF KNOWN PHOTOREACTIONS IN *R. RUBRUM*

It would be well to compare the rates of $DPIPH_2$ photooxidation with other photoreactions which have been reported in the literature. Table 5 reports the rates we have observed for both *R. rubrum* and *Chromatium*. The rates reported are average values, and the fastest photooxidation rate observed to date was the fast reaction with NAD present, which amounted to 553 μmoles $DPIPH_2$ oxidized per hour per mg of BChl. This reaction, then, is one of the fastest reactions reported for the photochemical system of the bacteria. In general, *Chromatium* particles were less active on a chlorophyll basis. The data again show the lack of a coupled slow reaction with either NAD or fumarate.

The rates for photophosphorylation reported in the literature usually fall between 100-300 μmoles per hour per mg of chlorophyll (16,41,42, 43). However, M. Baltscheffsky has reported a photophosphorylation rate of 620 μmoles per hour per mg of chlorophyll (44). The rates of the two reactions, the photooxidation of $DPIPH_2$ and ATP formation under the influence of light, are certainly very similar. Again this lends support to the idea that the photooxidation of $DPIPH_2$ observed is mediated by the central closed electron transfer system contained in *R. rubrum* chromatophores, and that the same light-activating system is active in both cases.

The rates of other photooxidation and photoreduction reactions reported in the literature for *R. rubrum* chromatophores have been com-

TABLE 5.

Rates of $DPIPH_2$ photooxidation. The values given for R. rubrum *are average values obtained from 50 experiments, with the exception of the aerobic reaction. The values given for* Chromatium *are average values taken from 5 experiments.*

	μmoles/hr/mg Chlorophyll	
	R. rubrum	*Chromatium*
Fast Reaction with NAD	404	224
Fast Reaction with Fumarate	220	287
Slow Reaction with NAD	12	0
Slow Reaction with Fumarate	7.3	0
Aerobic Reaction	47	16.5

piled in Table 6. Perusal of this table shows that the fastest rate reported is that by Amesz for the photoreduction of NAD by whole cells (21). This rate of 360 μmoles per hour per mg of BChl places this photoreduction within the same range as photophosphorylation, and just below the rate of the fast $DPIPH_2$ photooxidation reported in this paper. The rate reported by Amesz was the initial rate obtained when the cells went from a nonilluminated to an illuminated condition, and represents the maximal rate at which electrons could be transferred from cytochrome through chlorophyll to NAD.

The rate of 145 reported by Ash et al. for the photoreduction of methyl red in the presence of quinacrin is higher than the usual reaction reported. It appears from the evidence at hand that methyl red photoreduction (in the presence of ascorbate and DPIP as electron donor) is one of the more stable reactions catalyzed by chromatophores and involves only a portion of the chromatophore system. None of the ordinary enzymatic components involved in NAD photoreduction and fumarate photoreduction are required in the case of methyl red (17). The remainder of the photoreactions have rates between the range of 4 to 45 μmoles per hour per mg of BChl. The slow photooxidation reactions coupled to NAD and fumarate reported in this investigation fall in this range also. These apparently, then, are the photooxidation reactions which are coupled to the enzymatic components within the electron transfer system.

The information available on rates of the various photoreactions is consistent with the idea that an initial fast photooxidation of $DPIPH_2$ is representative of the fast photochemical reactions induced following the absorption of light quanta by the chromatophore. It is generally thought that the bacterial chromatophore contains a closed electron

TABLE 6.

Rates of other photoreactions of R. rubrum *chromatophores.*

Photoreaction	Rate and Reference µmoles/hr/mg Chlorophyll	
Oxidations		
H_2 Oxidation (with DPIP)	4	(Bose and Gest, 39)
$FMNH_2$ Oxidation (with NAD)	9.5	(Frenkel, 7)
Reductions		
NAD (with $FMNH_2$)	9.1	(Frenkel, 7)
NAD (with Succinate + CN^-)	45	(Vernon and Ash, 8)
NAD (with Succinate)	24	(Nozaki et al., 16)
NAD (whole cells)	360	(Amesz, 21)
Reductions Coupled with Ascorbate and DPIP		
NAD	37	(Nozaki et al., 16)
Disulfide	11	(Newton, 18)
Methyl Red	24	(Ash et al., 17)
Methyl Red + Quinacrine	145	(Ash et al., 17)
Sulfate	43	(Ibanez and Lindstrom, 5)

transfer system in the sense that electrons travel along this electron transport chain in the usual fashion (from the compound with the lowest oxidation potential to that with the highest potential) and are then recycled through the light-activated chlorophyll back to the low potential compound once again. Isolated chromatophores have the capacity to carry out the process of photophosphorylation with no added cofactors, and this phosphorylation is very sensitive to the extent of reduction of the various components within the chain, since either overreduction or overoxidation inhibits the cyclic phosphorylation process (42,55, 57). Thus, we can imagine the electron transport system of the chromatophore as consisting of a closed electron transport system which yields ATP when electrons are cycled around the system. There must be several points of entry onto the system to allow the photochemical reactions to be coupled with the chemical oxidation and reduction reactions which must take place in the whole cell (NAD reduction and substrate oxidation).

The fastest rates observed with isolated chromatophores involve the photophosphorylation process and the photooxidation of $DPIPH_2$ in the initial fast reaction. The fast reaction of $DPIPH_2$ must be coupled to the photoreduction of components contained within the cyclic electron transfer system. The most likely candidate for such a photoreduction is ubiquinone. The secondary slow reactions coupled to $DPIPH_2$ oxidation appear to involve other enzymatic components on the chromato-

phore which are not ordinarily associated with the cyclic electron transfer process. This accounts for the slower rates observed in general for the photooxidation and photoreduction reactions with *R. rubrum* chromatophores.

The same general pattern applies to *Chromatium*, with the exception that in this case the slow enzymatic reactions are not observed at all. Some essential cofactor must be lost or destroyed during preparation of the chromatophore.

There is an alternative explanation for the data presented above. There is ample evidence for "reverse electron flow" in mitochondrial systems, in which case the electrons are transferred from either succinate or cytochrome to NAD (45). This transfer being against the thermodynamic gradient, energy is required and the energy is supplied by ATP. Thus the requirements for electron transfer in the reverse direction are the necessary enzymatic components plus energy supplied in the form of ATP. In the present case, these conditions are present. Thus one could explain the photooxidation of $DPIPH_2$ as being due to reversed electron transport via the reagents contained in the chromatophore with the energy for this reverse electron transport being supplied by the ATP formed in the process of photophosphorylation. Although on the surface this explanation appears to be tenable, there are a number of reasons which lead me to believe that this cannot be the case. These reasons are as follows:

1. The photophosphorylation process is very sensitive to the respiratory inhibitors, antimycin A and HQNO. At 10^{-6} M inhibitor concentration, the photophosphorylation process is almost completely inhibited, while at this concentration of inhibitor the photooxidation reactions proceed with very little depression in rate. A more striking example is given by the compound quinacrine which inhibits photophosphorylation by 80 per cent at a concentration of 5×10^{-4} M (40), yet has no appreciable effect on the $DPIPH_2$ fast reaction at this concentration and actually stimulates the coupled slow reaction with fumarate.
2. When *R. rubrum* chromatophores are heated to 60°C, their ability to carry out photophosphorylation is completely inhibited. Nevertheless, such chromatophores still have the ability to catalyze the fast oxidation of $DPIPH_2$ as shown in Fig. 14.
3. The photoreduction of NADP by $DPIPH_2$ in the plant system (utilizing the long wavelength system in chloroplasts) does not appear to proceed by reverse electron flow. Thus, the photoreduction of NADP by $DPIPH_2$ results in the formation of ATP, and removal of plastoquinone from the chloroplasts prevents the ATP formation but does not prevent NADPH formation (46).
4. Addition of ATP in the dark to the system containing chromatophores, $DPIPH_2$ and NAD does not result in the formation of NADH and DPIP. The results of the experiments are shown in Fig. 15. Whereas

ATP was inactive, the subsequent illumination caused the immediate photooxidation of $DPIPH_2$ in the typical fast reaction. The possible objection to this reasoning is that ATP may not enter into the chromatophore and be able to affect the reactions as does ATP formed directly in the photophosphorylation process. This argument would seem invalid since the chromatophores used in this experiment are capable of coupling with external ADP and Pi to form ATP which is contained in the medium. Thus the phosphorylation site is available to added reagents and a reversed electron flow, if operative in the above experiments, should have been capable of demonstration.

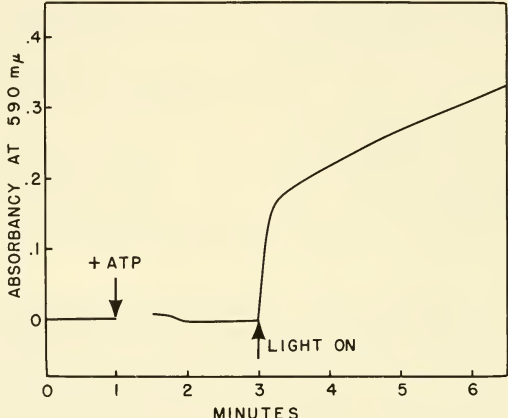

Fig. 15. Effect of ATP addition upon $DPIPH_2$ oxidation with NAD in the presence of *R. rubrum* chromatophores. The experimental conditions given for Fig. 1 were employed with 0.21 mg BChl present. At the arrow, 8 μmoles of ATP were added to the reaction system.

The reasons given above for supporting a direct electron transfer from $DPIPH_2$ to NAD are based on the fact that ATP formation and $DPIPH_2$ photooxidation can be separated by means of inhibitors and various treatments. The possibility remains, however, that the reaction does proceed via reverse electron transfer, with the energy being supplied not by ATP itself, but by some high energy intermediate which under normal circumstances would lead to ATP formation. If the inhibitors act at a late stage in ATP formation, and allow the formation of intermediate high energy compounds, then the conclusions based on

these inhibitor studies are not valid. However, one cannot resolve this problem at present, since it is not possible to detect and experimentally manipulate such high energy intermediates.

ELECTRON TRANSFER SEQUENCE

Since there is considerable information becoming available on the various photooxidation and photoreduction reactions catalyzed by *R. rubrum*, it would be well to attempt to correlate all this information. Figs. 16, 17, and 18 are an attempt to do this and are presented here not with the intent of being an authoritative statement on this matter, but rather with the idea of using this means to bring the information to the attention of workers in the field with the hope that it may stimulate thinking and experimentation to solve some of the problems which now face us. Fig. 16 is a representation of the components of the chromatophore electron transfer system arranged in a probable sequence for electron transfer. Fig. 17 has imposed upon this basic electron transfer system the sites of action of the various compounds known to be effective in photooxidation and photoreduction reactions. Fig. 18 lists the possible sites of action of the inhibitors known to affect the photochemical reactions in *R. rubrum* chromatophores.

It immediately becomes apparent that the various photochemical reactions demonstrated by *R. rubrum* chromatophores are couched in terms of only one electron transfer system. In discussing Fig. 17 it will be pointed out that a portion of this electron transfer scheme could be operative in the respiratory reactions which take place in the dark with this organism.

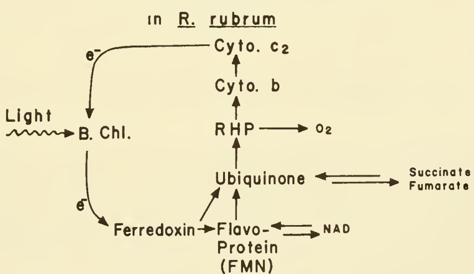

Fig. 16. Electron transfer components and sequence of reaction in *R. rubrum* chromatophores.

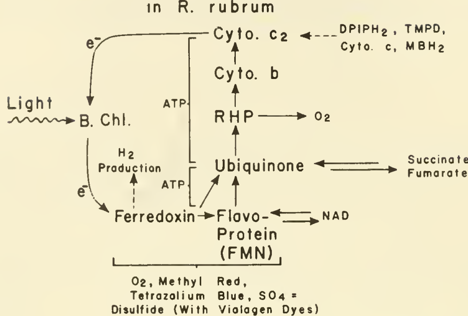

Fig. 17. Reaction sites for photochemical reactions observed with *R. rubrum* chromatophores.

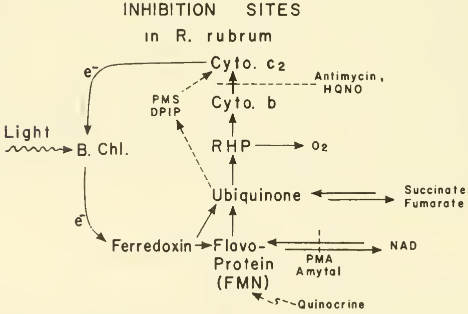

Fig. 18. Sites of inhibitor action with *R. rubrum* chromatophores.

The components contained within the *R. rubrum* chromatophores are presented in Fig. 16 in a logical sequence. There is ample evidence that cytochrome c_2 contained in this organism is photooxidized in a primary photochemical reaction by the BChl (9-13,28). This organism also contains a compound which appears to be similar to the ferredoxin isolated by Tagawa and Arnon from *Chromatium* (47). Preliminary evidence indicates that the ferredoxin is tightly bound to the chromatophore in the case of *R. rubrum* and is not readily extracted. The first compound shown to be reduced by the action of light in the case of plant

chloroplasts is the corresponding compound photosynthetic pyridine nucleotide reductase (48). For these reasons ferredoxin is presented as the compound being reduced by light-activated chlorophyll. It should be mentioned, however, that in the experiments designed to demonstrate a ferredoxin in *R. rubrum* fractions, the assay employed was the photoreduction of NADP by spinach chloroplasts. No stimulation has been observed in the *R. rubrum* photoreactions by addition of *R. rubrum* ferredoxin fractions.

The position of the other compounds in this scheme is less certain. The inclusion of a flavoprotein between ferredoxin and NAD is made on the basis that the NAD-coupled photooxidation of $DPIPH_2$ is stimulated by FMN at levels which are consistent with its functioning as a coenzyme to a flavoprotein. In a similar system involving the ferredoxin from *Clostridium*, a flavoprotein was shown to be required for the reduction of NADP (47).

Fig. 16 represents two pathways leading to ubiquinone, one coming from ferredoxin and the other from the flavoprotein. The evidence presented above on the slow photooxidation of $DPIPH_2$ coupled to fumarate and to NAD indicates separate pathways for these two reactions. Thus, FMN stimulates the NAD-supported reaction and has no effect on the fumarate-supported reaction. Quinacrine inhibits the NAD reaction and stimulates the fumarate-supported reaction.

There is considerable evidence that ubiquinone occupies a central position in accepting electrons on the reducing side of the scale. Thus Clayton has shown that in the case of *Chromatium* one of the primary reactions which occurs following illumination is most likely the photoreduction of ubiquinone (22). The high level of ubiquinone contained in *R. rubrum* chromatophores (33), and especially the large increase observed in light-grown cells over dark-grown cells indicates a prominent role for this compound (35). The scheme presents ubiquinone as transferring electrons to RHP. RHP is unique to photosynthetic bacteria, is oxidized by molecular oxygen (49), and has been shown to be essential for the photophosphorylation process (36). The assignment of sequence of electron transfer from ubiquinone to RHP is not in agreement with the reported oxidation potentials of -0.008 volts for RHP (49) and 0.098 volts for ubiquinone (50). The potentials observed in the isolated compound may not accurately represent the potential of the same compound *in situ* in the chromatophore structure, however. The main reason for choosing this sequence is that it allows ubiquinone to act very near the chlorophyll system, which is in agreement with present information.

Cytochrome *b* is included in the pathway between RHP and cytochrome c_2. This is the usual sequence encountered in mammalian tissues, and the spectroscopic evidence obtained by Nishimura, although not conclusive, indicates that cytochrome *b* contained in *R. rubrum* chromatophores can be reduced under the influence of light

when inhibitors such as antimycin A are present to block electron transfer between cytochrome *b* and cytochrome c_2 (23,24).

Fig. 17 presents the basic electron transfer system proposed in Fig. 16, and has included the possible sites of action of the various compounds which enter into the photochemical reactions demonstrated by these particles. For discussion purposes, let us begin at the lower end of the potential scale. The experiments of Tagawa and Arnon implicate ferredoxin in the hydrogen metabolism of selected bacteria, both photosynthetic and nonphotosynthetic (47). To date, no evidence has been presented to show that hydrogen evolution can be produced with isolated *R. rubrum* chromatophores, although it is made in copious amounts by the intact cell under various conditions (51). Because of the implication of ferredoxin in other hydrogen-producing systems, and because of the suitability of the potential of the two systems, it is indicated as the precursor of hydrogen in Fig. 17.

A number of compounds are shown as reacting with the chromatophore system at the level of ferredoxin and/or flavin. These compounds include oxygen, methyl red, tetrazolium blue, sulfate ion, and the disulfide bond contained in DTNB. The primary reason for including these compounds at this position is that the photoreductions involving these compounds are all heat stable and also relatively insensitive to the inhibitors which affect the other photoreactions. Lindstrom has shown that the photooxidation of $DPIPH_2$ in the presence of air is very stable to heat (6). Information presented above shows that aerobic photooxidase is also insensitive to the inhibitors tried, such as antimycin A, HQNO, and quinacrine. The methyl red and tetrazolium blue photoreductions have also been shown in our laboratory to be stable to heat. Heating chromatophores to 60°C for five minutes destroys the ability of these particles to carry out photophosphorylation and the secondary slow photooxidation of $DPIPH_2$ coupled with NAD and fumarate, but does not appreciably affect the methyl red photoreduction. Likewise, the photoreduction of methyl red and tetrazolium blue is also relatively insensitive to inhibitors known to affect the other photoreactions (17). Photoreduction of sulfate and DTNB are also fairly stable to heating, although heating does have more effect upon these reactions than upon the others in this group (5,18).

RHP is shown in Fig. 17 as reacting with molecular oxygen (49). Succinate and fumarate are shown as reacting with the electron transfer system through ubiquinone. This assignment is logical in view of the known reaction of succinate with ubiquinone in mitochondrial systems (52) and in view of the recent report that ubiquinone can be reduced enzymatically in the dark by succinate with chromatophores from *R. rubrum* (53).

The portion of the electron transfer chain up to RHP could very well be functioning as an NADH oxidase system in the dark respiratory activities of this organism. The phosphorylation coupled to this span

would allow for the phosphorylation accompanying aerobic oxidation of substrate molecules via NAD. Since this organism is lacking a cytochrome oxidase in the traditional sense, one looks for another compound to complete the electron transfer span to molecular oxygen, and the logical candidate is RHP (36). For reasons discussed above, the flavin listed in this scheme may well be common to both the photochemical reduction of NAD and the various enzymatic reductase activities observed in extracts of *R. rubrum* in the dark (36). It has been reported by Cohen-Bazire and Kunisawa that the particles called chromatophores, which can be isolated after the cell is broken, are actually segments of the cell membrane which has been ruptured during the process, and that in the intact cell the photosynthetic apparatus is laid down upon this limiting membrane (54). The implication is obvious, therefore, that this same membrane is involved in both the oxidative reactions and the photosynthetic reactions. One would expect, therefore, to find both of these functions combined in the one structural component, and the utilization of a portion of the photosynthetic electron transfer chain in the oxidative metabolism would meet this goal.

The compounds which are photooxidized in the presence of NAD and fumarate are shown to interact with the electron transfer scheme at the cytochrome c_2 level. One of the prime reasons for proposing this site of entry into the chain is that the photooxidation of these compounds is not affected appreciably by the respiratory inhibitors antimycin A and HQNO at concentrations which almost completely inhibit the photophosphorylation process. The data of Nishimura point toward the site of action of these inhibitors somewhere between cytochrome b and cytochrome c_2 (23,24). The entry of $DPIPH_2$ and the other photooxidizable compounds at the cytochrome c_2 locus is also consistent with the fact that this cytochrome is not firmly bound in the chromatophore system and can be removed by relatively easy treatments such as extraction with citrate buffer, etc. This indicates that the cytochrome is exposed to the aqueous medium in the chromatophore and would logically be a site of action for these compounds. Furthermore, Jacobs has shown that in a rat-liver mitochondrial system, TMPD reacts by reducing the cytochrome c on the particle (27).

Fig. 17 shows two sites for ATP formation along the electron transfer chain. This is in accord with the data of Baltscheffsky and Arwidsson (56), who have studied the effect of the inhibitor valinomycin upon *R. rubrum* chromatophores in the photophosphorylation reaction. Also, the data of Nishimura (34) on the amount of ATP formed per flash of light with *R. rubrum* chromatophores indicate that two sites for ATP formation are to be found. If PMS overcomes antimycin A inhibition by means of serving as a bypass for the inhibited site between cytochrome b and cytochrome c_2, then there must be a phosphorylation site before the site where PMS is reduced. The scheme in Fig. 17 accommodates this.

Fig. 18 shows the probable sites of action of various inhibitors which are effective with *R. rubrum* chromatophores. PMA is shown as inhibiting NAD reduction, since it was shown to be an effective inhibitor in the photoreduction of NAD by both succinate and ascorbate-DPIP by Nozaki et al. (16). Also, as shown above, this was an effective inhibitor of the NAD-supported photooxidation of $DPIPH_2$. Amytal would also be expected to inhibit at this position. Geller showed amytal to be an inhibitor of the dark aerobic oxidation of NADH by *R. rubrum* chromatophores, while it has no effect upon the photophosphorylation reactions (35).

Since quinacrine has an inhibitory effect upon the photophosphorylation process, it probably acts at another site in addition to the flavoprotein designated in Fig. 18. Quinacrine is shown as an inhibitor at the flavoprotein level, since it inhibits the photooxidation of $DPIPH_2$ coupled to NAD, yet stimulates the reaction coupled to fumarate. The stimulating effect of FMN on the NAD-supported photooxidation of $DPIPH_2$ also indicates a flavoprotein acting at this site.

The spectroscopic evidence of Nishimura and Chance (23,24) points to the site of action of antimycin A and HQNO as being between cytochrome b and cytochrome c_2. In addition, PMA has been shown to result in a photooxidation of cytochrome c_2 and a photoreduction of cytochrome b in *R. rubrum* cells, and probably acts at this locus also. Since PMS has been shown to overcome the inhibition of photophosphorylation by antimycin A, it bypasses the site which is inhibited by antimycin A. The most logical mechanism for this requires that PMS be reduced by ubiquinone and reoxidized by cytochrome c_2.

Recent evidence obtained by Bose and Gest (57) indicates that DPIP, when present in the oxidized form, can also serve as a bypass and overcome the inhibition of antimycin A upon photophosphorylation. This again is consistent with its photooxidation at the cytochrome c_2 level and its photoreduction at a prior point, most likely at the ubiquinone point in the electron transfer chain.

It is anticipated that some of the reaction sequences and components listed in Figs. 16-18 will be changed as additional information becomes available. Not all of the evidence available could be reconciled with the scheme as presented. Thus, Nishimura says that RHP is probably not located between ferredoxin and cytochrome b, since carbon monoxide (which does combine with RHP) does not affect the reduction of cytochrome b (23,24). Nishimura also states that quinacrine does not have any effect upon the absorption changes caused by illumination of *R. rubrum* chromatophores, as would be expected if quinacrine does inhibit at some site other than the flavoprotein designated in Fig. 16.

One main question to be resolved is whether there is one electron transport system serving both the photochemical and oxidative pathways in *R. rubrum*, or whether separate pathways are involved. As stated above, the schemes outlined in Figs. 16-18 accommodate the

idea that one electron transport chain functions in both areas, with the oxidative chain involving those components from NAD through RHP. Although Geller favors separate electron pathways for the photochemical and oxidative metabolism (35), his data on the effect of inhibitors correlate very nicely with the scheme proposed above. Thus, oxidative phosphorylation was not affected by antimycin A or HQNO, whereas photophosphorylation was strongly inhibited. Furthermore, amytal strongly inhibited NADH oxidation aerobically, but did not affect the photophosphorylation process.

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EFFECT OF REDUCED 2,6-DICHLOROPHENOLINDOPHENOL UPON THE LIGHT-INDUCED ABSORBANCY CHANGES IN *RHODOSPIRILLUM RUBRUM* CHROMATOPHORES: A COUPLED REDUCTION OF UBIQUINONE[1]

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The light-induced absorbancy changes in the photosynthetic system of *Rhodospirillum rubrum* have been investigated extensively. Whole cells of *R. rubrum* have been investigated by Duysens (1), Chance and Smith (2), and Smith *et al.* (3,4). Experiments with cell-free extracts or chromatophores from *R. rubrum* have been performed by Smith and Baltscheffsky (5), Clayton (6), and Nishimura (7,8). In general, the absorbancy changes noted can be correlated with three classes of compounds contained within the chromatophore, *i.e.*, the various forms of bacteriochlorophyll, the carotenoids, and the cytochromes. An assignment of the various absorbancy changes associated with these compounds has been made by Clayton (6).

The purpose of the present communication is to report on the effect of $DPIPH_2$ upon the absorption changes observed with the *R. rubrum* chromatophore fraction. This leuco dye, $DPIPH_2$, has been shown to be rapidly photooxidized by chromatophores of *R. rubrum* in a reaction which appears to be coupled to the photoreduction of components contained within the chromatophore. It was of interest, therefore, to examine the effect of $DPIPH_2$ upon the absorption changes in order to see if some correlation can be made between the photooxidation of $DPIPH_2$ and the observed absorbancy changes induced by light.

Fig. 1 presents the absorption spectrum of *R. rubrum* chromatophores. Fig. 2 presents the absorbancy changes observed when such chromatophores are illuminated. Considering the system with chromatophores only, the absorption changes previously noted by others (6, 7,9) are shown in the present case also. There are some differences between the difference spectrum for chromatophores shown in Fig. 2 and similar spectra reported by others, particularly in the region from 250 to 300 mμ. These differences most likely arise from the fact

[1] Contribution No. 109 from the Charles F. Kettering Research Laboratory.

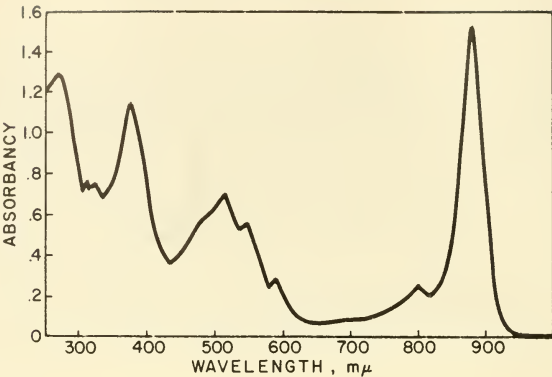

Fig. 1. Absorption spectrum of *Rhodospirillum rubrum* chromatophores. The chromatophores were prepared by treatment for 2 minutes in a 10KC Raytheon sonic oscillator. After removal of the cell debris by centrifugation at 20,000 x *g*, the chromatophores were sedimented by centrifugation for one hour at 100,000 x *g* and then washed twice by centrifugation in Tris buffer. Complete details concerning the growth of the bacterium and chromatophore preparation are given in reference 10. The chromatophores were suspended in 33 mM Tris buffer, pH 8.0 and .1 M sucrose, and contained 10 μgrams of BChl per ml of solution. Anaerobic conditions were obtained by three evacuations interspaced with flushing by argon gas (10). The absorption spectra were obtained with a Cary Model 14 recording spectrophotometer. For this experiment the absorption was measured with water in the reference cell.

that in the present case a slow scan was made under continuous illumination. Thus, the spectrum so obtained would include any "slow" and/or irreversible changes that occurred in addition to the rapid changes which usually serve as the basis for other reported difference spectra. The prominent features of the difference spectrum shown in Fig. 2 are the minimum at 600 mμ, the minimum around 550 mμ (most likely due to carotenoids), the broad maximum around 430 mμ (due to an unknown component in the chromatophores), a minimum around 387 mμ with a corresponding maximum around 360 mμ (due to a blue shift of a BChl absorption band) and a minimum around 260 mμ, which is most likely due to ubiquinone reduction.

When the absorbancy changes obtained in the presence of $DPIPH_2$ are compared with those due to chromatophores alone, two main differences are apparent. The obvious difference is the absorption band

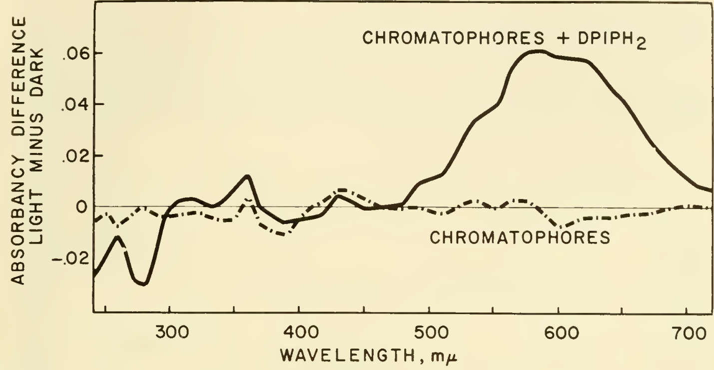

Fig. 2. The effect of $DPIPH_2$ upon the light-induced absorbancy changes in *R. rubrum* chromatophores. The suspension contained 10 μgrams BChl per ml and Tris buffer and sucrose as shown for Fig. 1. Where indicated, 66 μM $DPIPH_2$ (reduced with ascorbic acid) was also present. The 0 to .1 absorbancy scale was used, and illumination was obtained by means of two 6-volt, 18-ampere tungsten lamps situated above the cell compartment. Light was transmitted through a No. 2403 Corning filter to the circular absorption cells by means of a lucite plastic tube which served to guide the light rays to the absorption cell. The intensity of illumination was saturating for the reactions studied.

of DPIP, which has become oxidized by the photochemical system contained in the chromatophores. The changes due to chromatophores themselves are superimposed upon this broad absorption band of DPIP. The second important change is in the ultraviolet region, where a pronounced decrease in absorption around 280 mμ occurred. The most logical compound to be related to the band at 280 mμ is ubiquinone (9). Clayton has recently shown that illumination of *Chromatium* chromatophore suspensions results in changes corresponding to a photoreduction of the ubiquinone contained within these chromatophores. The discrepancy between the observed minima in the two cases (270 mμ reported for *Chromatium* and around 280 mμ for *R. rubrum* in this investigation) is due to the fact that there are some absorption changes in this region accompanying the oxidation of $DPIPH_2$. The conversion of $DPIPH_2$ to DPIP results in slight increases in absorbancy in the range from 260 to 290 mμ. The difference spectrum obtained with the dye itself (oxidized minus reduced) shows a minimum at 285 and a maximum at 270 mμ. It is the superimposition of this DPIP difference spectrum upon that due to chromatophores that shifts the observed

minimum to 280 mμ when $DPIPH_2$ is present in the illuminated chromatophore suspension.

The kinetics for the absorption changes at three important wavelengths have been determined and the results are shown in Fig. 3. A comparison is made between a system containing only chromatophores and one containing chromatophores plus $DPIPH_2$. At 600 mμ the

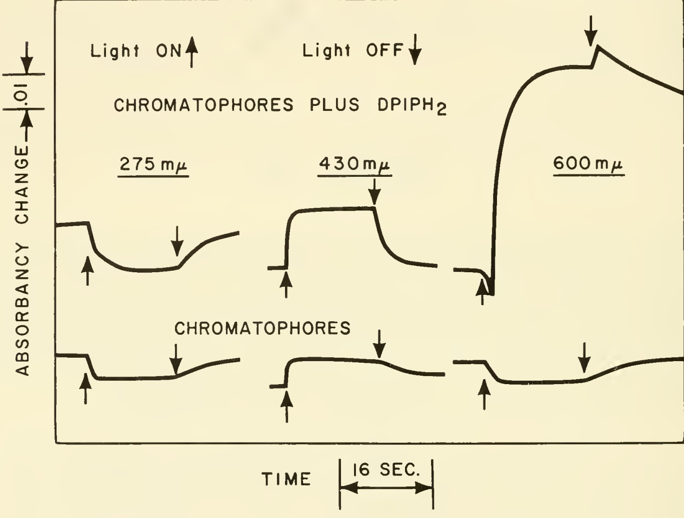

Fig. 3. Kinetics of the light-induced absorption changes observed with *R. rubrum* chromatophores in the absence and presence of $DPIPH_2$. Experimental conditions were the same as for Fig. 2, except that only one tungsten lamp was used for illumination, and a No. 2600 Corning filter was used to filter the actinic light. Corning filters were positioned in front of the phototube opening for the experiments run at 275 (No. 9863) and 430 mμ (No. 4784).

chromatophores alone show a decrease in absorbancy; with $DPIPH_2$ present, the rapid photooxidation of this reduced dye is shown by the increase in absorbancy. When the light is turned off, a slow decay or back reaction of the DPIP takes place. It is evident, however, that the intrinsic changes associated with the chromatophore component are faster than those associated with $DPIPH_2$ oxidation.

The experiments performed at 430 mμ show that the presence of $DPIPH_2$ results in a greater increase in absorbancy upon illumination and also a much faster decay time for the absorbancy difference. The

data taken at 275 mμ, which presumably corresponds to ubiquinone photoreduction, show that the presence of $DPIPH_2$ results in a more extensive decrease in the absorbancy which can be correlated with increased reduction of ubiquinone in the presence of $DPIPH_2$.

It is apparent that the changes at 275 mμ resulting from illumination are slower than those at 430 mμ, which are faster than the recorder can follow. This would relate the 430 mμ changes to a substance involved in the primary photochemistry resulting from light absorption, while the 275 mμ changes are consistent with a subsequent chemical reaction leading to ubiquinone reduction. The nature of the chromatophore component responsible for the increased absorption at 430 mμ upon illumination is not known. From a detailed analysis of the kinetics of the 430 mμ change under the influence of light and oxidizing agents, Clayton has concluded that this change is associated with one of the primary photochemical reactions taking place in the bacterial chromatophore (6). Since $DPIPH_2$ does affect the 430 mμ band, showing a marked effect upon the decay of this absorption band, this indicates that $DPIPH_2$ effectively supplies electrons to the oxidized component which produces the 430 mμ band upon illumination.

SUMMARY

The absorption changes occurring upon illumination of *R. rubrum* chromatophores in the absence and presence of $DPIPH_2$ have been investigated. The presence of $DPIPH_2$ resulted in the appearance of a minimum in the difference spectrum at about 280 mμ, which is thought to be due to a coupled reduction of the ubiquinone contained in the chromatophore and the oxidation of added $DPIPH_2$. The effect of $DPIPH_2$ upon the kinetics of the absorption changes was also studied.

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ELECTRON TRANSPORT SYSTEM IN FACULTATIVE PHOTOHETEROTROPH: *RHODOSPIRILLUM RUBRUM*

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INTRODUCTION

Regardless of their nature—strict aerobe, facultative anaerobe, or strict anaerobe—photosynthetic tissues contain subcellular portions, chloroplasts in green plants or chromatophores in photosynthetic bacteria, where the photosynthetic pigments and all the hematin compounds are present (1). The photosynthetic pigments are the main constituents of the photochemical apparatus (2,3), and the hematin compounds are parts of a special electron transport system coupled to the absorption of light energy in the photochemical apparatus. In green plants this system is wholly separate from the mitochondrial respiratory system, but in the photosynthetic bacteria this system is available for respiration as part of a common system for both anaerobic photosynthetic metabolism and aerobic nonphotosynthetic metabolism. The primary photochemical act gives electrons a driving force to reduce one terminus of the electron transport system, and the consequent oxidation-reduction reactions in the system take them back to their starting point, coupling to phosphorylation in a manner analogous to oxidative phosphorylation in mitochondria (4,5). This series of reaction has been designated as "cyclic" photophosphorylation by Arnon (6). In green plants, but not in the photosynthetic bacteria, there exists an additional system capable of drawing an electron photochemically out of a water molecule to reduce the electron transport system on the one hand and to evolve molecular oxygen on the other. In experiments with chloroplasts, the photosynthetic oxygen evolution (Hill reaction) can be demonstrated in the presence but not absence of an appropriate oxidation-reduction substance (Hill reagent). This overall reaction couples to a phosphorylation designated as "noncyclic" photophosphorylation. It is not known yet whether in chloroplasts electrons migrate through the electron transport system in such a cyclic fashion as in chromatophores.

The outline of photosynthetic electron transport mentioned above has been derived from the pioneering works published so far (7,8,9,1, 6). Most current research is aimed at elucidating the nature of the

photochemical apparatus (2,3,10,11,12) and the photophosphorylation coupled with the electron transport system.

The present speech will deal with the photosynthetic and nonphotosynthetic electron transport systems and related phosphorylation in a facultative photoheterotroph, *Rhodospirillum rubrum*.

PHOTOSYNTHETIC AND NONPHOTOSYNTHETIC RESPIRATION

Photosynthetic bacteria, whether facultative anaerobes or strict anaerobes, consume molecular oxygen in the presence of substrates (respiration with broad meaning). There is a dramatic effect of illumination on the respiratory activity of the facultative photoheterotrophs. This was first observed by Nakamura (13), who noted that illumination suppressed oxygen consumption with a cell suspension of *Rhodopseudomonas palustris*. Using *R. rubrum*, Johnston and Brown (14) demonstrated that oxygen is not produced in detectable amounts during the light-inhibited oxygen respiration, providing a strong argument against Nakamura's postulate that the light inhibition may be due to a light-stimulated production of oxygen. On the other hand, Sistrom, Griffiths, and Stanier (15) have found that cells of *R. rubrum* grown aerobically in darkness (dark-grown cells) are largely devoid of the photosynthetic pigments (bacteriochlorophyll and carotenoids) and that respiration of the cells is not influenced by light.

As expected, cells of *R. rubrum* grown anaerobically in light (light-grown cells) and suspended in a growth medium consumed oxygen faster in darkness than in light (Fig. 1). However, the ratio of dark-to-light respiration varied from culture to culture, with values as high as 4.5. Optimal pH values were 7.7 for dark (total) respiration, 8.0 for light (light-insensitive) respiration, and 7.5 for respiration which could be inhibited by light (light-sensitive) (18). Respiration of the dark-grown cells was not influenced by light and showed optimal pH at 8.0 either in light or in darkness; the respiration rate of *R. rubrum* was much slower than that of the ordinary aerobic bacteria possessing cytochrome oxidases; the Q_{O_2} at 30°C and pH 7 was 15 to 20 mm.^{3}/hour/mg. for dark respiration of the light-grown cells and somewhat greater (25-45 mm.^{3}/hour/mg.) for respiration of the dark-grown cells. With light-grown cells, the Q_{O_2} for dark respiration and the ratio of dark-to-light respiration declined gradually as the cells were aged or aerated.

n-Butanol and ethanol, tested as possible narcotics, inhibited all of the light-sensitive respiration at 0.15 M and 1.2 M, respectively, but had little effect on the light-insensitive respiration (Fig. 2). The effect of narcotics could be easily removed by a simple washing of the cells. Allyl alcohol inhibited both respirations. Ethylene glycol also inhibited the dark respiration to a lesser extent, but the ratio of dark-to-light

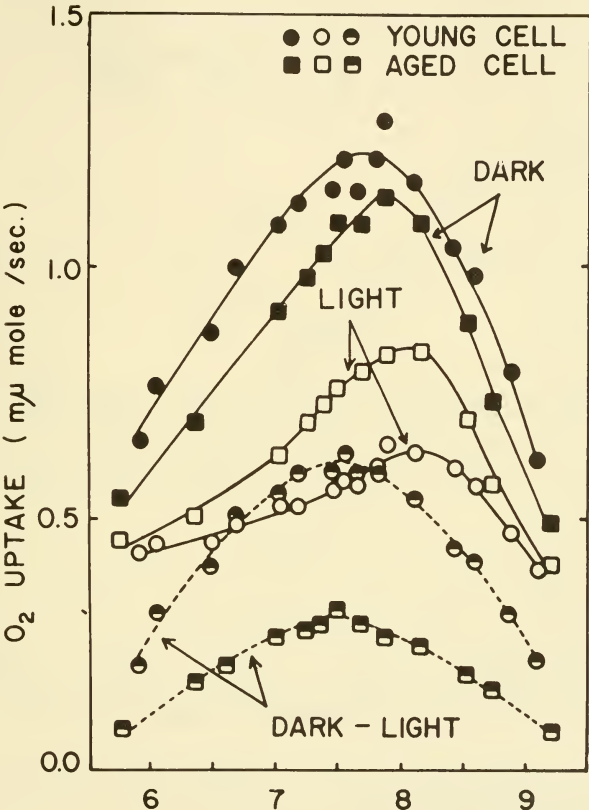


Fig. 1. Effect of pH on oxygen consumption by light-grown cells of *R. rubrum*. The cells of *R. rubrum* were grown anaerobically under continuous illumination at 30°C in the medium described by Gest, Kamen, and Bregoff (16). Oxygen uptake was measured by the oxygen-electrode method which had been improved by Hagihara (17). Reactions were carried out at 22°C in darkness and in light (approximately 150 ft-candles) with the cell suspension in the growth medium (approx. 5 mg. dry weight in 2 ml. of reaction mixture). Originally the terms "young" and "aged" cells were adopted to describe cells immediately after incubation for 3 to 5 days and cells allowed to stand for some days in a refrigerator, respectively. At the present time, "aged" cells describes those which had been appropriately aerated in darkness at low temperature (18).

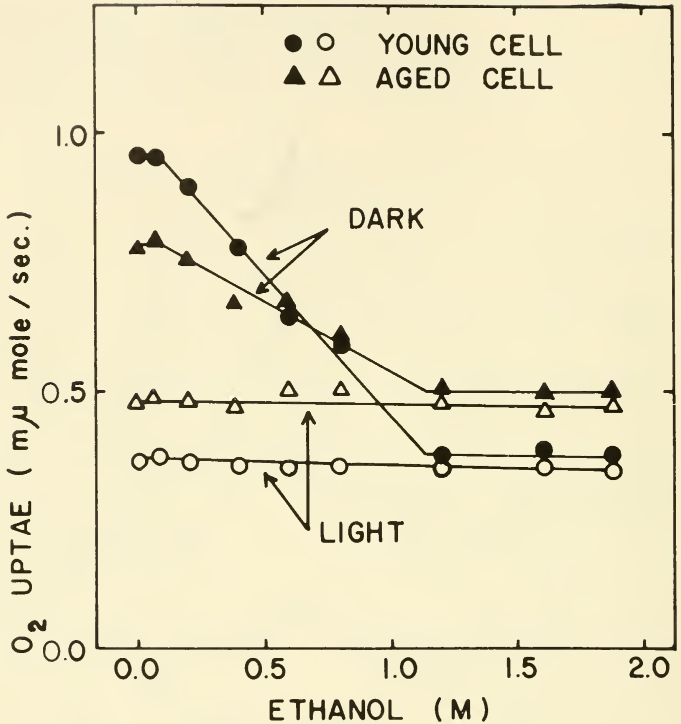


Fig. 2. Effect of ethanol on oxygen consumption by light-grown cells of *R. rubrum*. Experimental conditions were the same as for Fig. 1 except that ethanol was added as indicated.

respiration was never less than 1.7 (Fig. 3). This accords with the well-known fact that ethylene glycol is an efficient solvent for storage of photophosphorylating chromatophores below 0°C. In contrast, respiration of the dark-grown cells was hardly influenced in the presence of 0.15 M *n*-butanol.

Carbon monoxide and cyanide, as typical respiratory inhibitors, markedly inhibited respiration of either the dark-grown or the light-grown cells. With the light-grown cells, dark and light respiration both were inhibited by cyanide, but the ratio of dark-to-light respiration

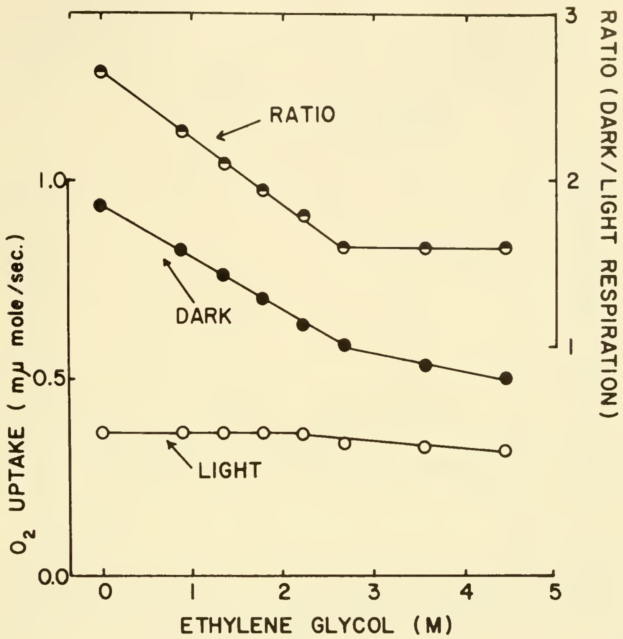


Fig. 3. Effect of ethylene glycol on oxygen consumption by light-grown cells of *R. rubrum*. Experimental conditions were the same as for Fig. 1, except that ethylene glycol was added as indicated (18).

rose when the concentration of cyanide was increased from 10^{-4} to 10^{-3} M (Fig. 4). The light-insensitive respiration appeared to be more cyanide-sensitive than the light-sensitive respiration. This indicates that the system for oxygen uptake is at least in part different between the light-grown and the dark-grown cells, and it is probable that the light-insensitive respiratory system of the light-grown cells is the same in kind but varies in activity from the respiratory system of the dark-grown cells.

Respiration of light-grown cells of *R. rubrum* in growth medium was hardly influenced by heating below 45°C for 5 minutes (Table 1). After heating at 50°C, the dark (total) respiration decreased approximately 20 per cent in rate, the light-insensitive respiration increased approximately 30 per cent, and the light-sensitive respiration decreased

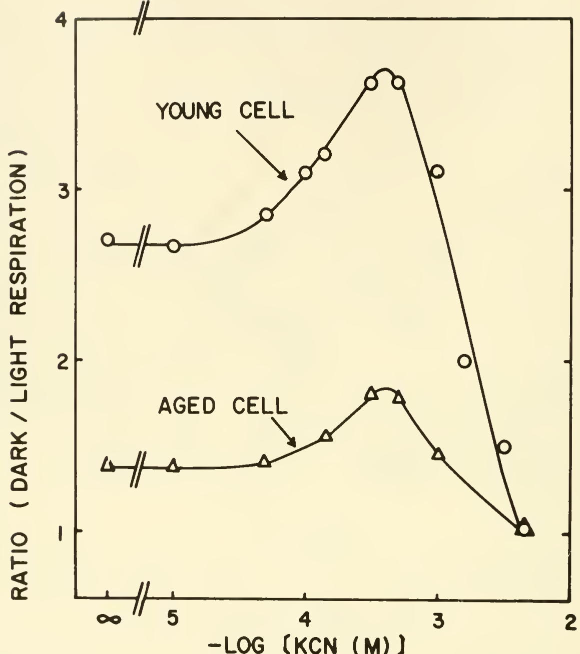


Fig. 4. Effect of cyanide on oxygen consumption by light-grown cells of *R. rubrum*. Experimental conditions were the same as for Fig. 1, except that potassium cyanide was added as indicated.

by half. Respiration of the cells heated at 55-60°C showed no response on illumination, while the cells heated at higher temperature consumed oxygen faster in light than in darkness.

Chromatophores prepared from *R. rubrum* according to the ordinary method for the photophosphorylation assay and suspended in growth medium consumed oxygen at rates comparable to those found for the intact cells. At 24°C the chromatophores showed oxygen uptake *stimulated* by light, the rate in light being approximately twice as fast as that in darkness. At 4°C the slowed oxygen uptake was *inhibited* by light. The chromatophores, if heated at 95°C for 5 minutes, coagulated and still exibited oxygen uptake in light but none in darkness.

TABLE 1.

Effect of temperature treatment on respiration of light-grown cells of R. rubrum

	O_2 uptake (mμ-mole/2 min.)		Ratio
	Dark	Light	Dark/Light
Untreated	163	78	2.1
Five minutes at			
45°	161	83	1.9
50°	133	99	1.3
55°	87	87	1.0
60°	77	78	1.0
65°	19	50	0.4
70°	3	17	0.2
Freezing-thawing two times	107	107	1.0

Experimental conditions were the same as for Fig. 1, except that the suspensions of light-grown cells of *R. rubrum* were heated or frozen and thawed at pH 7.2 (18).

After centrifugation of the resulting chromatophore suspension, neither the sediment nor the supernatant consumed oxygen in light, while the mixture of the two showed the light-dependent oxygen uptake. It was found that fresh growth medium or reduced cytochrome c_2 (see below) could not substitute for the supernatant, providing a strong argument against the postulate that this light-stimulated oxygen uptake resulted from an enzymic reaction. It was noted that this light-dependent oxidation still was cyanide sensitive, although only 30 per cent of oxygen uptake could be inhibited by a high concentration (5×10^{-3} M) of cyanide. The cyanide inhibition on oxygen uptake became less efficient with increasing temperature.

The light-grown cells, if aged, also showed the same light-*stimulated* respiration as for the nontreated chromatophores. This change was accelerated by allowing the cell suspension to stand under aerobic conditions, especially in light. The light-stimulated respiration was, in all likelihood, a photooxidation of some reducing substances catalyzed by bacteriochlorophyll, probably in its modified form, analogous to the photooxidation reactions described by Vernon and Kamen (19).

An action spectrum of light *inhibition* on respiration of light-grown cells was measured (20) (Fig. 5). It shows that bacteriochlorophyll and carotenoids mediate this action, and it closely resembles the action spectrum for photosynthesis obtained by Thomas (22) and Thomas and

Nijenhuis (23). Comparison of the action spectrum with the absorption spectrum indicates that the efficiency of light absorbed by carotenoids is approximately 40 per cent of that absorbed by bacteriochlorophyll, in good agreement with Goedheer (24).

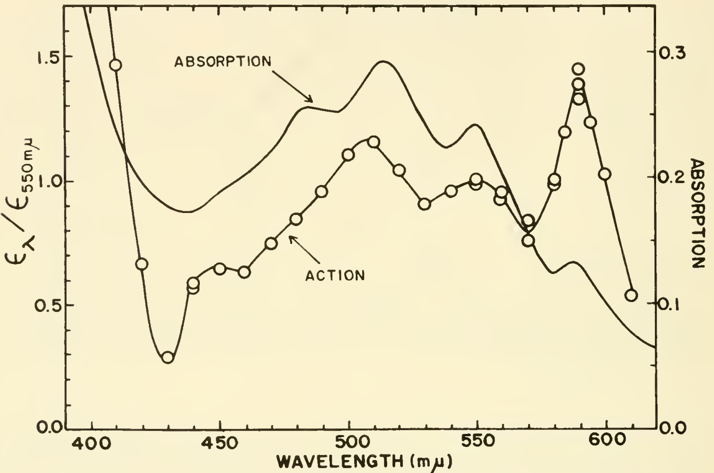


Fig. 5. Action spectrum of light inhibition on respiration of "young" light-grown cells of *R. rubrum*. Before and after this experiment, it was determined with the use of the oxygen-electrode method that the ratios of dark-to-light respiration were from 1.9 to 2.1 at 27°C. The action spectrum was measured by an apparatus which was devised by Castor and Chance (21). Other experimental conditions were the same as for Fig. 1, except that pH of the cell suspension was 7.2. The absorption spectrum was measured with a Cary, model 14R, spectrophotometer, using a chromatophore suspension prepared by the conventional 90-second sonication method (chromatophores (S)) (20).

The cells, particularly if aged under aerobic conditions or disrupted, started to show the oxygen uptake which was *stimulated* under illumination. Taylor (unpublished results) has found that the action spectrum for light-stimulated respiration with the aged, light-grown cells shows peaks between 420 mμ and 430 mμ, much as in Fig. 6. He also obtained an action spectrum with the aged, blue-green mutant (devoid of carotenoids) of *Rhodopseudomonas spheroides*, which showed a prominent shoulder at 410 mμ on a peak somewhere in the near ultraviolet and a small symmetrical peak at 520 mμ. It may be worth noting that these

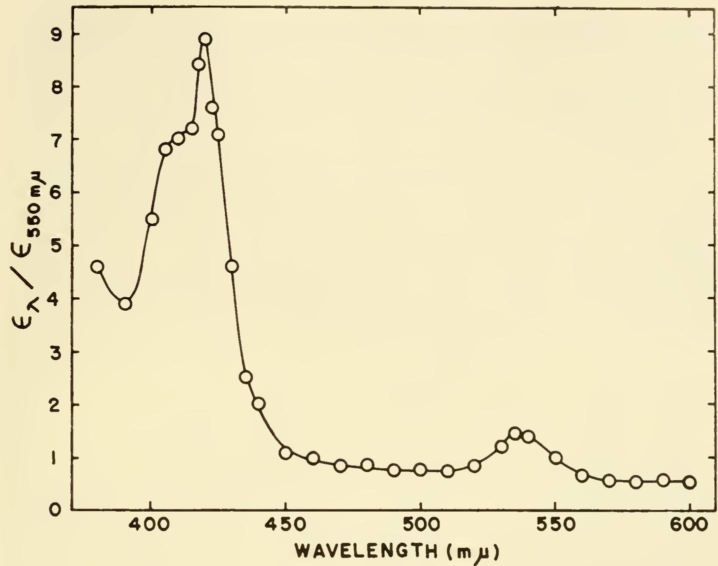


Fig. 6. Action spectrum of light-stimulated respiration of "young" light-grown cells of *R. rubrum* whose light-sensitive respiration had been depressed approx. 95 per cent with 0.15 M *n*-butanol. The ratio of dark to light respiration was determined to be 2.4. In this experiment, the cell suspension was previously mixed with 0.15 M n-butanol and the assay was carried out under air or a gas mixture of CO : O_2 = 4:1. In assay, irradiation by scanning lights (variable in wavelength) was immediately followed by diphasic response under the gas mixture containing CO, but monophasic under air. During the time required for arrangements in this assay, the respiration was altered in response to light so that light stimulation was observed. The action spectrum of the light-stimulated respiration thus altered was measured with some experimental difficulties. In assay under the gas mixture, the null balance between the scanning and control (550 mμ) lights was made for only the initial phase of response. Using air instead of the gas mixture gave similar results, but the response was monobasic (20). Taylor (unpublished) obtained similar action spectra, using cells which were so aged that their respiration was stimulated by light (see text).

action spectra for light-stimulated respiration with aged cells resemble the absorption spectrum of bacteriochlorophyll treated with ferric chloride or iodine (25). It seems likely that oxidation products of bacteriochlorophyll cause the oxygen uptake stimulated by illumination. Geller and Lipmann (26) have reported that some pigments having peaks between 420 mμ and 435 mμ are reduced when the chromato-

phores are illuminated *anaerobically*. It was found that with the chromatophore suspension under aerobic conditions a difference spectrum of "illuminated" minus "nonilluminated" exibited a new trough at 415-435 mμ. It is not known whether the peak and the trough correspond to the peaks in the action spectrum for light stimulation on respiration of the aged cells.

Respiration of the dark-grown cells was inhibited by carbon monoxide, and the inhibition was removed by illumination. An action spectrum for this light relief was found to be of the type designated "cytochrome *o*" by Castor and Chance (21, also 20) (Fig. 7). It is similar to that with a nonphotosynthetic bacterium *Micrococcus pyrogenes* var. *albus* (27,28). The action spectrum is the same as the absorption spectrum of a variant hemoprotein from *R. rubrum* (abbreviated as RHP) in its reduced form bound to carbon monoxide (29).

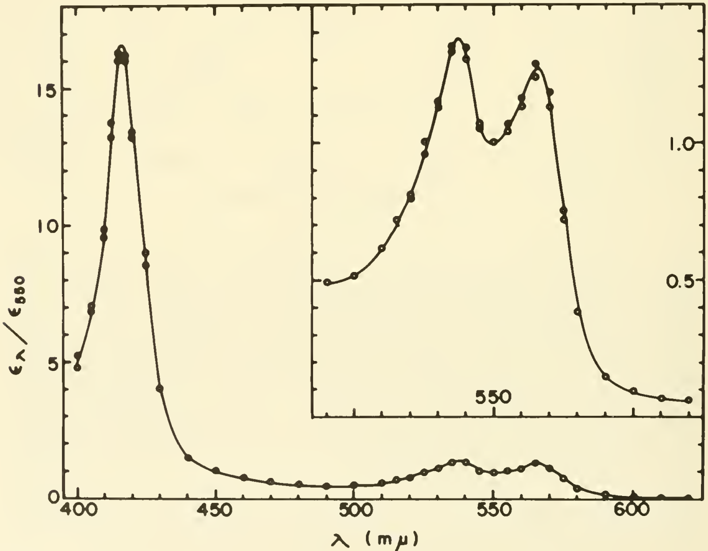


Fig. 7. Action spectrum of light relief on CO-inhibited respiration of dark-grown cells of *R. rubrum*. *R. rubrum* cells which had been grown for three days aerobically in darkness (approximately fivefold multiplication in dry weight) were used. Care was taken so that pH of the growth medium did not exceed 8 during incubation. Before this experiment, it was determined with the use of the oxygen-electrode method that the respiration of the cell suspension was not influenced by alternate darkness and light, although the cells possessed bacteriochlorophyll and carotenoids, as seen with a microspectroscope (20).

In view of its high autoxidizability (29), it is supposed that RHP (cytochrome *o*) is functional as an oxidase in respiration of *R. rubrum* as well as in the nonphotosynthetic bacterium *Micrococcus pyrogenes*. On the other hand, Bartsch and Kamen (30) have purified RHP from the photoautotroph *Chromatium*, which never grows aerobically. It may be, therefore, rational to consider that RHP has another anaerobic function, perhaps as one of the oxidation-reduction components in the photosynthetic electron transport system.

In the action spectrum on the light relief of carbon monoxide-inhibited respiration of dark-grown cells, the absorption peaks which correspond to those of the complex of typical cytochrome oxidases with carbon monoxide are not seen (31,32,33). This indicates that the cytochrome oxidases, if present, are not functional. This indication may apply to respiration of light-grown cells which show a lower respiratory activity, in good accordance with Smith (34).

ELECTRON TRANSPORT SYSTEM

Chromatophores from *R. rubrum* catalyze a reduction of cytochrome c_2 in its oxidized form by appropriate substrates such as succinate. This bacterium contains succinic dehydrogenase (19,35) and coenzyme Q_{10} (Rudney, H., and Sugimura, T., quoted by Sugimura and Okabe (36)). It was found that cytochrome c_2 reduction by succinate with chromatophores was completely inhibited in the presence of a low concentration (10 γ/ml) of antimycin A. Geller and Lipmann (26) have found that photophosphorylation in chromatophores is antimycin A sensitive. Sugimura and Okabe (36) have reported that the coenzyme Q_{10} present in chromatophores is reduced by succinate. Geller (37) has reported that the photophosphorylation induced in the presence of phenazine methosulfate (PMS) is hardly influenced by antimycin A.

Although all attempts to disrupt chromatophores into active but much smaller fragments with the use of some detergents (digitonin, cholate, and Triton x-100) have failed, it was noted that cytochrome c_2 reduction by succinate in darkness was inhibited in the chromatophores which had been treated with an appropriate concentration of Triton x-100, in the same manner as ascorbate-induced photophosphorylation (below) in the chromatophores with antimycin A. According to current considerations on the antimycin A-sensitive site in the mitochondrial respiratory system, the coenzyme Q_{10} present in chromatophores may be the site sensitive to antimycin A as well as Triton x-100.

From cells of *R. rubrum*, RHP and cytochrome c_2 have been extracted in a water-soluble state and purified (38,39) to crystalline states (40). Both heme proteins have been characterized in detail (29, 40,41,42), and their E_0' has been determined to be +0.31 V for cytochrome c_2 (39) and -0.01 V for RHP (29). Horio and Kamen (43) de-

termined the concentrations of these compounds in chromatophores to be 8.0 x 10^{-5} M for cytochrome c_2 and 7.4 x 10^{-5} M for RHP. Little work has been done on the isolation and characterization of *b*-type cytochromes from photosynthetic bacteria (1,44), although Vernon and Kamen (39) have detected the existence of a *b*-type cytochrome in *R. rubrum*. The cytochrome *b* is autoxidizable and bound firmly to the chromatophores (45). There are NADH-linked reductases which are capable of reducing the Cyt *b* (39). Geller (46) has found that the ratio of Cyt *b* content of light-grown to dark-grown cells is 1:3. Using chromatophores freed of photosynthetic pigments by being well washed with acetone containing ether, Cyt *b* was found to be a one-electron carrier of E_0' at pH 7 of 0 $\pm$ 0.04 V (Horio and Kitahara, unpub).

Horio and Kamen (47,18) have highly purified a flavoenzyme which is capable of reducing RHP and cytochrome c_2 in the presence of NADH. In addition, it was found recently that the flavoenzyme could catalyze reduction of the cytochrome *b* present in chromatophores which had been well washed with acetone. The reduction of RHP and cytochrome *b* by the enzyme could be observed under anaerobic but not aerobic conditions, the anaerobic reductions being nearly equivalent in rate to both aerobic and anaerobic reductions of cytochrome c_2. Under anaerobic conditions the enzyme could also reduce externally added flavins (FAD, FMN and riboflavin), and under aerobic conditions it could oxidize NADH by molecular oxygen. These findings raise the possibility that the cells consume molecular oxygen at the levels of flavins, cytochrome *b*, and RHP. When cytochrome c_2 was present, the flavoenzyme could not catalyze the NADH oxidation by molecular oxygen until all the cytochrome c_2 became reduced. This phenomenon enables us to speculate how respiration of the light-grown cells are inhibited under illumination; the flavoenzyme does not react with molecular oxygen so long as cytochrome c_2 is oxidized by the illuminated photochemical apparatus and is partly in its oxidized form. It may be rational to extend this speculation to the respiratory activity of RHP. This may be strengthened by the findings of Vernon (48), who noted that the aerobic NADH oxidation by chromatophores was remarkably inhibited under illumination.

Analogous to the mitochondrial electron transport system constructed by Green's school (4), cytochrome *b* has been placed outside the photosynthetic, cyclic electron transport chain of the *R. rubrum* chromatophores, but was located on a site connecting both the succinic and NADH dehydrogenases with the cyclic chain in the hypothetical scheme for the electron transport system in light-grown *R. rubrum* (see below).

Oxygen uptake with chromatophores from *R. rubrum* is faster in light than in darkness, either in the presence or absence of externally added substrate. The chromatophores, if dialyzed, lose most of the

activity of endogenous oxygen uptake in darkness as well as in light, doubtless because of the lack of endogenous, oxidizable substances. Under dark, aerobic conditions, dialyzed chromatophores slowly oxidize reduced cytochrome c_2 externally added; in most cases the dark cytochrome c_2 oxidation proceeds at approximately one-twentieth the rate of the aerobic, dark oxidation of succinate, which agrees with Smith (34).

Under aerobic conditions at room temperature, cytochrome c_2 oxidation is stimulated approximately tenfold upon illumination, but not under anaerobic conditions at all. Both the cytochrome c_2 oxidations in darkness and in light are inhibited by cyanide, with a Km for cyanide of approximately 5×10^{-6} M and 8×10^{-3} M, respectively. Cytochrome c from bovine heart muscle can be oxidized in the same manner as Cytochrome c_2. The aerobic, dark oxidation of cytochrome c is about twice as fast as that of cytochrome c_2.

Using *R. rubrum*, Frenkel (49,50) has shown that chromatophores can catalyze photoreduction of NAD in the presence of either $FMNH_2$ or succinate with an accumulation of NADH. His findings have been extended by Vernon (51) and Vernon and Ash (52). Many investigations have also shown that a heme protein(s) and NAD present in the cells are oxidized and reduced, respectively, upon illumination with actinic light (53,37,54,55).

In the presence of succinate or ascorbate, chromatophores from *R. rubrum* reduce NAD under anaerobic conditions, as expected (Fig. 8). The NAD photoreduction was inhibited by ADP in the presence and absence of Pi, with a Km for ADP of approximately 5×10^{-3} M (Fig. 9) (56). ATP and pyrophosphate (PP) inhibited to the same extent as ADP, while AMP and adenosine were less active. On the other hand photosynthetic ATP formation from ADP and Pi in chromatophores was enhanced in rate with an increasing concentration of ADP (the highest concentration tested was 10^{-2} M), the Km for ADP ("bound" plus "free") being about 10^{-5} M (57)), providing a strong argument against the postulation that NAD might be part of the photosynthetic, cyclic electron transport chain.

In response to variations in concentration of succinate or NAD, the photoreduction of NAD reached a steady state in which about half of the NAD present was reduced, indicating the E_0' value of the photochemically produced reductant to be around -0.32 V. In the presence of NADH/NAD (1:1) (E_0', -0.32 V), the NADH was oxidized by succinate/ fumarate (E_0', + 0.02 V) anaerobically in darkness, providing that the E_h value of the succinate/fumerate system was more positive than -0.04 V. This suggests there is an enzyme system which catalyzes oxidation of NADH by fumarate anaerobically in darkness. It seems, therefore, most likely that there is a regulatory mechanism in the enzyme system which catalyzes anaerobic oxidation of NADH/NAD by

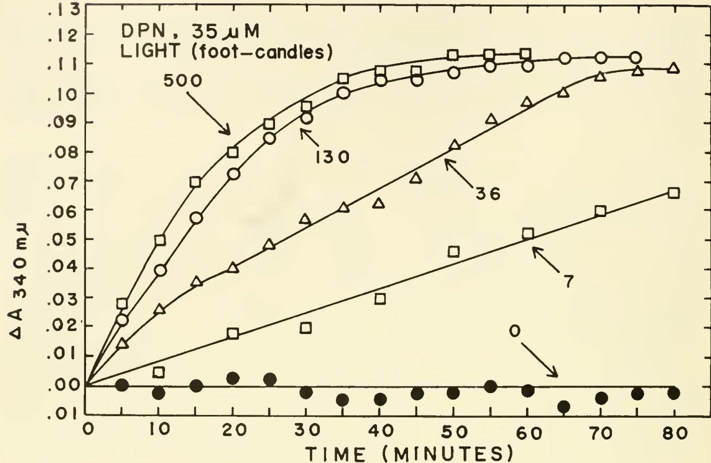


Fig. 8. Effect of light intensity on NAD (DPN) reduction by *R. rubrum* chromatophores. Reduction of NAD was followed by measuring the absorbancy increase at 340 mμ. Reactions were carried out at 20°C in vacuum in Thunberg-type cuvette (1-cm. optical path). The standard components of the reaction mixture were as follows: 0.1 M Tris containing 10% sucrose (Tris-sucrose buffer) of pH 8.0, 1.00 ml.; 0.1 M $MgCl_2$, 0.20 ml.; 5.3 x 10^{-4} M NAD, 0.20 ml.; *R. rubrum* chromatophores (S) (A_{880} mμ/ml., approx. 50), 0.20 ml. The total volume of cuvette was adjusted to 3.00 ml. with water, and in the side chamber was 0.1 M succinate (pH 8), 0.20 ml.

succinate/fumerate in darkness, so that when the E_h value of the oxidant is -0.04 V or more negative, electrons do not flow from NADH to fumarate. If the E_h value is more positive, it may exceed the capacity of the regulatory mechanism, possibly for phosphorylation.

Using cell-free extracts from *Rhodopseudomonas palustris*, Katoh (58) has found that oxygen uptake with succinate in darkness and in light was remarkably inhibited by ADP (half inhibition at 1.25 x 10^{-3} M). In contrast, with the use of *R. rubrum* it was recently found that the light-stimulated oxidation of cytochrome c_2 by chromatophores was markedly accelerated by the addition of ADP, and to a lesser extent by the addition of Pi (Fig. 10) (Horio and Yamashita, unpublished). The addition of both ADP and Pi further accelerated the oxidation, which was not influenced by antimycin A.

Duysens (59) and Goedheer (60) have reported that bacteriochlorophyll is oxidatively bleached in light. Using ferri-ferrocyanide, the

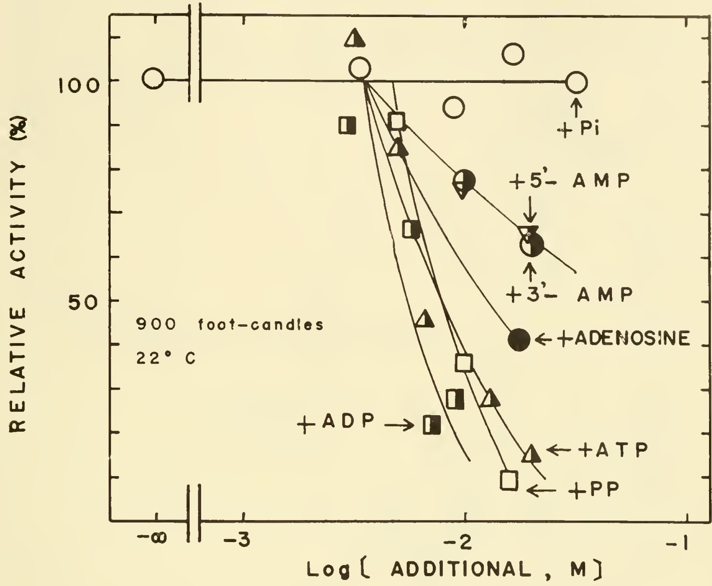


Fig. 9. Effect of various substances on photoreduction of NAD by *R. rubrum* chromatophores. Experimental conditions were the same as for Fig. 8, except that reactions were carried out at 22°C in light (approx. 900 ft-candles) in the presence of 3.3×10^{-4} M NAD (56).

normal oxidation-reduction potential (E'_0) of bacteriochlorophyll present in the cells was estimated to be 0.4-0.515 V (see also ref. 61). This value might correspond to the E'_0 value of the nonilluminated bacteriochlorophyll. Spikes *et al.* (62) and Vishniac (63) have demonstrated that cytoplasm preparations from leaves of the wild sunflower, and a mixture consisting of chlorophyll *a* and an aqueous-extract from an acetone-dried preparation of spinach leaves, respectively, brought about significant reductions in redox potential upon illumination. In fact, an ethylene glycol solution of bacteriochlorophyll prepared from *R. rubrum* showed a more negative value in light and upon cessation of illumination the potential became more positive if assayed anaerobically in the presence of ascorbate with the use of platinum and calomel electrodes (Fig. 11). Duysens, Huiskamp, Vos and van der Hart (64) have suggested the following reactions:

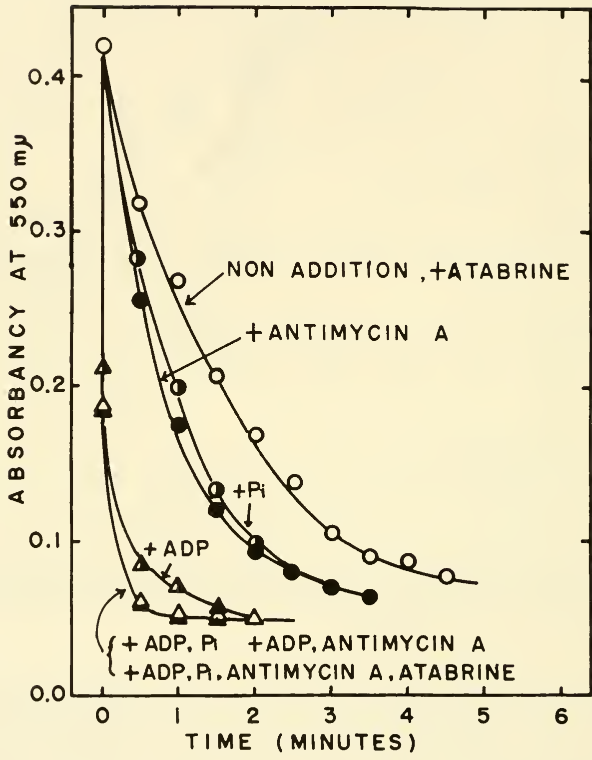


Fig. 10. Effect of various substances on photooxidation of cytochrome c_2 by *R. rubrum* chromatophores. Reaction mixture (1.20 ml.) contained components as follows: 0.033 M Tris (pH 8.0), 3.3% sucrose, 0.008 M $MgCl_2$, 0.008 M ADP, 0.008 M Pi, 4.1×10^{-4} M KCN, 8×10^{-5} M atabrine, 1 γ antimycin A, approx. 2×10^{-5} M cytochrome c_2 and *R. rubrum* chromatophores ($A_{880\ m\mu} = 0.42$). Reactions were carried out at 20°C in light of approximately 1,000 ft-candles. Difference absorbancy was made between the two cuvettes containing the reaction components with and without cytochrome c_2.

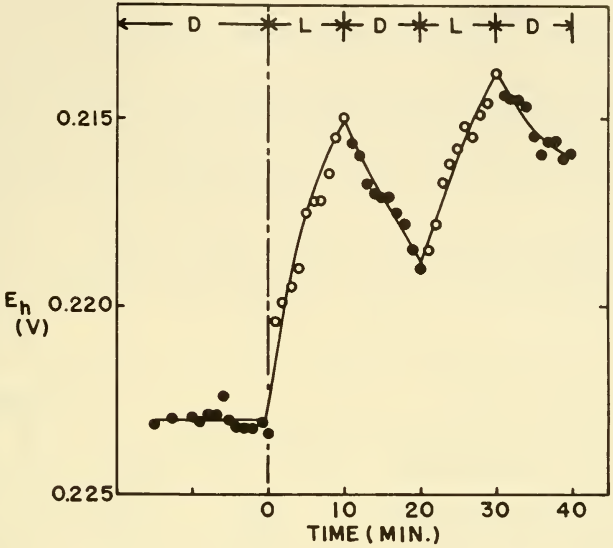


Fig. 11. Effect of light on oxidation-reduction potential of bacteriochlorophyll. A crude sample of bacteriochlorophyll from *R. rubrum* was dissolved in a small volume of acetone, and then diluted with a large volume of ethylene glycol. The resulting solution was further diluted with one-half volume of 0.01 M Tris buffer (pH 8) containing 0.01 M ascorbate. E_h was measured at 24°C in helium gas in alternate periods of darkness and light (approx. 1,500 ft-candles) by a Radiometer, type pH 4.

Excited BChl + X ⟶ oxidized BChl (bacterioviridin?)

+ XH (photosynthetic reductant)

oxidized BChl + reduced cyt + proton(s) ⟶ BChl + oxidized cyt

One of the simplest explanations might be that bacteriochlorophyll, when illuminated by actinic light, is converted into its new form which has an E_0' value low enough (around -0.32 V, perhaps with the aid of a nonheme iron protein such as photosynthetic pyridine nucleotide reductase (65,66,67) to reduce the opposite terminus of the electron transport system and thus to complete a cyclic electron transport system.

PHOTOPHOSPHORYLATION COUPLED WITH PHOTOSYNTHETIC ELECTRON TRANSPORT SYSTEM

Earlier, Gest and Kamen (68) demonstrated that illumination greatly accelerates both the uptake of Pi^{32} by intact cells of *R. rubrum* and the turnover of Pi between soluble and insoluble cellular fractions. Frenkel (69) was the first to clearly demonstrate light-induced phosphorylation by cell-free preparations (chromatophores) of photosynthetic bacteria. He found (70) that the washed chromatophores actively esterify Pi in the presence of ADP or IDP but not with AMP or IMP. ADP generally appeared to be a better acceptor than IDP.

Photophosphorylation by bacterial chromatophores appears dependent on catalytic amounts of reductants (37,71). A number of ex-

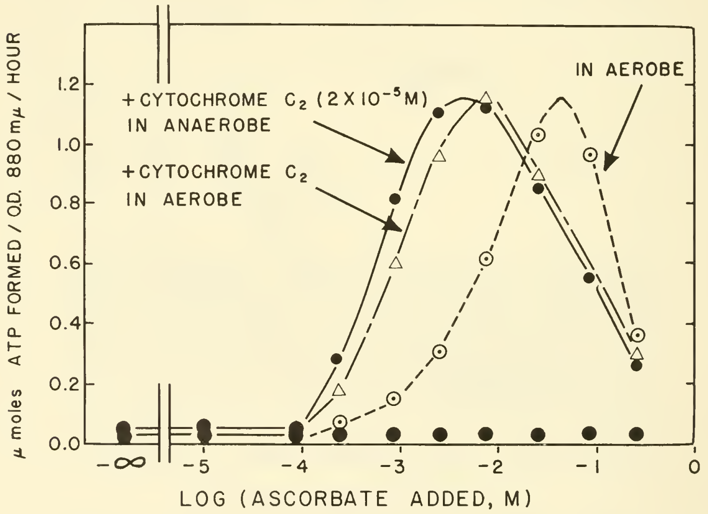


Fig. 12. Effect of cytochrome c_2 on photophosphorylation by *R. rubrum* chromatophores. ATP formation was followed by measuring the radioactivity incorporated from Pi^{32} into ATP in the presence of ADP. Standard components of the reaction mixture were as follows: Tris-sucrose buffer (0.2 M, 10% pH 8.0), 0.50 ml.; 0.1 M $MgCl_2$, 0.10 ml.; 0.1 M Pi^{32} solution (pH 8), 0.10 ml.; 0.1 M ADP solution (pH 8), 0.10 ml.; suspension of washed *R. rubrum* chromatophores (S) (A_{880} mµ/ml., approx. 50), 0.10 ml.; total volume adjusted to 1.50 ml. by addition of the buffer. In some cases a fixed concentration of oxidized cytochrome c_2 (2×10^{-5} M) was present. Open symbols, in light (450 ft-candles) (43).

planations have been offered, one of which is that there are optimal values for the oxidation-reduction potentials of intrachromatophore components for maximal coupling of photoactivated electron transport to phosphorylation processes (71,72,26). As expected, washed chromatophores of *R. rubrum* photosynthesize ATP from ADP and Pi in the presence of appropriate concentrations of ascorbate (ascorbate-induced photophosphorylation) (Fig. 12). Under aerobic conditions, photophosphorylation reached its maximal rate in the presence of approximately 5 x 10^{-2} M ascorbate.

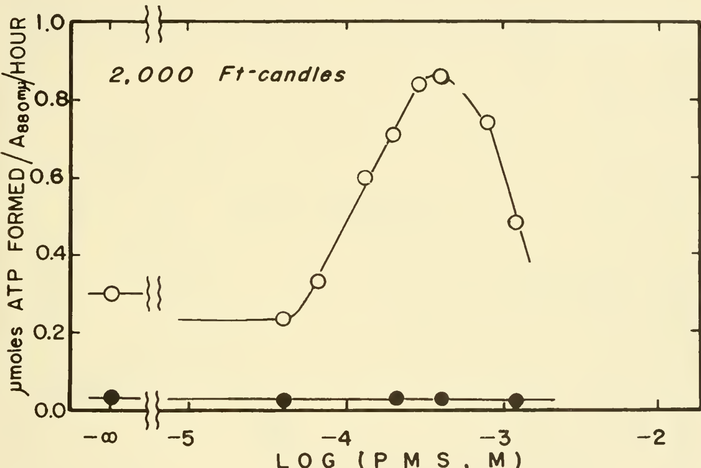


Fig. 13. Effect of phenazine methosulfate on photophosphorylation in *R. rubrum* chromatophores. Experimental conditions were the same as for Fig. 12, except that PMS was substituted for ascorbate.

Photophosphorylation was induced in the presence of PMS (Fig. 13). The concentration of PMS capable of inducing the maximal rate of photophosphorylation was notably influenced by the intensity of illumination, which was not true of ascorbate. When anaerobic conditions were maintained, the rate of phosphorylation was maximal in the presence of a much lower concentration (10^{-2} M) of ascorbate. In spite of the difference in effective ascorbate concentrations, the maximal rates of photophosphorylation were the same under both conditions. It follows that the presence of oxygen influences the photophosphorylation rate by lowering the reducing capacity of ascorbate through oxidation. Although

the maximal rates of photophosphorylation varied from one preparation of washed chromatophores to another, the relationship between photophosphorylation rate and ascorbate concentration remained unaltered with either kind of chromatophores, active or less active. Under aerobic conditions, the addition of 2×10^{-5} M oxidized cytochrome c_2 lowered the concentration of ascorbate required for maximal photophosphorylation from 5×10^{-2} M to 8×10^{-3} M. The maximal rate of photophosphorylation was, however, not altered significantly. Apparently, the addition of cytochrome c_2 mimics anaerobic conditions.

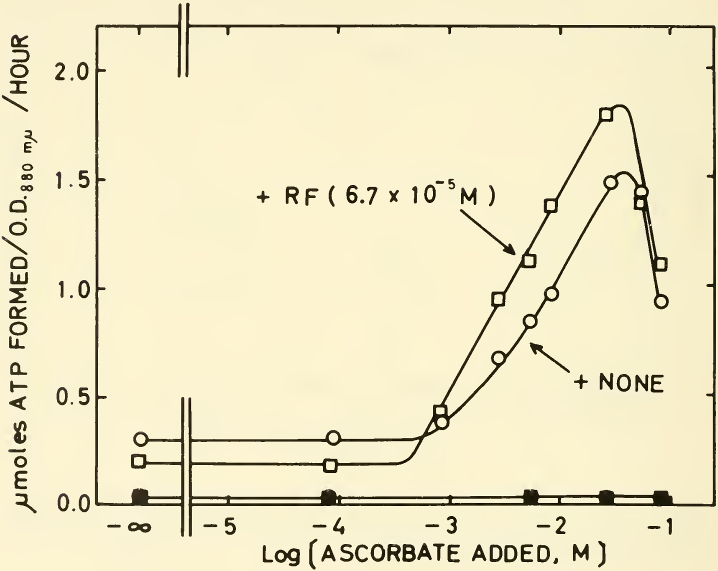


Fig. 14. Effect of riboflavin (RF) on photophosphorylation in *R. rubrum* chromatophores. Experimental conditions were the same as for Fig. 12, except that riboflavin was added as noted. Open symbols, in light (450 ft-candles); black symbols, in darkness (43).

Baltscheffsky (73) has reported that atabrine dihydrochloride (quinacrine hydrochloride) remarkably inhibits the succinate-induced photophorylation in chromatophores, and that the inhibition is neutralized by the addition of a high concentration (mM order) of FAD, but not FMN. He has claimed that FAD is functional in the photosynthetic electron transport chain. As we have shown, when 7×10^{-5} M riboflavin

(RF) is present, the ascorbate-induced photophosphorylation is maximally accelerated (Fig. 14), but the rate is depressed in the presence of higher concentrations of riboflavin (43). FMN and FAD accelerate photophosphorylation in the same manner, but at much higher concentrations. The ascorbate-induced and the PMS-induced photophosphorylations are also inhibited by atabrine to the same extent, with a Km for atabrine of 2 x 10^{-5} M.

ATP and PP were found to be powerful inhibitors of the photophosphorylation; the lower the concentration of ADP present, the greater was the inhibition by either ATP or PP (Fig. 15) (56). Kinetic studies showed that the inhibition by ATP was competitive with Pi, but uncompetitive with ADP, while PP was competitive with both ADP and Pi.

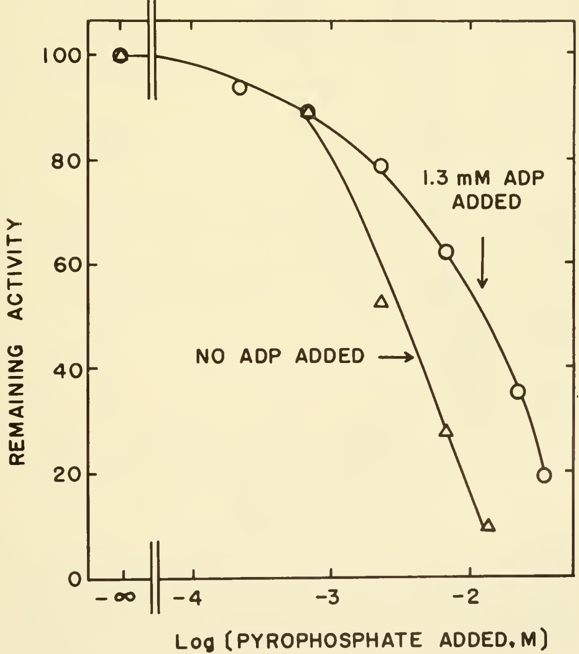


Fig. 15. Effect of varied concentration of pyrophosphate on photophosphorylation by *R. rubrum* chromatophores. Experimental conditions were the same as for Fig. 12, except that the concentration of ascorbate was fixed at 0.07 M.

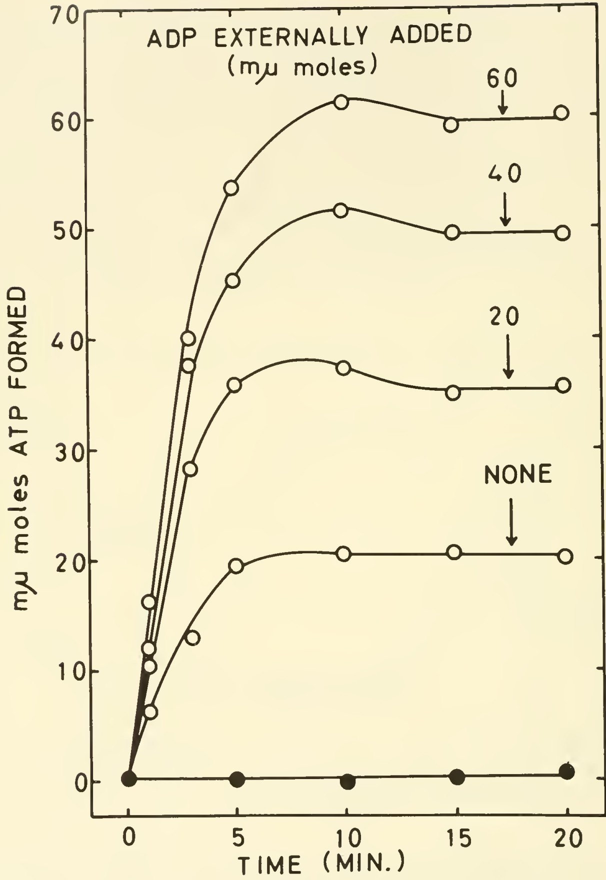


Fig. 16. Effect of externally added ADP on steady state of photophosphorylation with *R. rubrum* chromatophores. Experimental conditions were the same as for Fig. 12 with some exceptions as follows: The chromatophores (G) were prepared by grinding of cells with alkaline aluminum oxide and washing five times. Ascorbate, 4.5 mM; ADP, varied from 0 to 60 mμ-moles as indicated. Open and solid symbols show reactions in light and in darkness, respectively. The dark reactions were carried out in the presence of 60 mμ-moles of the ADP externally added (57).

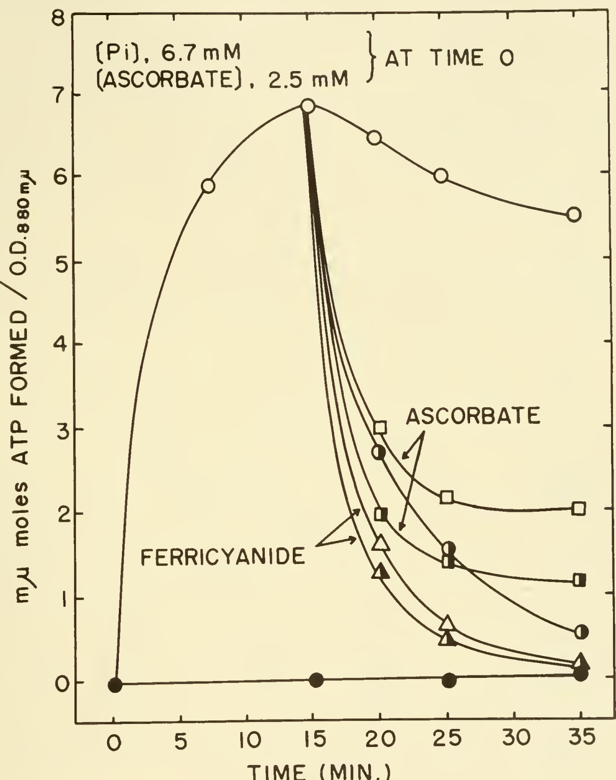


Fig. 17. Effect of oxidant and reductant on steady state photophosphorylation of "bound ADP" with *R. rubrum* chromatophores. Experimental conditions were the same as for Fig. 16 with exceptions as follows: *R. rubrum* chromatophores (G), washed three times; ADP, not added; reactions were started in the presence of 2.5 mM ascorbate in light, and after 15 minutes 0.10 ml. of water, 0.01 M ferricyanide or 1.0 M ascorbate was added, then reactions were further carried out in continuous "light on" or in "light off." Half-closed and solid symbols represent reactions in light-off and in continuous darkness, respectively (74).

It was found that *R. rubrum* chromatophores contained a small but definite amount of "bound" ADP which could not be washed out by repeating the centrifugal washing procedures (57). Without the addition of external ADP, well-washed chromatophores could photosynthesize ATP^{32} from the "bound" ADP and added Pi^{32} (Fig. 16). Most of the "bound" ADP present in the chromatophores appeared to be esterified. Chromatographic surveys have failed to detect photosynthetic formation of $IDP\text{-}P^{32}$ in extracts from well-washed chromatophores which had been illuminated with Pi^{32}. These negative data favor the postulation that ADP is the direct phosphate acceptor. It should be noted that photophosphorylation of the "bound" ADP is optimal in the presence of a much lower concentration of ascorbate (about 10^{-3} M) than photophosphorylation of externally added ADP.

The ATP formed from the "bound" ADP ("bound" ATP) in the light appeared to be rapidly replaced with externally added ATP, but was also rapidly decomposed into "bound" ADP and free Pi when

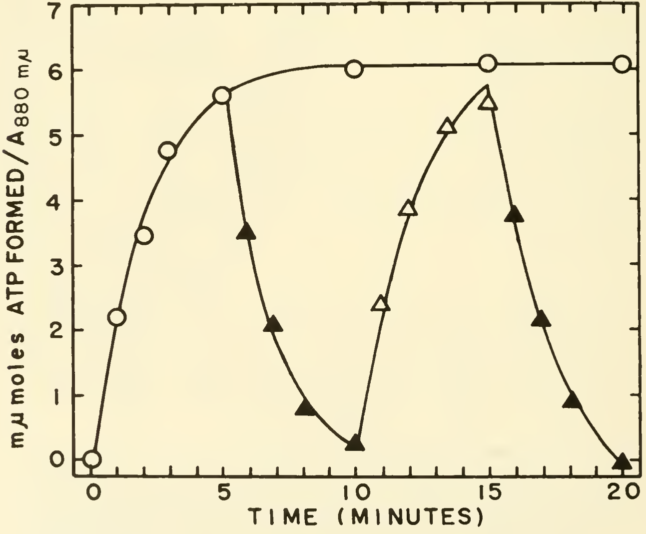


Fig. 18. Effect of "light on" and "light off" on ATP formation from bound ADP in "intact" chromatophores of *R. rubrum*. Experimental conditions were the same as for Fig. 17, except that the concentration of ascorbate was fixed at 1 mM. Control runs were carried out in darkness, and differences between tests and controls were plotted, although the values for control were hardly altered (74).

illumination ceased (Fig. 17). This dephosphorylation of "bound" ATP is distinct from dephosphorylation of externally added ATP since washed chromatophores dephosphorylated externally added ATP into ADP and Pi at a negligible rate (less than one-tenth) either in light or in darkness. Also, the dephosphorylation of the "bound" ATP in the dark period was influenced by the addition of either oxidant (ferricyanide) or reductant (ascorbate) in the same manner as photosynthetic ATP formation was. It is probable that, when the light is turned off, the "bound" ATP is converted into "bound" ADP and free Pi by the backward reaction of photophosphorylation. The backward reaction of

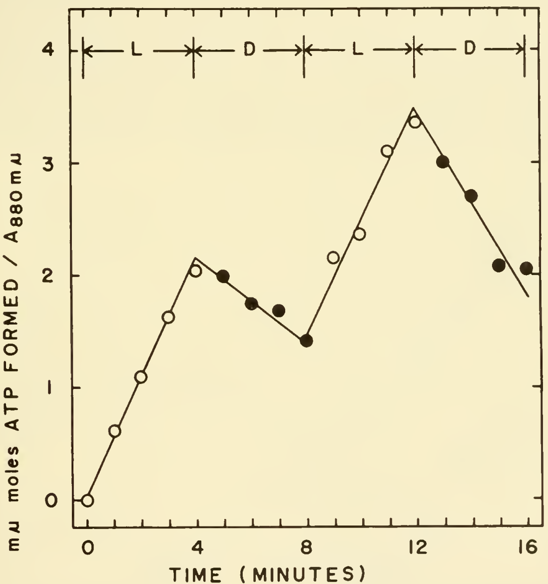


Fig. 19. Effect of "light on" and "light off" on ATP formation from bound ADP in "less intact" chromatophores of *R. rubrum*. Experimental conditions were the same as for Fig. 18, except that *R. rubrum* chromatophores (S) were used instead of chromatophores (G). Pi^{32} was incorporated into the organic phosphate fraction by the reactions standing in darkness throughout the reaction time. The compound capable of incorporating Pi^{32} in darkness liberated all the incorporated Pi^{32} by boiling in N-HCl for 7 minutes, but was not ATP. Identification of this compound is under investigations.

photophosphorylation could be demonstrated with the washed chromatophores prepared by grinding the cells with alkaline aluminum oxide (Fig. 18) and appeared to be more or less uncoupled in the washed chromatophores (showing negligible ATPase activity) which had been prepared by the conventional method of sonication (Fig. 19).

With *R. rubrum*, it was found that photophosphorylation in chromatophores was inhibited in the presence of Triton x-100, a nonionic detergent. Triton influenced the ascorbate-induced photophosphorylation to a much greater extent than the PMS-induced photophosphorylation (Fig. 20). Antimycin A completely inhibited ascorbate-induced photophosphorylation but not the PMS-induced photophosphorylation, clearly indicating that the antimycin A-sensitive site in the electron transport chain is by-passed in the presence of PMS. PMS-induced photophosphorylation probably consists of phosphorylations at more than one phosphorylating site, one of which is the same as the phosphorylating site (flavin level) of the ascorbate-induced photophosphorylation (43). It seems likely that the PMS-induced photophosphorylation takes place also at the cytochrome c_2 level.

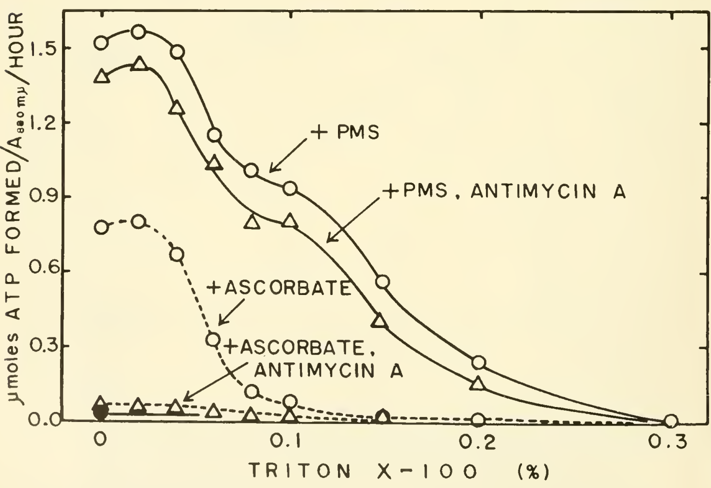
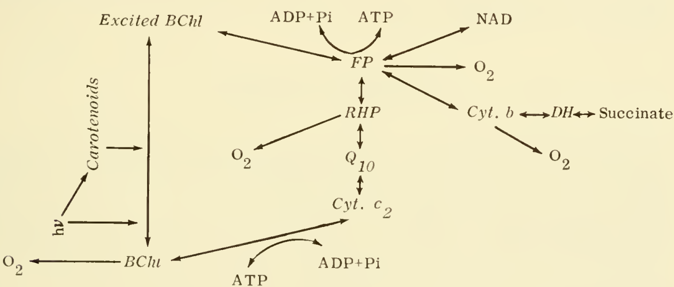


Fig. 20. Effect of Triton X-100, nonionic detergent, on photophosphorylation by *R. rubrum* chromatophores. Experimental conditions were the same as for Fig. 13, except that PMS or ascorbate was added at optimal concentration, and the reactions were carried out in the presence or absence of antimycin A.

HYPOTHETICAL SCHEME ON PHOSPHORYLATING, PHOTOSYNTHETIC ELECTRON TRANSPORT SYSTEM

In summary, a hypothetical scheme for the electron transport system in "intact" chromatophores of *R. rubrum* is presented (Fig. 21). Under anaerobic conditions, electrons migrate through the electron transport system in a cyclic fashion at the expense of the energy absorbed from light in bacteriochlorophyll (BChl) and its accessory pigment, carotenoids. The oxidation-reduction reactions around both flavoprotein (FP) and cytochrome c_2 are able to couple phosphorylating reactions. Differences in E_0' between the excited bacteriochlorophyll (around -0.32) and FP (0 to -0.2 V), and between cytochrome c_2

1) In "young" cells:



2) In "aged" cells:

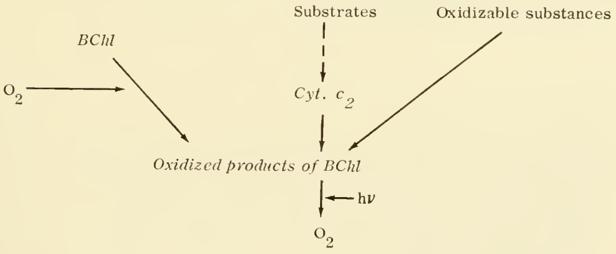


Fig. 21. Hypothetical scheme for electron transport system in chromatophores present in young or aged cells of light-grown *R. rubrum*.

(+0.31 V) and the bacteriochlorophyll (+0.4 to +0.52 V) may account for ATP formations coupled with these oxidation-reduction reactions. Although the difference in E'_0 between FP and cytochrome c_2 is sufficient, it is not known yet whether there is a coupled phosphorylation. Under aerobic conditions, it is certain that RHP is functional as an oxidase. It seems, however, unlikely that RHP is the only factor capable of reacting with molecular oxygen. Other possible oxidizing sites are FP and cytochrome *b*. The light inhibition of respiration of "young" cells results from the same photochemical act as does photosynthetic ATP formation, confirmatively suggesting that the photochemical act completes the cyclic electron transport system and thus makes oxygen uptake at some of these oxidizable components depressed. The light-stimulated respiration with chromatophores or the "aged" cells is caused by some function of the 410 to 420 mμ component(s), possibly an oxidized product(s) of bacteriochlorophyll, which is absent or not functional in the "young" cells.

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PHYSIOLOGY OF BACTERIAL CHROMATOPHORES

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Several years ago a systematic investigation of the antigenic components of bacterial chromatophores was initiated with the hope of detecting structural relationships between the bacterial particles and other cellular constituents, as well as identifying any unique antigens which might characterize the photochemical apparatus (1-3). The results of these investigations have provided some leads to possible new enzymatic systems in chromatophores, especially to those systems which may possess some mechanoenzymatic properties. Furthermore, some of the structural data from the immunochemical work contain implications for the problem of energy transfer in the photochemical apparatus.

To summarize the earlier work, it was found that photosynthetic bacteria elaborated unique antigenic constituents during photosynthetic growth and that these new antigens were attached to the bacteriochlorophyll-bearing components of the cell and were serologically related to the cell wall-membrane complex. Furthermore, the antigenic substructure of the chromatophore was disulfide bonded; i.e., the antigenically reactive groups were spaced between pairs of disulfide bonds in the chromatophore. When isolated chromatophore particles were treated with reagents known to cause scission of disulfides, the particles were quantitatively cleaved to yield serologically univalent derivatives of the parent chromatophore. These results indicated that chromatophores contained a repeating substructure with the "monomers," in an antigenic sense, placed between disulfide bridges in the particles.

The wealth of literature on radiation effects on simple and complex disulfides, including proteins, indicates that these bonds are very susceptible to radiation cleavage (4,5). It seems possible that the structural disulfides interspaced in the chromatophore particle could serve as energy "sinks" for photochemical processes. Calvin and coworkers (6) proposed such a mechanism for the simple disulfide lipoic acid some years ago, and although the lipoic acid hypothesis has re-

ceived little direct experimental support, the theoretical considerations upon which it was based are of considerable utility. Of more immediate relevance are studies by Massey and collaborators (7-9) on the enzyme, lipoyl dehydrogenase. This enzyme is a protein in which disulfides involved in maintenance of the intact tertiary structure of the enzyme also participate in the catalytic cycle (7). The enzyme is a flavoprotein, and interactions between the enzyme sulfur and flavin contribute to the formation of a variety of spectral peaks during the catalytic cycle (8). Of greatest interest for bacterial photosynthesis, however, is the presence of certain long wavelength bands in the enzyme, recently attributed to charge transfer complexes between

$$\text{B-ChL} \xrightarrow{\text{Light}} \text{fp C.T. Complex}\left[\text{Chromatophore-S}\right]_N\text{S}$$

$$\left[\text{Chromatophore-S}\right]_N\text{S} \longrightarrow \text{Chromatophore-S}^{\cdot}$$

$$\text{Chromatophore-S}^{\cdot} + P_i \longrightarrow \text{Chromatophore-S}{\sim}PO_3H_2$$

$$\text{Chromatophore-S}{\sim}PO_3H_2 + \text{ADP} \xrightarrow{-e} \text{ATP} + \text{S}\left[\text{Chromatophore-S-S-Chromatophore}\right]_N\text{S}$$

Fig. 1. Speculative scheme of energy transfer and phosphorylation in a chromatophore. Transfer of excitation energy from bacteriochlorophyll to flavoprotein charge transfer complex, formation of sulfur radical in interchromatophore disulfide bridge, and oxidative phosphoryl transfer from thiol phosphate formed by radical.

reduced enzyme flavin and oxidized pyridine nucleotide (9). These long wavelength bands appear in the 900 mμ region, and are consequently desirably situated for energy capture via inductive resonance transfer from bacteriochlorophyll. It is relevant, furthermore, that photosynthetic cells contain relatively large amounts of lipoyl dehydrogenase (diaphorase). One might envisage, as outlined in Fig. 1, a process in which a disulfide-bonded chromatophore flavoprotein subunit, forming a charge transfer complex analogous to that in lipoyl dehydrogenase, accepts excitation energy from bacteriochlorophyll. This energy is transferred to the interchromatophore disulfide to form a sulfur radical "sink" that dehydrates orthophosphate to form a chromatophore-thiol phosphate; i.e., a "high energy" phosphate linked to the enzyme complex. Subsequent electron flow, in which the phosphate is transferred in an oxidative step by nucleophilic attack of ADP,

regenerates the disulfide and forms ATP. While this scheme is entirely speculative, it is backed by work with other related systems and chemical models (10-15). Our own work on inhibition of photophosphorylation by disulfide exchange processes suggested that coupling might take place through intervention of a sulfur-containing species (16).

It was the initial immunochemical work that led to a search for an enzymatic system capable of photoreducing disulfides in the chromatophores of *Rhodospirillum rubrum* (17). Some recent experiments with this system have led to the view that the bacterial particles are capable of undergoing light-induced conformational changes, and that

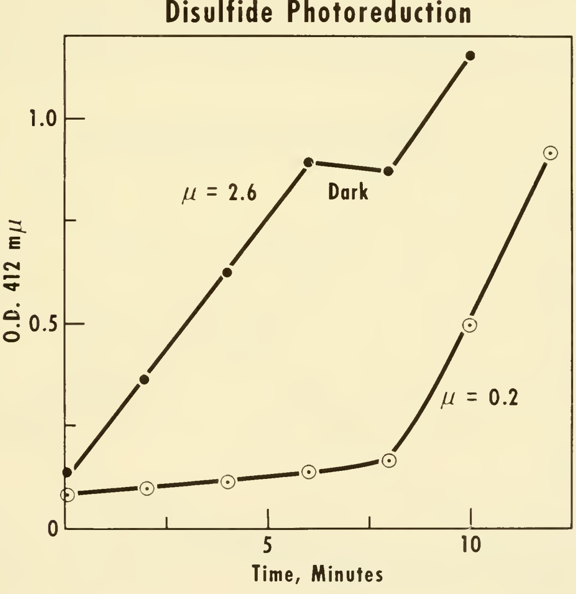

Fig. 2. Photoreduction of the aromatic disulfide, DTNB, by *Rhodospirillum rubrum* chromatophores. The reaction mixtures contained in 3 ml. 0.2 M tris buffer, pH 7.5, 0.1 ml. chromatophores (O.D. 880 = 40), 20 μmoles ascorbate, 0.1 μmole dichlorophenolindophenol, 2 μmoles methyl viologen, and 1 μmole 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). Ionic strength (μ) was altered by addition of $(NH_4)_2SO_4$. Atmosphere, helium.

such changes precede certain electron transport processes in chromatophores. Data in Fig. 2 illustrate a typical assay of *R. rubrum* chromatophores for photoreduction with the aromatic disulfide 5,5-dithiobis-2-nitrobenzoic acid (DTNB) as a thiol-trapping system. The assays were performed using media of two different ionic strengths. Both preparations carry out the light-activated photoreduction at identical rates, under anaerobic conditions, when the ascorbate-indophenol system is used as the electron donor and methyl viologen as catalyst. However, preparations in media of low ionic strength invariably display induction periods in photochemical activity, which is eliminated by salts. Induction is freely reversible and demonstrable with a large variety of di- and monovalent anions and cations; it is an effect of ionic strength on the particles. That elimination of the induction period is not solely the result of a general solute or osmotic process is indicated, because neither sucrose, urea, nor alcohols at relatively high concentration eliminate induction. In fact, nonionic solutes extend

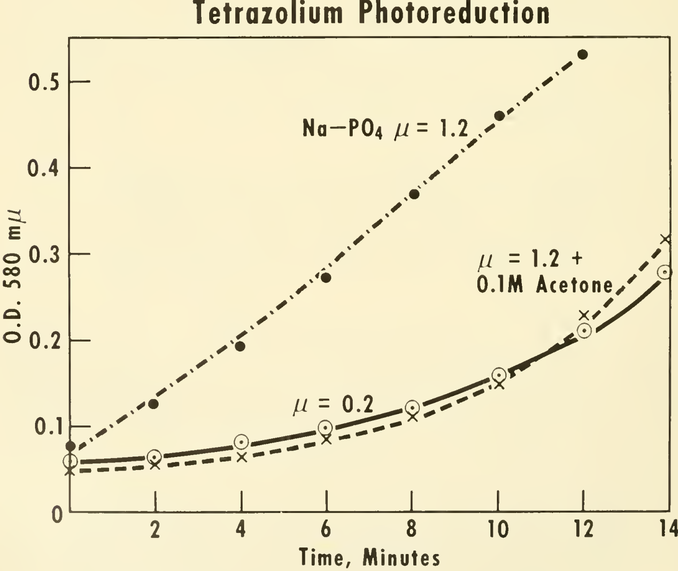

Fig. 3. The effect of acetone on photoreduction of tetrazolium blue by *R. rubrum* chromatophores in media of high ionic strength. Ascorbate-indophenol system is the electron donor.

induction, as illustrated by the effect of acetone (Fig. 3). The induction period is characteristic of a large number of light-activated redox processes in chromatophores, including photooxidation and photoreduction reactions, and is freely reversible; i.e., particles carrying out photoreactions undergo induction periods again when diluted with

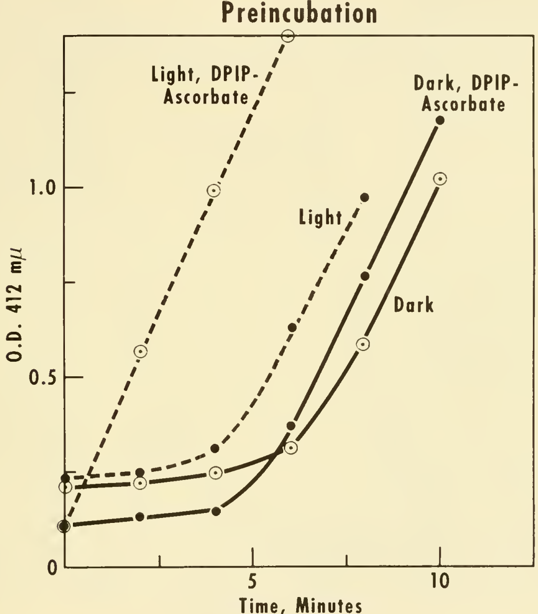

Fig. 4. Preincubation effect on induction period during disulfide photoreduction. Chromatophores were preincubated in light and dark with and without an ascorbate-indophenol system for 10 minutes. The methyl viologen-DTNB acceptor system was then added and the reaction followed spectrophotometrically in light. Atmosphere, helium.

media of low ionic strength. Finally, the induction period is inversely proportional to ionic strength over a wide range.

The reversibility of the induction period suggested an "active" process associated with photochemical electron transport in chromatophores. Consequently, the effects of preincubation of chromatophores in light and dark under various conditions on subsequent photochemical activity of the particles were examined. Data in Fig. 4 show that in a

manner entirely analogous to the effect of high ionic media, preincubation of chromatophores in light, but not in the dark, and with an electron donor system completely eliminates induction. These results provide the first indication of a possible coupling of photochemical electron flow to processes occurring during induction. It should be mentioned that media of high ionic strength reversibly suppress photophosphorylation.

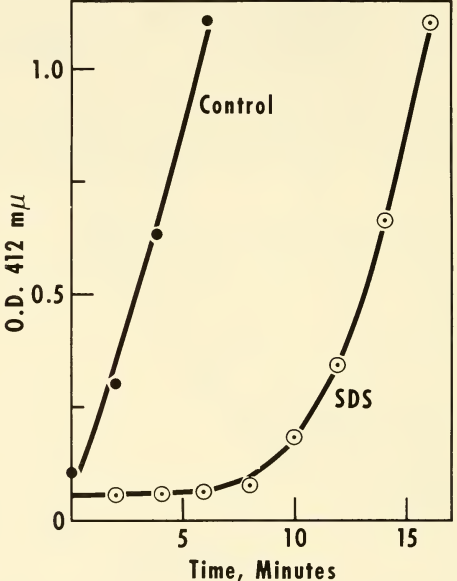

Fig. 5. Effect of sodium dodecyl sulfate (SDS), 0.3 mg./ml. on disulfide photoreduction in medium of high ionic strength (μ = 2.5, $(NH_4)_2SO_4$ in tris buffer).

Finally, additional information about the nature of induction comes from a study of other agents promoting the process in media of high ionic strength. For example, the data in Fig. 5 show that the anionic detergent, sodium dodecyl sulfate (SDS) is a very effective agent for producing an induction period; whereas cationic detergents were not. It should be noted that SDS dissolves chromatophores and destroys the pigment system in media of low ionic strength, and that chromatophores

are protected against inactivation by preincubation in light with an electron donor system.

DISCUSSION

The inclination is to interpret these observations as a result of light-induced conformational changes taking place in the bacterial chromatophore during induction. The nature of the interactions is suggested by the types of reagents inducing and eliminating the lag period. Salts, which would be expected to strengthen hydrophobic interactions among nonpolar chromatophore components (18), eliminate the lag. Nonionic solutes and detergents, which would weaken hydrophobic interactions, extend or produce induction. These results have opened up a new area of research currently under investigation.

Some of our earlier immunochemical studies, and the intriguing observations of Tuttle and Gest (19), have suggested that bacterial chromatophores are associated with the cytoplasmic membrane in *R. rubrum*. If so, then one might expect light-induced electron flow to alter the mechanical properties of the membrane and its permeability. Indications of this are already provided by recent experiments of Bose, Gest, and Ormerod (20). Further work, relevant to our own work on disulfides, shows that membrane transport processes, both in the mitochondrial (21) and toad bladder systems (22), are closely linked to thiol-disulfide interactions in the membranes involved. If these phenomena are all interrelated in the membranous bacterial chromatophore, then the induction period in chromatophore-photochemical-redox reactions might indicate a relationship between the mechanical properties of the membrane and photochemical electron transport in this subcellular system. Such a relationship has already been indicated in work on oxidative phosphorylation systems and predicted to exist in all phosphorylating systems by the elegant theoretical work of Mitchell (23).

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NONHEME IRON PROTEINS AND *CHROMATIUM* IRON PROTEIN[1]

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Relatively little attention has been given nonheme iron proteins as such in photosynthetic bacteria. Heme proteins, especially the cytochromes, are produced in large amounts by these bacteria (1) and, at least in *Chromatium*, nonheme iron is also found in appreciable amounts. Newton and Newton (2) showed the acid-soluble iron content of *Chromatium* chromatophore fractions to be in the range of 0.7 to 1.7 mg/g protein. On a comparative biochemical basis these electron transfer particles might be expected to contain nonheme iron proteins which play a role in electron transfer reactions. Enzymes of the iron flavin type such as succinic dehydrogenase (3) and NADH-cytochrome *c* reductase (4), as well as the less completely characterized iron-containing proteins hydrogenase (5) and ferredoxin (6-8), may be chromatophore constituents. The first three enzymatic activities are found in various chromatophore preparations, while ferredoxin has been isolated from photosynthetic bacteria in soluble form (8). In this report is described a nonheme iron protein isolated in relatively large amounts from *Chromatium*.

EXPERIMENTAL PROCEDURE

Purification of Protein.

Chromatium iron protein has been purified as a byproduct in the course of *Chromatium* cytochrome purification (9-11); no comprehensive purification procedure is yet available. The general procedure we have used is as follows. A crude cell-free extract, usually prepared by sonication, was centrifuged at 25,000 x*g* for 1 hour to remove cell debris plus large chromatophores. The protein precipitated by 30-60 g ammonium sulfate per 100 ml extract was collected, dissolved in buffer, and dialyzed free of salts. The protein fraction was chroma-

[1] The work reported here was performed in the laboratory of Professor M. D. Kamen with support from the National Institutes of Health (Grant No. C-5992), National Science Foundation (Grant No. G-19642), and the C. F. Kettering Foundation.

TABLE 1.

Spectroscopic properties of Chromatium *iron protein*

Extinction coefficients*/ (E mg/ml, d = 1.0 cm)

λ_{max} (mμ)	Reduced	Oxidized
277	4.0	3.7
283	4.1	3.9
292	3.7	3.5
374	—	1.8
388	1.6	—

Extinction coefficients*/ ($\Delta E_{ox.-red.}$)

λ_{max} (mμ)	ΔE mg/ml, d = 1.0 cm
283	-0.23
335	+0.32
480	+0.675

*/ Spectra were measured in 50 mM phosphate buffer, pH 7, d = 1.0 cm, with a Cary Model 14 spectrophotometer.

tographed on a DEAE-cellulose column previously equilibrated with 20 mM Tris, pH 8. The greenish-brown colored iron protein was eluted with buffer plus 40 mM NaCl. Two cytochromes were eluted at higher salt concentrations; RHP with buffer plus 80-100 mM NaCl, and cytochrome 552 with buffer plus 140-160 mM NaCl. In one preparation these three proteins were recovered in the following approximate yields per 100 g dry weight of cells:

Iron protein	15 μmoles	(10,000 MW, assumed)
RHP	6 "	(36,000 MW)
Cytochrome 552	2 "	(97,000 MW)

A pooled sample containing 1300 mg iron protein, purity index A_{283}/A_{388} = 3, was rechromatographed as above, but on A-25 DEAE-Sephadex. The main fraction, which was concentrated on a short DEAE-cellulose column and then desalted on a G-25 Sephadex column, yielded 1200 mg iron protein, purity index = 2.52. At this stage the protein appeared to be homogeneous by the criteria listed below.

The three *Chromatium* proteins were recovered in about the same proportions from an acetone powder of washed small chromatophores

isolated by the procedure of Newton and Newton (2), implying that the chromatophores are the functional intracellular location of all three proteins.

Assay Procedure.

The only assay methods so far used for any of the colored proteins of *Chromatium* depend on direct spectrophotometric measurements. The iron protein lacks distinctive strong absorption bands and therefore could not be recognized when mixed with the strongly absorbing cytochromes. Once separated from the cytochromes, the concentration of iron protein was determined by measuring the absorbancy of the 388 mμ absorption band. As a purity index, the ratio of absorbancy at 283 mμ to that at 388 mμ was used. Extinction coefficients of these and other absorption bands of the protein are recorded in Table 1.

RESULTS

Spectroscopic Properties.

Spectra of the oxidized and reduced iron protein in 50 mM phosphate buffer, pH 7, are compared in Fig. 1. The protein was isolated in the reduced form. To prepare the oxidized form excess potassium ferricyanide was added to the reduced protein dissolved in 50 mM phosphate buffer, pH 7, after which the solution was passed through a G-25 Sephadex column to remove salts from the brown-colored oxidized iron protein. To obtain the reduced spectrum shown in Fig. 1, the oxidized sample was reduced by adding to the solution an approximately 1 mg droplet of pure mercaptoethanol. In the visible region this reduced spectrum was identical to that obtained by reducing the protein with dithionite. In the ultraviolet region the mercaptoethanol did not interfere at wavelengths greater than 250 mμ.

The oxidized-minus-reduced difference spectrum in Fig. 2 was obtained by using oxidized and reduced samples treated like those used for the spectra in Fig. 1. Oxidation of the iron protein resulted in a small decrease in absorption in the UV region, and in an increase in absorption throughout the near UV and visible regions of the spectrum, with the maximum change at 335 mμ and 480 mμ, as shown in Fig. 2. Minor inflections in the absolute spectrum at about 620 and 690 mμ and a low, wide absorption band centered at about 1050 mμ were noted with high concentrations of the reduced protein. The spectrum of the reduced protein was unchanged in the presence of carbon monoxide or 10 mM cyanide. No change in the visible spectrum was observed when reduced iron protein was treated with excess dithionite in an anaerobic Thunberg cuvette after the solution had been repeatedly evacuated and then flushed with high purity argon. Similarly, when the protein was treated with hydrogen gas plus 5 per cent palladium

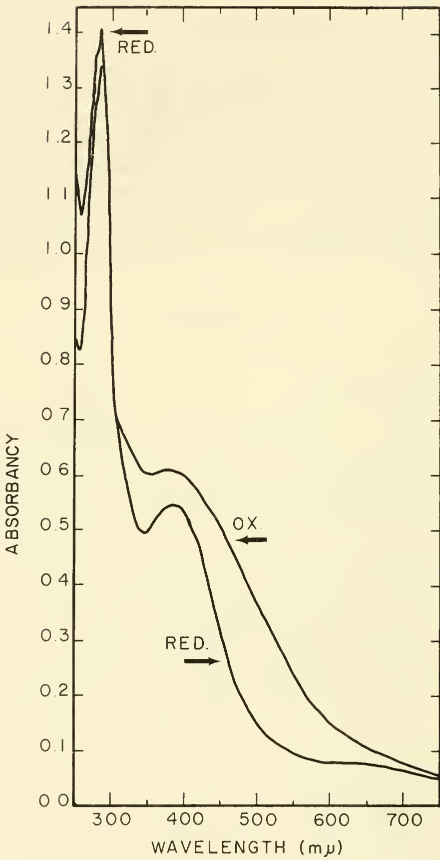

Fig. 1. Absorption spectra of oxidized and reduced *Chromatium* iron protein. The sample cuvette contained 0.34 mg/ml oxidized iron protein in 50 mM phosphate buffer, pH 7. Approximately 1 mg mercaptoethanol was added to reduce the protein.

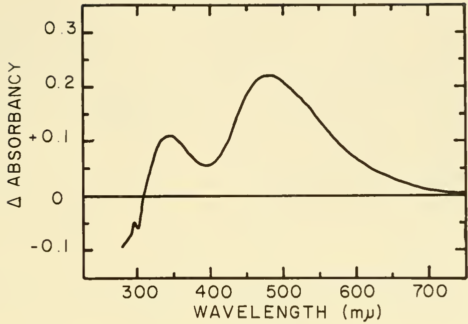

Fig. 2. Difference spectrum (oxidized-minus-reduced) of *Chromatium* iron protein. The sample cuvette contained 0.34 mg/ml oxidized iron protein in 50 mM phosphate buffer, pH 7; the reference cuvette contained the same mixture plus approximately 1 mg mercaptoethanol added to reduce the protein.

on asbestos in an anaerobic system there was no change in absorption spectrum, indicating that, unlike the ferredoxins (8), the protein cannot be bleached by reduction with a powerful reductant.

Physical and Chemical Properties.

By several criteria the protein appeared to be homogeneous. The sedimentation coefficient of the protein dissolved in 50 mM phosphate buffer, pH 7, was measured in the Spinco Model E analytical ultracentrifuge; the values obtained, extrapolated to infinite dilution, gave a value of $S_{20,w}$ = 1.7. A single sedimenting peak was observed over a concentration range of 0.15 to 0.7 per cent protein. Freshly prepared samples traveled as a single band when subjected to electrophoresis on paper or cellulose acetate foil using 50 mM barbital buffer, pH 8.5, although a small amount of oxidized protein was found after the sample had been stored frozen at -20°C for several months. A single, nearly symmetrical elution curve, with slight tailing, was obtained when purified protein was eluted at constant ionic strength (20 mM Tris plus 40 mM NaCl, pH 8) from an A-25 DEAE-Sephadex column.

An oxidation-reduction potential determined by titration with buffered ferri-ferrocyanide (12) gave the value, E'_m, pH 7 = +0.35 volt. The ratio of oxidized to reduced iron protein was determined from the absorbancy change at 480 mμ. A one electron change was indicated for the interreaction between the protein and titrant. A commercial grade of cytochrome *c* became partly reduced by the reduced iron

protein, but no interaction between purified *Chromatium* cytochrome 552 (E'_m, pH 7 = +0.01 volt) and the iron protein was found to occur.

The protein contained 2 per cent iron, as found by ashing samples with hot fuming nitric acid and measuring the iron colorimetrically with o-phenanthroline (13). Spectrographic analysis of the iron protein was generously performed by Dr. Bert L. Vallee. No metal other than iron was found in significant amounts.

In unpublished experiments performed with Dr. Helmut Beinert, EPR spectroscopy of a several month's old preparation of the iron protein showed a broad signal centered at g = 2 and a minor signal at g = 4.3. The signal at g = 4.3 is certainly due to Fe^{+3} and the signal at g = 2 is in all likelihood also due to Fe^{+3}. The first signal at g = 4.3 represents a relatively small amount of high spin Fe^{+3} and the second signal represents a relatively large amount of Fe^{+3}, probably of the low spin form. The g = 2 signal was extremely sensitive to temperature and broadened rapidly as the sample temperature increased. On addition of ferricyanide the g = 2 signal increased manyfold, indicating that the iron in the starting sample was largely in the reduced state. The signals were abolished by addition of reducing agents such as ferrocyanide and dithionite.

As noted with other iron-containing proteins such as succinic dehydrogenase (14) and PPNR (photosynthetic pyridine nucleotide reductase) (15), acidification of the iron protein to pH 1 or less caused the liberation of hydrogen sulfide as well as iron, and precipitated a colorless denatured protein. The stoichiometric relation between the H_2S evolved and the Fe liberated by such treatment of the iron protein was not determined.

Amino Acid Analysis and Chemical Constitution.

Results of amino acid analyses performed according to standard methods (16) by Dr. Dus in the laboratory of Dr. Helen Van Vunakis are summarized in Table 2, where the minimum integral number of amino acid residues is listed. A total of 84 residues were counted, indicating a minimum polypeptide molecular weight of 8842.

Nonpeptide material in addition to iron and hydrogen sulfide precursor was detected in the protein. Mild acid hydrolysis of the protein in 1 N HCl at 100°C for 4 hours liberated a trace of unidentified carbohydrate, as detected by aniline hydrogen phthalate spray test (20), plus a yellow-colored material which exhibited blue-white fluorescence under 254 or 366 mμ irradiation. Similar fluorescent material, which coincided with ninhydrin-stainable material in paper chromatograms, was released by enzymatic hydrolysis with trypsin, leucine amino peptidase plus carboxy peptidase, and by pronase. An absorption maximum at 260-270 mμ of this still impure material dissolved in ethanol decreased after the addition of sodium borohydride in a manner reminiscent of the behavior of quinones (21).

TABLE 2.

Chromatium *iron protein amino acid analysis*

Amino acid	Minimum # residues	Amino acid	Minimum # residues
Try*/	3	Gly (C term.)†/	6
Lys	7	Ala	19
His	1	½ Cys	2
Arg	2	Val	3
Asp	10	Met	1
Thr	4	iLeu	2
Ser (N term.)**/	2	Leu	5
Glu	9	Tyr	1
Pro	5	Phe	2
	Total # residues:		84
	Minimum peptide MW = 8842		

*/ Tryptophan was determined by differential titration with N-brom-succinimide according to the method of Patschornik et al. (17). A value of 3 μmoles tryptophan per 9 mg protein was obtained.

**/ Amino terminal amino acid was determined by the DNP method of Sanger (18).

†/ Carboxyl terminal amino acid was determined by the modified hydrazinolysis method of Bradbury (19).

Interaction Between Iron Protein and Chromatophores.

When a mixture of washed, dialyzed *Chromatium* chromatophores approximately 7 μM in bacteriochlorophyll, together with approximately 0.4 mg/ml reduced iron protein in phosphate buffer, was illuminated in the spectrophotometer by far red light (λ 720 mμ, 10 mwatt/cm^2), a change in the absorption spectrum occurred which indicated that the iron protein had been converted to the oxidized form, as shown in Fig. 3. The analogous difference spectrum for illuminated-minus-dark anaerobic reaction mixture is shown in Fig. 4. The initial reaction rate in the first experiment was about 15 per cent of the total absorbancy change per minute, but the rate rapidly decreased and about 45 minutes were required for the reaction to reach the state where nearly quantitative photooxidation of the iron protein had occurred. When the samples were placed in the dark the starting spectrum slowly returned. The light and dark cycle was repeated several times with apparent complete reversibility. Evidently the iron protein underwent anaerobic

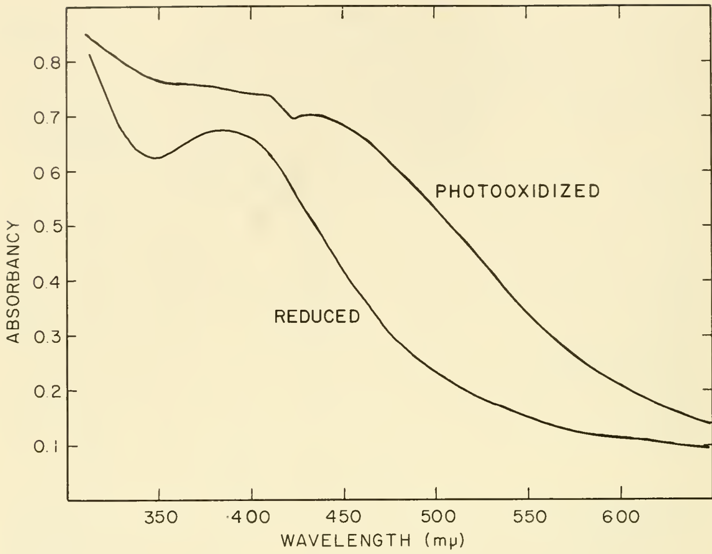

Fig. 3. Spectra of reduced and photooxidized *Chromatium* iron protein. The photooxidation was performed with an anaerobic reaction mixture containing washed, dialyzed *Chromatium* chromatophores (7 μM in bacteriochlorophyll) plus 0.4 mg/ml reduced iron protein in 50 mM phosphate buffer, pH 7. The iron protein was omitted from the reference cell. The sample cuvette was illuminated for 45 minutes with far-red light from a 100-watt tungsten filament lamp, filtered through 3 cm distilled water and a Wratten 88A filter which transmitted light of wavelength longer than 720 mμ. The incident light intensity at the cuvette was 10 mwatt/cm^2.

photooxidation in the presence of illuminated chromatophores, and in the dark after illumination the photooxidized protein was reduced back to the original state.

The photooxidation of *Chromatium* iron protein could not be demonstrated when chromatophores of *R. rubrum*, *Rps. spheroides* or *Rps. palustris* were tested.

Interaction between reduced iron protein and *Chromatium* chromatophore cytochrome(s) was observed spectrophotometrically. Of the total particle-bound cytochromes detected by a difference spectrum of dithionite reduced-minus-oxidized chromatophores, approximately 10 per cent was reduced by added excess iron protein. This observation may indicate that there exists in the chromatophore a c_2 type

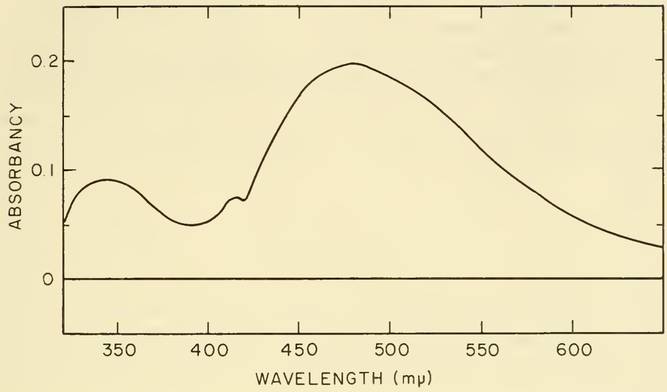

Fig. 4. Difference spectrum (photooxidized-minus-reduced) of *Chromatium* iron protein. The reaction conditions described under Fig. 3 were used except that reduced iron protein was included in both reference and sample cuvettes.

cytochrome which can be readily reduced by an oxidation-reduction agent with as positive a potential as that of the iron protein (E'_m, pH 7 = +0.35 volt).

In experiments to be published elsewhere, performed with Dr. J. M. Olson, rapid and reversible photooxidation of the cytochromes of *Chromatium* chromatophores supplemented with reduced iron protein was observed. The light-minus-dark difference spectrum obtained with a double beam spectrophotometer resembled spectra of light-induced changes in whole cells of *Chromatium* reported by Olson and Chance (22).

DISCUSSION

The evidence presented here seems to implicate *Chromatium* iron protein as a component of the chromatophore electron transfer system in an as yet unknown role. The iron protein can be isolated from chromatophores together with cytochromes which are believed to participate in light-induced reactions in the chromatophore. Added iron protein undergoes a reversible anaerobic photooxidation in the presence of illuminated chromatophores. However, the anaerobic photooxidation of particle-bound iron protein has not yet been observed because, unlike the cytochromes, the protein lacks distinctive absorp-

tion bands which would make direct observation feasible. Added iron protein can reduce chromatophore cytochrome(s), which can then undergo light-induced changes. Interaction of reduced iron protein with photooxidized particle cytochrome would result in the observed anaerobic photooxidation of the iron protein. No evidence was obtained as to the nature of the reductant which seems to accumulate as the iron protein is photooxidized and then reduces the oxidized iron protein in the dark.

There are similarities between properties of *Chromatium* iron protein and the PPNR and ferredoxin types of iron-containing proteins; here are emphasized some differences which may indicate that the two types of proteins are functionally different. A primary difference is the widely separated oxidation-reduction levels at which the proteins appear to function. E_m' for ferredoxins and PPNR is reported to be -0.42 volt (8), whereas *Chromatium* iron protein seems to operate at $E_m' = +0.35$ volt. If this discrepancy is substantiated by further investigation, then the two kinds of proteins must perform different functions.

The ferredoxin type proteins are more strongly adsorbed by DEAE-cellulose (6-8) than is the *Chromatium* protein and they require 4-5 times higher salt concentration for elution from the adsorbent. This difference in acidic properties of the proteins may merely reflect a difference in charged amino acid content, or may indicate that some major difference in chemical constitution exists between the two kinds of proteins.

Drs. H. Mower and J. E. Carnahan reported in a private communication that *Chromatium* iron protein showed less than 1 per cent the activity of a comparable amount of authentic ferredoxin when tested in the *C. pasteurianum* pyruvate phosphoroclastic assay for ferredoxin (6). The iron protein is either a ferredoxin with quite different specificity from the clostridial protein or it is contaminated with a small amount of an effective ferredoxin. A definitive test for ferredoxin function would involve a reversible oxidation-reduction reaction between the iron protein and the homologous *Chromatium* hydrogenase. An assay based on this principle has not been attempted.

Chromatium iron protein contains a chromophore in addition to the iron. Inasmuch as the spectra of the iron protein resemble reported spectra of ferredoxins and PPNR (8), the latter types of proteins may contain a functional group similar to the fluorescent chromophoric substance found in the *Chromatium* protein.

An estimate may be made of the minimum molecular weight of the complex iron protein. If all the iron found in the protein is functional, then a molecule containing only 3 Fe would be too small, but one with 4 Fe (MW = $4 \times 55.5/0.02 = 11{,}100$ g/mole) could easily accommodate the minimum peptide of approximately 8900 MW, plus iron and chromophore material.

With the evidence at hand it is not possible to specify the role of the iron protein in *Chromatium* electron transfer processes. It seems unlikely that the protein can serve as a ferredoxin. Inasmuch as no protein with properties like those of the *Chromatium* iron protein has been extracted from the nonsulfur purple bacteria, *R. rubrum*, *Rps. spheroides* and *Rps. palustris*, the iron protein may play a special role in *Chromatium* metabolism. Characterization of an iron protein reductase should help clarify the possible function of *Chromatium* iron protein.

SUMMARY

A nonheme iron protein, which contains an unidentified chromophore with properties of a substituted quinone, has been isolated from *Chromatium*. The minimum estimated molecular weight of the protein is about 11,000. The iron protein with an oxidation-reduction potential of E'_m = +0.35 volt is implicated in chromatophore electron transfer reactions. In the dark, reduced iron protein can reduce a part of the *Chromatium* chromatophore cytochromes which can undergo light-induced photooxidation. Thus illuminated chromatophores can cause anaerobic photooxidation of added reduced iron protein. The specific role of *Chromatium* iron protein in electron transfer reaction of the organism remains to be determined.

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THE RESPIRATORY SYSTEM OF *RHODOMICROBIUM VANNIELII*

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The phenomenon of oxygen uptake by a variety of photosynthetic bacteria grown anaerobically in the light has been well documented by a variety of investigators (1). In almost all cases studied, this oxygen uptake was inhibited by light, indicating a close relationship between electron transport involved in respiration and photosynthetic processes. It was observed, however, that oxygen uptake by *Rhodomicrobium vannielii* was stimulated, not inhibited, by light (2). This process was therefore examined in detail, in the hope that the interrelationship, if any, of electron transport occurring during respiration and photosynthetic processes could be more clearly defined.

RESULTS

Oxygen Uptake.

Oxygen uptake by intact cells of *R. vannielii* could be detected both in light and darkness. In the presence of endogenous or added substrate (i.e., succinate) the rate of oxygen uptake in the light was significantly greater than that observed in the dark. Photoinhibition of oxygen uptake was not observed, although in some instances, light had only a small effect on the rate of oxygen uptake. The results are summarized in Table 1.

TABLE 1.

Respiratory activity of intact cells of R. vannielii
(μmoles O_2/hr/mg. protein)

	Endogenous	With Succinate
Light	0.170	0.223
Dark	0.148	0.175

Cell-free extracts were prepared by exposure of intact cells to sonic oscillation or by passage through a French press. The rate of oxygen uptake in the dark decreased to approximately 5 per cent of the level observed in the intact cells prior to rupture. Addition of the supernatant fluid and various cofactors, including mammalian cytochrome *c*, cytochrome c_2 (isolated from *R. vannielii*), DPN, FMN, and FAD, did not increase the rate of oxygen uptake. In contrast to this, oxygen uptake by cell-free extracts in the light was equal to or greater than that observed in the intact cells (Table 2).

TABLE 2.

Oxygen uptake by intact cells and separated components of R. vannielii

	Fraction I	II	III	IV	Cell Free Extract	Intact Cells
chl./prot.[1]	12	16.8	36	15	17	—
O_2 uptake[2]						
Dark	2.5	0.5	0	0	3.0	57
Light	12.0	6.4	1.8	2.0	21.0	60

[1] chl./prot. = moles of bacteriochlorophyll x 10^9/mg. protein.
[2] O_2 uptake = mμmoles O_2/min. in the presence of 0.001 M Na succinate.

Utilizing procedures previously described (3) a variety of fractions were derived from intact cells. The degree of separation and enrichment has been described elsewhere (3) and is illustrated in Table 2. Using these preparations, oxygen uptake was measured both in light and darkness. As can be seen in Table 2, oxygen uptake in the dark was carried out only by the fractions containing the least pigment. Oxygen uptake in the dark was not observed with the most highly pigmented fractions. The total rate of oxygen uptake in the dark by the various fractions was equivalent to that observed in the original cell-free extract. It thus appears that separation of oxygen uptake in the dark from light-induced oxygen uptake was achieved, since a high rate of oxygen uptake by the highly pigmented fraction occurred only when the light was turned on.

Oxidation of Cytochrome by Oxygen or Light.

Illumination of intact cells under anaerobic conditions resulted in oxidation of the cytochromes. The light-induced cytochrome changes also occurred under aerobic conditions (Table 3) (4). Difference spectra (anaerobic vs. aerobic) in the light were quite similar to those obtained in the dark (Fig. 1).

TABLE 3.

Effect of light and oxygen on cytochrome oxidation in intact cells of R. vannielii (4)

	Anaerobic, light minus dark	Aerobic, light minus dark	Dark, aerobic minus anaerobic
ΔO.D.$_{(422.5\text{-}440\ m\mu)}$	-0.0027	-0.0019	-0.0018

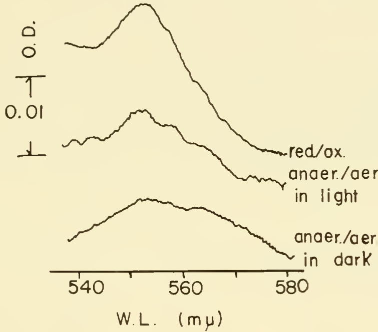

Fig. 1. Difference spectrum, anaerobic *vs.* aerobic, of intact cells of *R. vannielii* in the light and dark.

Dark Oxidase.

Carbon monoxide and cyanide inhibited oxygen uptake in the dark. A mixture of carbon monoxide and oxygen (9:1, v/v) caused a 55 per cent inhibition. The concentration of cyanide required for 50 per cent inhibition was $10^{-3.7}$ M.

The inhibitory effect of carbon monoxide and the presence of a CO-binding hemeprotein in this organism (3) suggests that this hemeprotein is acting as the terminal oxidase in dark respiration. It is of significance that the CO-binding hemeprotein is localized primarily in the components of the cell containing the least pigment.

The difference spectra shown in Fig. 1 indicate that cytochromes *c*-553 and *b*-563 are involved in oxygen uptake in the dark.

Light-Induced Oxygen Uptake.

Lyophilized preparations of the most highly pigmented fraction separated from cell-free extracts were tested for oxygen uptake. Oxygen uptake was not observed in the dark; however, light-induced activity was observed with or without the addition of exogenous substrates. Addition of succinate or ascorbate stimulated oxygen uptake, the latter substrate being the most effective.

Oxygen uptake by suspensions of the lyophilized preparation was also measured after isooctane extraction. The activities of various preparations, i.e., (a) the original lyophilized preparation, (b) isooctane-extracted material, and (c) extracted material with the addition of the isooctane extract, were measured and compared in the presence of ascorbate. As shown in Table 4, the highest rates were observed in the isooctane-extracted preparation whereas the unextracted preparation had the lowest rate of oxygen uptake. The isooctane extract contained material which had an absorption maximum at 270 mμ which disappeared upon reduction with sodium borohydride. PCMB (10^{-4} M) did not affect the rate of oxygen uptake by the unextracted lyophilized preparation whereas treatment with digitonin doubled the rate.

TABLE 4.

Effect of isooctane extract upon the light-induced oxidation of ascorbic acid

	Exp.	Extracted	Extracted, then Extract back Added	Original
Rate of Oxygen Uptake, μmoles/min./	#1	.083	.077	.058
O.D. 800 mμ	#2	.076	.040	.013

The stimulation by light of oxygen uptake by intact cells of *R. vannielii* and *Rhodopseudomonas palustris* can be readily observed in the presence of ascorbate. It is clear, however, that the effect is considerably increased by using cell-free extracts. Since extraction with isooctane or digitonin treatment also increases the rate of oxygen uptake, it may well be that breakage of the cells disrupts in some manner the normal electron transport chain that exists *in vivo*. The increase in light-induced oxygen uptake by isooctane extraction also indicates that quinone(s) are an integral part of the electron transport chain. The lack of inhibition by PCMB indicates also that PPNR or a PPNR-like factor is not involved in photooxidase activity. From these data, and the results of recent experiments, it appears that light-

induced oxygen uptake is the result of a nonenzymatic process. Details of the material presented in this paper will be described elsewhere.

ACKNOWLEDGMENTS

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Dr. Morita is on leave of absence from the Department of Biophysics and Biochemistry, University of Tokyo, Toyko, Japan.

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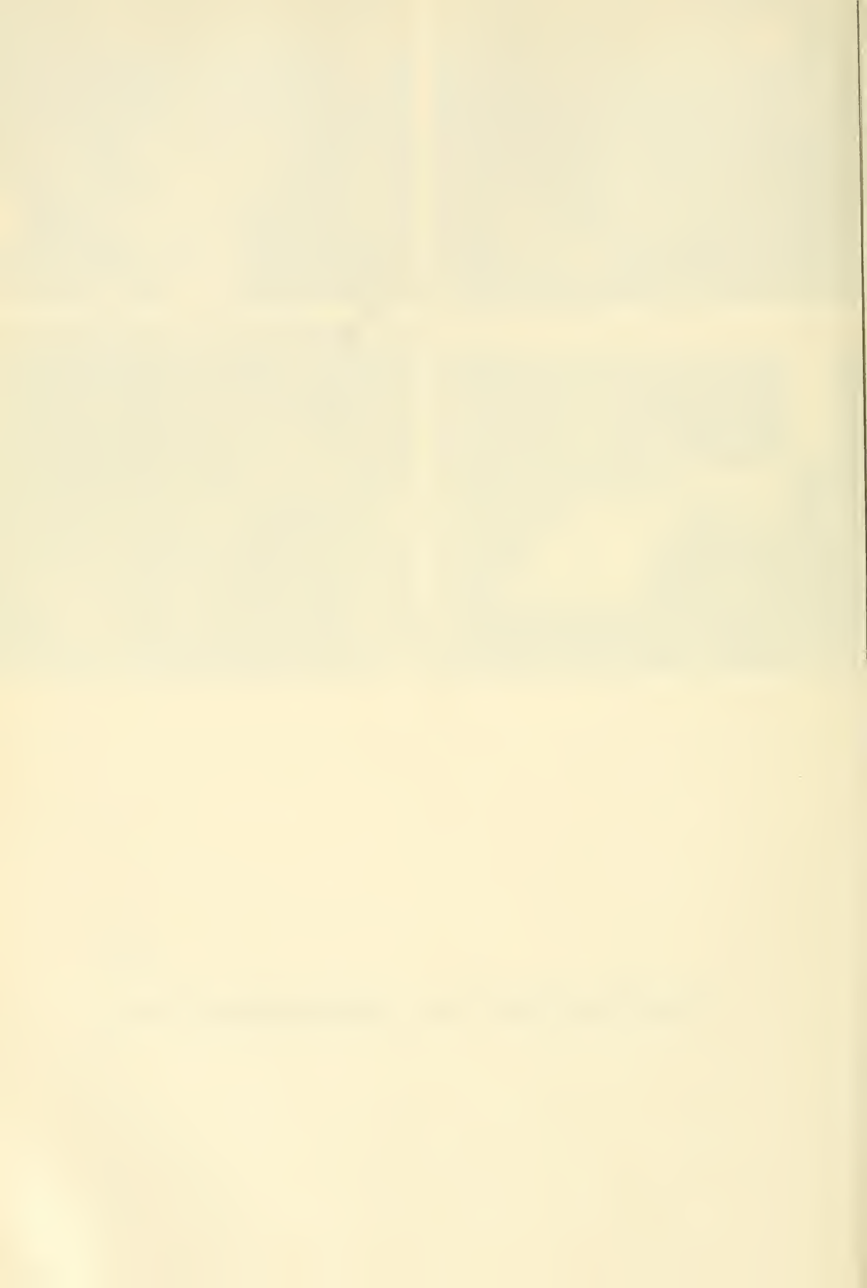
Upper left: R. Clayton; *upper right:* J. C. Goedheer, C. Sybesma, W. Butler; *lower left:* R. W. Treharne, N. Good, R. Miller, G. Seely, B. Ke, W. Butler; *lower right:* J. M. Olson, R. M. Smillie, S. Morita.

IV

PHOTOCHEMICAL CONSIDERATIONS



PRIMARY QUANTUM CONVERSION: ELECTRON SPIN RESONANCE EVIDENCE[1]

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In this paper we will discuss evidence provided by EPR on the nature of the primary quantum conversion process in bacterial photosynthesis. We shall not discuss, except by reference, the peripheral evidence offered by investigations of model systems, such as triplet state studies or charge-transfer studies, but will try to demonstrate evidence which may be obtained to identify the species producing the observed light-induced EPR signal in living photosynthetic systems.

There exist excellent discussions of EPR technique in general (1, 2,3), with reference to application in biological systems (4,5,6,7,8), and with particular application to photosynthetic systems (9,10). A discussion of the technique and its early application to the problems of photosynthesis is not warranted here.

A MODEL

One desires to choose a model with which as wide a range of observed phenomena as possible may be explained. We shall discuss a model suggested previously (2) which encompasses the photosynthetic acts of both bacteria and green plants. We define the act of primary quantum conversion as the series of events occurring between the absorption of electromagnetic radiation and the appearance of the primary transient oxidant and reductant which participate in the chemical reactions of photosynthesis. Fig. 1 shows the assigned redox relationships of species in the proposed electron transport pathway adjoining the primary quantum conversion act. Only the step involving $h\nu_1$ is assumed to apply to photosynthetic bacterial systems, in which oxygen is not a product. The high efficiency of the photosynthetic process requires that the oxidized species returns to its initial state by mechanisms which allow only for small losses of energy to heat or electromagnetic radiation. This requirement led to a physical depic-

[1] The work described in this paper was sponsored, in part by the U. S. Atomic Energy Commission.

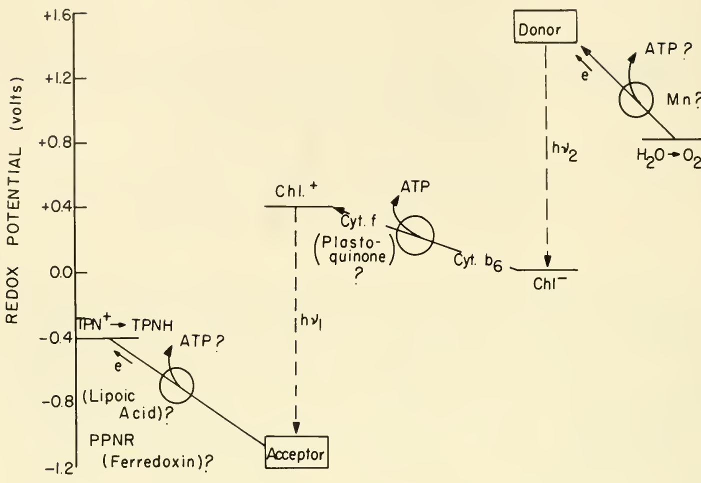

Fig. 1. Schematic diagram showing the approximate redox relationships of some species proposed as involved in the primary quantum conversion act or acts.

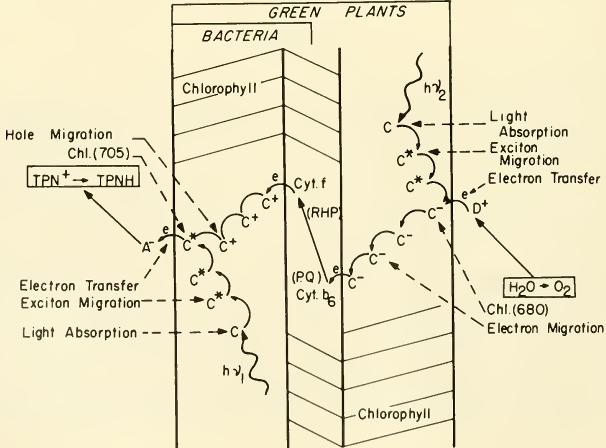

Fig. 2. Schematic diagram of the proposed mechanism for the primary quantum conversion act or acts.

tion of the quantum conversion process as shown in Fig. 2. This model provides a temperature-independent mechanism for the quantum conversion act. The steps are as follows: 1) Absorption of light by the active pigment system(s), resulting in formation of an exciton; 2) migration of the exciton to the site of electron transfer; and 3) production of a plus radical ion (hole) in a matrix of identical molecules and a reduced acceptor at low oxidation potential.

Chromatophores, the primary pigment-containing fragments of bacterial photosynthetic systems, are considered to represent the smallest unit that retains photosynthetic activity as defined by the ability to do photophosphorylation. The photoinduced EPR absorption line shape and the optical absorption spectra of chromatophores are the same as those of the parent whole cell. In *Rhodospirillum rubrum* chromatophores the EPR signals display better reproducibility than those evoked in the whole cells, and these were used as the sample for the experiments to be discussed. The chromatophores were prepared in the manner described by Androes, *et al.* (11).

REDOX EXPERIMENT

The measurement of the redox potential for oxidation of the active pigment is obtained by introducing an external redox couple into the system and attempting to vary the oxidation level of components in the electron transport chain in a controlled fashion (12). Changes in redox potential, using ferricyanide, induced dark EPR signals identical to those produced upon illumination. A complementarity between the chemically produced and light-induced signals was observed, as shown in Fig. 3. The oxidation potential for production of chemically produced signal up to one half of its maximal value corresponds to that for reduction of the light-induced signal to one half of its maximal value and occurs at an oxidation potential of $\sim$+0.46v. Recent evidence, obtained by optical absorption changes, which suggests BChl as the species initiating the electron-transfer process of photosynthesis is presented by Clayton (13). A value of $\sim$+0.46v for the oxidation potential is obtained by observing changes in the optical absorption spectra of BChl in *R. rubrum* resulting from a redox titration with the same couple (14).

In the application of EPR to physical systems the three observables which usually provide the most information about the physical environment of the electron are the g value, the line width and the appearance of fine and/or hyperfine structure. These parameters have been measured in photosynthetic systems, but unfortunately have not yielded evidence for precise identification of the site of the EPR signal. Until precise identification of the species producing the light-induced EPR

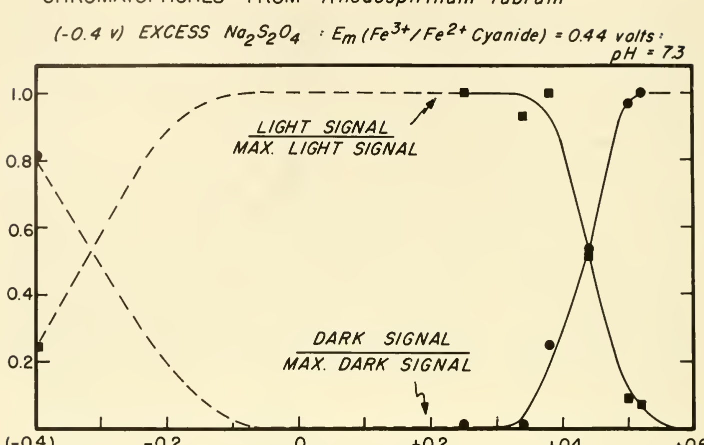

Fig. 3. Redox titration of the chemically induced and photoinduced EPR signal in chromatophores from *Rhodospirillum rubrum* ($-O_2$; pH = 7.3). Potential calculated: E_m (0.02 mole of Fe^{3+}/Fe^{2+} cyanide) = +0.44 volts.

signal is made, a direct test of any model by EPR experiment is not possible.

Three additional parameters, which depend on physical variables in the system, may be observed. These are the dependence of the EPR signal on the wavelength of irradiating light, the dependence on temperature, and the kinetics of signal production by light.

SPECTRAL RESPONSE

The magnitude of the light-induced EPR signal in *R. rubrum* has been observed to be dependent on the wavelength of light used to irradiate the sample (action spectra). BChl in *R. rubrum* has an optical absorption maximum at λ = 8800 Å. The maximum EPR signal also occurs at this wavelength (11), indicating that BChl is an important participant in the chain of events leading to the production of a light-induced EPR signal.

The photoproduction of spins by distinct absorption bands should show up as structural features in the action spectra. For example, it

has not been possible to observe either the presence or absence of a resolved peak in the action spectrum due to the secondary absorption peak at λ = 8000 Å in *R. rubrum*. It is felt that this is due to difficulties in experimental design. Androes *et al.* (11) have demonstrated that self-absorption effects may distort the action spectra when too high a sample concentration is used. Published action spectra have also suffered distortion from the use of light intensities capable of saturating the photoresponse. A better defined maximum at 8800 Å in the action spectrum has been obtained in *R. rubrum* by reducing the light intensity an order of magnitude below that used by Androes ($\simeq 10^{15}$

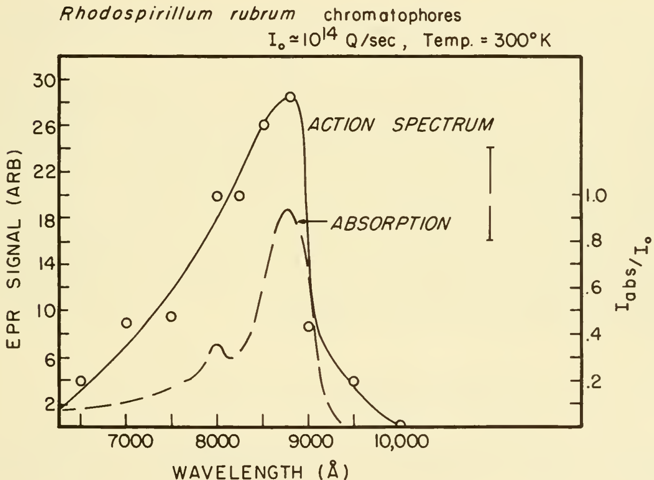

Fig. 4. Action spectrum of chromatophores from *R. rubrum*, taken at constant quanta/sec-cm^2 incident on the sample. The absorption spectrum is shown in dotted lines for comparison.

quanta/sec-cm^2), and is shown in Fig. 4. Distortions still mask possible contributions by the secondary peak. Further reduction of light intensity and/or concentration reduces the magnitude of the signal to nearly that of the noise observed in our spectrometer. Thus we face the need for a significant increase of the signal-to-noise ratio, which will require extensive instrumentation. In accordance with the above observation, Weaver (15) has recently published an action spectrum of *Chlamydomonas reinhardi* in which distortion was reduced by extrapolation of the light-induced signal to low light intensities.

If the observed EPR signal truly represents information about the primary conversion process, then the quantum yield of spins should be of the order of unity. The measurement of the quantum yield must await the solution of the difficulties mentioned above. Unless the quantum yield of spins can be observed under conditions in which it is independent of concentration and light intensity, the information obtained is misleading.

LOW TEMPERATURE

Reduction of the temperature at which signals are studied should allow the exclusion of those processes contributing to the EPR signal which require spatial migration of chemical species. Observations on *R. rubrum* at low temperature have been reported by Tollin, Sogo and Calvin (16). A reversible photoinduced signal with rapid kinetics was observed. Reversible photoinduced signals at these low temperatures suggest that at least part of the observed room temperature EPR signal results directly from a temperature-independent physical primary quantum conversion act, as postulated in the model. Optical absorption changes in *Rhodopseudomonas spheroides* at temperatures of 77°K and 1°K have been observed by Arnold and Clayton (17). We are proposing to associate the temperature independent component of the EPR signal with the mechanism producing the changes observed in the optical absorption spectrum by these investigators.

TRANSIENT RESPONSE TO A LIGHT PULSE

Preliminary experiments observing the response of the EPR signal when *R. rubrum* chromatophores are irradiated with a brief pulse of light have been performed. A square light pulse of 1 second duration was used, which had a rise time of $\simeq 10\ \mu$sec. The incident light intensity was approximately 10^{15} quanta/sec-cm^2, predominantly in the wavelength region 7000 to 8000 Å.

The EPR signal, i.e., the maximum value in the derivative of the absorption curve, occurred with a one-half rise time of the order of magnitude of 100 milliseconds. The decay time observed varied from several seconds to several hundred milliseconds. This decay time seemed to depend on aging effects in the sample, decreasing with increasing age.

Through this technique we are endeavoring to determine the kinetics of unpaired spin production by absorbed light and to relate the EPR observation to steps in, or subsequent to, primary quantum conversion.

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EFFECT OF REDUCED 2,6-DICHLOROPHENOLINDOPHENOL AND N,N,N',N'-TETRAMETHYL-*P*-PHENYLENEDIAMINE ON THE LIGHT-INDUCED ELECTRON SPIN RESONANCE SIGNAL OBSERVED WITH *RHODOSPIRILLUM RUBRUM*[1]

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Light shining on photosynthetic bacteria produces effects which may be followed by determining the optical absorption changes of the pigments, the oxidation-reduction reactions that ensue, and (more recently) the electron spin resonance signal (ESR). By correlating optical absorption changes produced by light and by chemical oxidation, Duysens (1) and Clayton (2) have suggested that certain of the absorption changes may be due to a photooxidation of bacteriochlorophyll. Calvin and Androes (3) have also suggested that the light-induced formation of the ESR signals in bacterial chromatophores is due to the oxidation of bacteriochlorophyll. This paper will present evidence that is compatible with the latter suggestion, showing that light-induced free-radical electrons can be influenced by the reducing agents $DPIPH_2$ and TMPD[2], which react with illuminated chromatophores (4,5).

EXPERIMENTAL PROCEDURES

R. rubrum (obtained from A. Frenkel) was cultured using a medium containing malate, glutamate, acetate, ammonium chloride, salts, trace elements and vitamins as previously described (6). The cells were grown in 2 or 4 liter bottles at 30-35°C and at a light intensity from incandescent bulbs of ca. 400 ft.-candles. A 4% inoculum was used and the cells harvested in their log phase after 40 hrs. of growth.

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[1] Contribution No. 110 from the Charles F. Kettering Research Laboratory.

[2] The abbreviations used in this paper are as follows: AA, ascorbic acid; and TMPD, N,N,N',N'-tetramethyl-*p*-phenylenediamine dihydrochloride. The term "chromatophore" refers to the photosynthetically active particle derived from the whole cell, as explained in reference No. 6.

Chromatophores were prepared by a method similar to that described by Vernon and Ash (7) with the following modifications: Tris buffer concentration was increased to 0.1 M, sonication time was decreased to 2 minutes, and the last two centrifugations each were increased to 100,000 x *g* for 1 hr. Chromatophores were stored unfrozen at 4°C in the dark under argon. The capacity of the preparation to produce a light-induced ESR signal remained at a high level for as long as two weeks. The procedure for reducing the O_2 tension in the ESR sample cell consisted of 5 evacuations alternated with N_2 flushings.

The chromatophore suspensions were routinely adjusted to have absorbancy values of 1.5 to 1.9 at 880 mμ in the ESR cell with a light path of ca. 0.03 cm. The volume within the microwave cavity was ca. 0.06 ml. This represents a bacteriochlorophyll concentration of from 100 to 120 mg BChl./ml. The ESR data were obtained with a Varian V4502 spectrometer equipped with 100 kc/sec field modulation. The preparations were illuminated by a focused 1000 watt projection lamp after filtering with 4.5 cm of H_2O and an interference filter which resulted in ca. 45% transmission at 880 mμ (λ max.) and a band width at one-half height of ca. 10 mμ.

RESULTS

Light induces an increase in the ESR signal of chromatophores at a rate that is not possible to follow with our present equipment. On the other hand, the decay of the ESR signal, when the light is turned off, is slower and differences with time can be resolved. The addition of $DPIPH_2$ (reduced with AA) to chromatophores caused an increase in the rate of decay (measured at one magnetic field value) as shown in Fig. 1. When the oxidized form of DPIP was added, it also increased the decay rate of the signal from chromatophores. However, larger concentrations of the oxidized form were required, and it was concluded that some of the dye became reduced in the presence of the chromatophores. Anaerobic conditions were employed, and separate experiments showed a slow endogenous reduction of DPIP under these conditions. The decay was accelerated to a greater extent with increasing concentrations of $DPIPH_2$.

A better resolution of the rates of formation and decay of the chromatophore ESR signal in the presence of $DPIPH_2$ was obtained with a fast oscillograph recorder as shown in Fig. 2. This shows that the decay and formation of the ESR signal in the presence of $DPIPH_2$ both have half-times on the order of 100 msec.

The symmetrical molecule TMPD, which readily forms relatively stable-free radicals upon partial oxidation, has been shown by Jacobs (8) to initiate coupled electron transport in rat liver mitochondria. When this molecule was added in the completely reduced form to il-

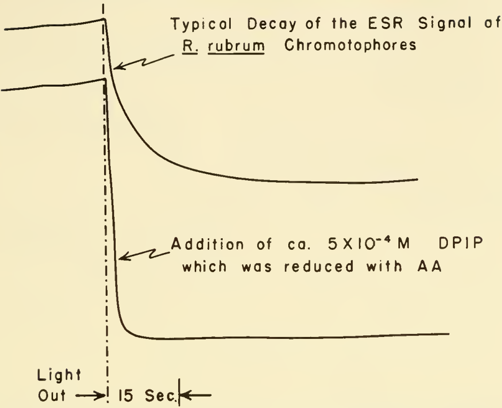

Fig. 1. Decay of the light-induced ESR signal from *R. rubrum* chromatophores in the absence and presence of $DPIPH_2$. The upper curve represents the control with ca. 5 x 10^{-4} M AA and is an average of seven determinations. The lower curve represents the decay of the ESR signal after addition of ascorbate-reduced $DPIPH_2$ for a final concentration of 5 x 10^{-4} M. The absorbancy at 880 mμ was 1.5 in both cases. Modulation amplitude 9 gauss.

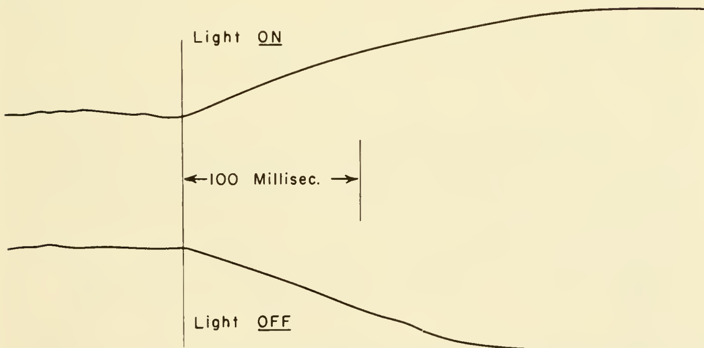

Fig. 2. The averaged curves of formation and decay of the light-induced ESR signal of *R. rubrum* chromatophores in the presence of $DPIPH_2$. The sample was at least an order of magnitude greater in chromatophore concentration than that for Fig. 1 and was opaque to light. Data were obtained with a Visicorder oscillograph and at spectrometer settings so that there was no instrumental limitation for time resolution.

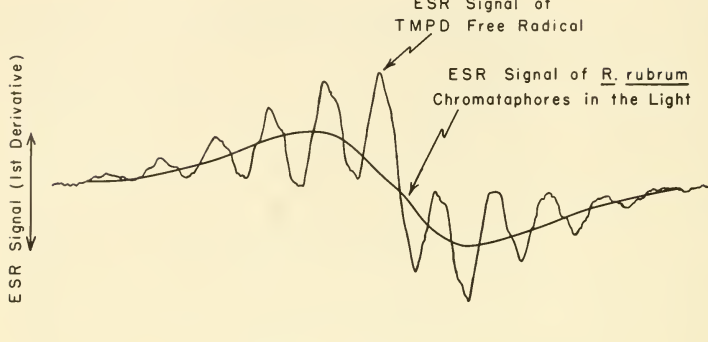

Fig. 3. A magnetic field scan of the first derivative ESR signals due to TMPD and *R. rubrum* chromatophores in the light. The modulation was sufficiently high (4 gauss) to reduce the resolution of the hyperfine lines of the TMPD radical ion so that only the 13 lines which make a larger splitting of 7.4 gauss are evident (10). The oxygen tension was reduced as described in the text.

luminated *R. rubrum* chromatophores, a complex relationship was established. Fig. 3 shows a magnetic field scan of the ESR signals in an anaerobic system containing TMPD and chromatophores in the light. The signals due to both TMPD and the chromatophores show a larger steady state value upon illumination. Light of 880 mμ, where only the bacteriochlorophyll absorbs, is effective in this reaction.

Taking advantage of the fact that the chromatophore signal is 2 to 3 times as broad as that of the TMPD (9,10), we made preliminary kinetic analyses of the two ESR signals. A span of magnetic field values was chosen where the TMPD first derivative ESR signal changed from one direction to the other, while the light-induced ESR signal from chromatophores had a constant direction. In Fig. 4, each of the curves represents a different magnetic field position arranged so the directions of the two signals first are additive then subtractive. When the light was turned on at an additive point, the total signal was the highest because it combined both signals. In this instance, an increase in ESR signal due to chromatophore is always reflected in the positive direction of the first derivative plot. The curves at the left of the "light-on" series show the increase in TMPD free radical concentration in the positive direction, while the magnetic fields used in those experiments on the right-hand side cause a negative deflection due to increased TMPD radicals. A similar but reverse phenomenon was observed when the light was turned off. In both series of experiments, it was observed that the TMPD ESR signal had a faster re-

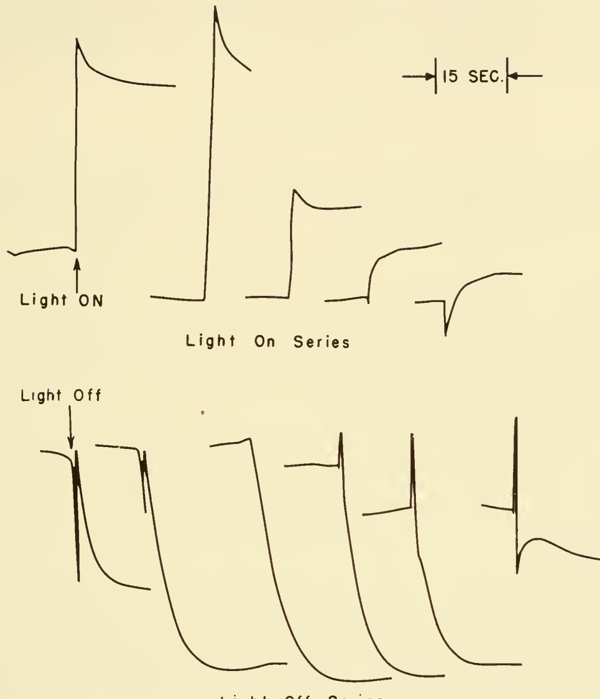

Fig. 4. The time dependence of superimposed ESR signals of TMPD and *R. rubrum* chromatophores at different magnetic field positions when the light was turned on and off. From left to right each curve represents an increasing magnetic field value, where an increase in TMPD radical ion concentration first adds to and then subtracts from the chromatophore ESR signal. An increase in the ESR signal due to chromatophores is always in the positive direction. Final absorbancy at 880 mμ was ca. 1.7, and the TMPD concentration was ca. 10^{-5} M. Modulation amplitude 9 gauss.

sponse time than the chromatophore signal, both for formation in the light and decay in the dark. The kinetics are complex, however, and do not allow any definitive statement to be made at this time concerning the interaction of TMPD and the system responsible for the ESR signal in *R. rubrum* chromatophores. This system is interesting for the reason that it allows experimental detection of both the component

responsible for the chromatophore ESR signal and a compound which has been shown to react with the chromatophore electron transfer components in a photochemical reaction.

DISCUSSION

The data presented above indicate that the reduced dyes $DPIPH_2$ and TMPD can interact with the chromatophore component responsible for the light-induced ESR signal in *R. rubrum* chromatophores. Moreover, we have confirmed Calvin and Androes' (3) and Beinert, Kok and Hoch's (11) observations that the addition of the oxidizing agent ferricyanide to chromatophores and chloroplast preparations produces a large ESR signal in the dark. This observation and the fact that both dried and wet chromatophores produce a light-induced ESR signal, which has kinetics similar to the chlorophyll-related optical absorption changes, support the contention that the ESR signal is due to the photooxidation of bacteriochlorophyll.

A plausible interpretation of the foregoing results is that light partially oxidizes the bacteriochlorophyll to produce free radicals which, in turn, give rise to the ESR signal of the chromatophore. A reduced dye, such as $DPIPH_2$, may then couple with the oxidized chlorophyll and, by donating an electron, promotes the decay of the ESR signal.

The interaction of the positive radical ions of TMPD and the free radicals produced by light in the chromatophore represents a more complex relationship. When the light is turned on, it appears that the time constant of formation of the TMPD free radicals is faster than that of the chromatophore component. Once the TMPD radicals have been formed, the kinetics at different magnetic field positions indicate that a decrease in the total signal occurs, probably due to the interaction of the TMPD free radicals with redox agents contained in the chromatophore. The rapid changes observed for the TMPD signal when the light is turned off are more difficult to explain. Knowledge of the exact relationship of the two free radicals must await further studies.

SUMMARY

1. The dye $DPIPH_2$ promotes the decay of the light-induced ESR signal from *R. rubrum* chromatophores. This effect is increased with increasing concentrations of dye.

2. A more complex relationship exists upon addition of TMPD to an anaerobic system. Both the formation and decay of TMPD positive radical ions in the light and in the dark are more rapid than those of

the light-induced free radical due to chromatophore components (bacteriochlorophyll).

ACKNOWLEDGMENTS

The authors gratefully acknowledge the aid of Mrs. F. Lubbers and Dr. S. Hood, who supplied the cultures and chromatophore preparations. We wish to thank Dr. H. Beinert and Mr. R. Hansen for the data of Fig. 2.

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THE LIGHT-INDUCED ELECTRON SPIN RESONANCE SIGNALS OBSERVED IN THE GREEN BACTERIUM *CHLOROPSEUDOMONAS ETHYLICUM*[1]

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Photosynthetic activity in a variety of organisms seems to be correlated with unpaired electrons generated by light. These unpaired electrons have been observed by electron spin resonance absorption (ESR) (1-9,13). In higher plant and algal systems the light-induced ESR consists of two signals, one narrow and fast decaying (*n,f*: signal I) with a g-value of ca. 2.002 and no hyperfine structure, and one broader and slow decaying (*b,s*: signal II) with a g-value of ca. 2.005 and a specific hyperfine structure (2,3). *Rhodospirillum rubrum* and other purple photosynthetic bacteria produce only one *n,f* signal which has no hyperfine structure (3) and a g-value of 2.003 (4) or 2.002 (5). While the origin of the *b,s* signal is not yet determined (the O_2 evolving system has been implicated (6), as well as the CO_2 fixing system (4)), there have been suggestions as to the origin of the *n,f* signal. Calvin and collaborators (4,7,8) indicated that in higher plant, algal, and bacterial systems the signal is generated by unpaired electrons in photogenerated positive chlorophyll radicals in the environment of a cytochrome. More specifically, Beinert, Kok and Hoch (9) have presented evidence that in the green alga *Anacystis nidulans* the *n,f* signal is due to a photooxidized form of the photoconverter "P700."

In the following paragraphs some preliminary data are given on the light-induced ESR in whole cells of the green photosynthetic bacterium *Chloropseudomonas ethylicum*, strain 2K, and in a sonic fraction of these cells. This organism, like the other green bacteria, contains, in addition to the main chlorophyll pigment, chlorobium chlorophyll, a small amount of another chlorophyll, chlorophyll-770, which has a striking resemblance to bacteriochlorophyll (10)[2]. The substantial

[1] The experiments were carried out at the Charles F. Kettering Research Laboratory, Yellow Springs, Ohio. Contribution No. 114.

[2] Bacteriochlorophyll is the chlorophyll of purple photosynthetic bacteria. Chlorobium chlorophyll-660 (bacterioviridin) is the main chlorophyll in most green bacteria and has maximum absorption at about 740-750 mμ *in vivo* and at 660 mμ in ether solution. Chlorophyll-770 is the minor chlorophyll in green bacteria and has maximum absorption at 809 mμ *in vivo* and at 771 mμ in ether solution.

energy transfer from chlorobrium chlorophyll to this minor chlorophyll, together with the fact that light absorbed by this minor chlorophyll is more effective in oxidizing cytochrome (11), leads to the suggestion that this pigment might be the unique terminal energy acceptor which couples absorbed light energy to photosynthesis. In view of this, observations were made on the light-induced ESR signals in cells and extracts of *Cps. ethylicum*, using light selectively absorbed by the two pigments.

MATERIALS AND METHODS

The sample of whole cells was taken from a two-day-old culture of *Cps. ethylicum*, grown as described previously (12). The subcellular fraction was obtained by washing the cells with a 0.1 M Tris buffer solution (pH 7.8) and rupturing at low temperature for about 4 min. with a 10 kc/s Raytheon sonic oscillator. The sonicate was centrifuged at 20,000 x *g* for 20 min. and the supernatant fluid was centrifuged at 140,000 x *g* for 3 hrs. The pellet was resuspended in a very small amount of Tris buffer and transferred to an ESR sample vessel as a thick suspension. This resulted in a sample which was totally light absorbing.

A Varian V-4500 spectrometer equipped with a 100 kc/s modulation unit was operated with a modulation amplitude of 12 gauss which resulted in a signal to noise ratio of ca. 6:1. The sample vessels used were 0.03 cm. thick and contained a volume within the wave guide of ca. 0.06 ml. Calculations of the g-values were made using the signals from a standard radical of peroxylamine disulfonate in conjunction with a dual sample accessory. Baird-Atomic interference filters were used to select the wavelengths of the actinic light; these had a half-value bandwidth of 9, 11.5 and 14.5 mμ for the $\lambda_{max.}$ of 703, 744 and 825 mμ, respectively. The light source was a 1000-watt projection lamp focused on a 50% T grid of the microwave cavity. The intensity of the incident light at the three wavelengths was measured with an Eppley thermopile and a Leeds and Northrup No. 2420B galvonometer. The integrations were carried out numerically.

RESULTS

Both the whole cells and the subcellular fraction exhibited light-induced ESR signals. Fig. 1 shows the signals as obtained by integration of the recordings from the spectrometer which were induced by light of $\lambda_{max.}$ 703, 744 and 825 mμ in whole cells of *Cps. ethylicum*. Although a slight asymmetry on the low magnetic field position was observed consistently, it was not of sufficient magnitude to infer the

existence of a second signal. The results for whole cells are summarized in Table 1. The relative heights of the signals are uncorrected for absorbancy and light intensity.

The signal obtained from the subcellular fraction by light of $\lambda_{max.}$ 825 mμ is shown in Fig. 2. This signal appears to be larger than that

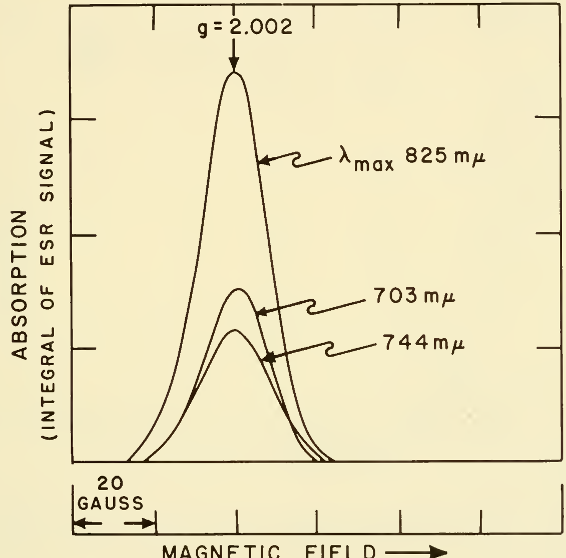

Fig. 1. Light-induced ESR signals in whole cells of *Chloropseudomonas ethylicum* for three wavelength regions with $\lambda_{max.}$ 703, 744 and 825 mμ. The signals are obtained by numerical integration of the spectrometer recordings (averaged over 5 spectra) and are uncorrected for differences in absorbance of the sample or for incident light intensities. Cells were obtained from a 2-day-old culture.

obtained with whole cells, but the chlorophyll content of the two samples was not compared. The g-value is 2.002 and the width at one-half height ($\Delta H_{1/2}$) is 17 gauss. After continued illumination, the signal decayed, possibly as a result of the aerobic conditions under which both the preparation and the measurements were made.

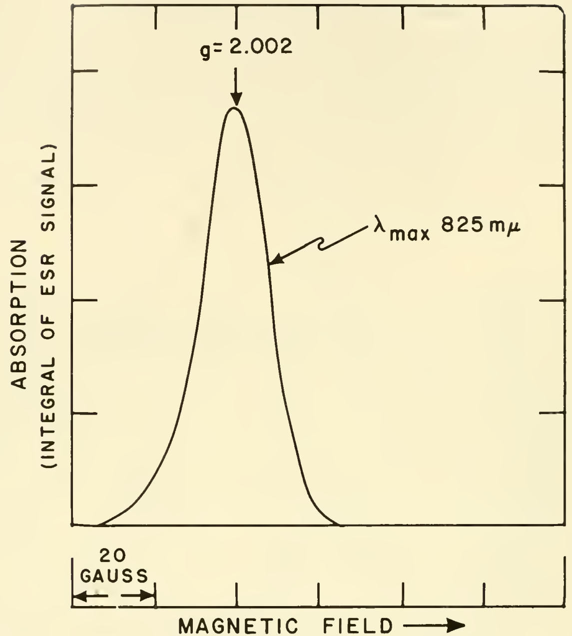

Fig. 2. Light-induced ESR signal in a sonic extract (cf. text) of *Chloropseudomonas ethylicum* for light of $\lambda_{max.}$ 825 mμ. The signal is obtained by numerical integration of the spectrometer recordings (averaged over 2 spectra). Material was extracted from a 6-day-old culture.

DISCUSSION

These preliminary results show that 825 mμ light absorbed exclusively by chlorophyll-770 in a green bacterium *in vivo* and in a sonic fraction is effective in generating a *n,f* ESR signal with a g-value comparable to that of purple photosynthetic bacteria. This substantiates the hypothesis that in this organism chlorophyll-770 is directly active in photosynthesis.

From Table 1 it might be concluded that light with $\lambda_{max.}$ 744 mμ and absorbed by chlorobium chlorophyll is less effective in producing unpaired electrons than is light with $\lambda_{max.}$ 825 mμ. This conclusion, however, would be justified only if the sample were not totally absorb-

TABLE 1

The ESR Signals Produced by Light of Different Wavelengths in Whole Chloropseudomonas ethylicum Cells

Absorbing Chlorophyll Component	Wave-length $\lambda_{max.}$ of Incident Light (mμ)	Relative Absorbancy of Chlorophyll Component	Quantum Intensity of Incident Light at Sample (Quanta sec^{-1} cm^{-2})	Relative Unpaired Electron Concentration (area of integrated ESR signal)	g-value	ESR Signal Linewidth $\Delta H_{1/2}$ (gauss)
Chlorophyll-770	825	0.5	2.1×10^{15}	1	2.002 ± 0.001	18
Chlorobium Chlorophyll (maximum)	744	10	2.2×10^{15}	0.37	2.002	20
Chlorobium Chlorophyll	703	3	1.5×10^{15}	0.43	2.002	22

ing as it was in this case. Calvin (13) and Heise (3) have shown that a totally absorbing sample shifts the ESR signal action spectrum maxima toward longer wavelengths. The layers of bacterial cells near the surface of incidence of the light absorb light at the absorption maximum to the extent that other layers are shaded. This results in minimum ESR signals at the absorption maxima. In the case of excitation with 825 mμ light, however, little or no shading was present, as is indicated by the observation that upon removal of the filters to produce intense white light the signal amplitude does not increase significantly with respect to the amplitude of the signal produced by 825 mμ light. Thus, the relative magnitudes of the ESR signals at 744 mμ and 825 mμ cannot be taken as significant.

To make a more reasonable comparison, incident light with $\lambda_{max.}$ 703 mμ was used. With less absorption by chlorobium chlorophyll at this wavelength (see Table 1), the production of unpaired electrons should occur through more layers of cells than is the case for light with $\lambda_{max.}$ 744 mμ, resulting in less shading. However, it is still impossible to state how much of the difference in amplitude between the signal produced at 825 mμ and that produced at 703 mμ is due to differences in shading. Taking into account the difference in quantum intensity at these two wavelengths (Table 1), the conclusion can be made that chlorobium chlorophyll is at least 60% as effective as chlorophyll-770 in producing unpaired electrons.

If unpaired electrons have a direct relation to the primary light conversion act in photosynthesis, these preliminary results can be interpreted as a substantiation of the hypothesis (11,12) that in the green photosynthetic bacteria, chlorophyll-770 is the terminal energy acceptor and that chlorobium chlorophyll serves as an accessory pigment, although the possibility of a direct participation of chlorobium chlorophyll in some photochemical reaction still cannot be discounted.

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Note added in proof. Recently, light-induced absorbancy changes with a maximum at about 840 mμ were observed in *Cps. ethylicum* (C. Sybesma and W.J. Vredenberg, *Biochim. Biophys. Acta,* in press), which give evidence for the existence of a reaction center, P 840, directly energy-fed by chlorophyll-770. The *n,f* ESR signal observed *in vivo* and in the sonic preparation may be due to this reaction center. This suggestion is consistent with the observation that no light-induced ESR signal was evident in a purified watery solution (10) of chlorophyll-770.

MECHANISMS OF LIGHT-ACTIVATED ELECTRON TRANSPORT IN BACTERIA: THE EFFECT OF VISCOSITY ON REACTION RATES[1]

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Mechanisms of electron transfer and electron transport in light- and oxygen-induced phosphorylation systems have been a matter of considerable discussion and speculation for some years (1-3). In photosynthetic bacteria, conditions are highly favorable for a detailed examination of the reaction mechanisms (4) which occur; and in this paper we wish to compare the effect of two parameters, temperature and viscosity, upon the light-induced reaction velocity (5,6). Where possible, similarities and differences of reaction mechanisms in light-induced systems will be compared with those observed in systems involving oxygen-induced electron transfer.

Configuration of electron transport chain.

It is appropriate to compare the nature of the electron transport chain of the chromatophore with that of the elementary unit of oxygen-linked electron transfer which has recently been christened "oxysome" (7). (See Fig. 1.)

The configuration employed here emphasizes the similarities between the two systems, at least insofar as the electron-accepting properties of oxygen and chlorophyll are concerned. A chief difference, of course, is found in the electron-donating properties of chlorophyll which, at least in the green plants, appear to lead to pyridine nucleotide reduction by an electron transport pathway involving the pyridine nucleotide reductase system. As a matter of fact, this difference is relatively superficial when we include in the oxygen-linked system the well-established observation that internal high energy intermediates generated in oxidative phosphorylation lead to a side pathway of pyridine nucleotide reduction as well. In fact, it is even possible that a similar pathway is highly active in the photosynthetic system, according to the results of Gest and Bose (8). In general, we see only one essential point of difference between the light- and oxygen-

[1] This research is supported in part by the National Science Foundation, Public Health Service, and Office of Naval Research.

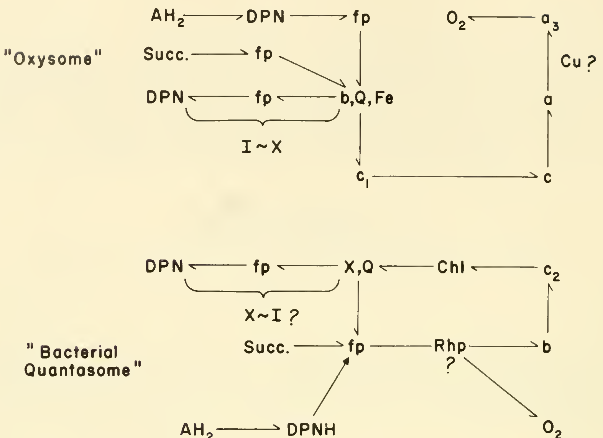


Fig. 1. Electron transfer and transport pathways in light- and oxygen-induced reactions (ME-115).

induced systems, namely, that the electrons, instead of flowing down a thermodynamic gradient to water as in the oxygen-induced system, are activated by the excited state of chlorophyll so that they leave cytochrome and flow to an electron acceptor.

Obviously, many structural similarities exist in the two systems, and these similarities are emphasized in the various attempts to isolate their "elementary assemblies": the chromatophore or "quantasome" of the photosynthetic system (9), or the "elementary particle" (10) or "oxysome" (7) of the oxygen-activated system. Electron micrographic data of fine structure in mitochondria now appear to be the most convincing evidence in favor of elementary structure. However, an objective study of the actual sizes of the units involved strongly suggests that fine structure of mitochondria is much more appropriately associated with the respiratory carriers themselves and their oxidative phosphorylation cofactors than with the complete unit of electron transport and phosphorylation. In fact, we envisage this fine structure to represent a continuum of bound, yet mobile, carriers interacting by rotational and collisional motions to transport electrons from substrate to oxygen with attendant phosphorylation (7). In this symposium a similar view of a continuum membrane structure is also suggested and it will be interesting to determine whether or not the chromatophore or quantasome shows a similar type of fine structure suggesting electron carrier mobility. With the possibility in mind

that physical-chemical studies of the reaction mechanisms in the chromatophore and, in fact, in the whole bacterial cell, might lead to a "preview" of what the electron micrographs may eventually reveal, we have most recently studied the effects of viscosity upon the kinetics of electron transport in *Rhodospirillum rubrum* and *Chromatium*, and have compared these results with those observed in the oxygen-linked systems.

EFFECT OF VISCOSITY UPON REACTION VELOCITIES IN ELECTRON TRANSPORT SYSTEMS

Effect of viscosity upon the cytochrome chain.

As a model for studies in photosynthetic systems, we have tested the effects of glycerol upon reaction rates in the cytochrome chain of intact cells and nonphosphorylating particles derived therefrom (Table 1). The data show almost complete inhibition of ethanol oxida-

TABLE 1.

Inhibition Of Electron Transfer By Glycerol
(Expt. 942 - II) (26°)

Material	Substrate	Glycerol (%)	Inhibition (%)
Bakers Yeast	Ethanol	40	>98
Keilin-Hartree Heart Muscle Preparation	DPNH	50	82
	Succinate	53	54

tion in a suspension of baker's yeast treated with 40 per cent glycerol. No specific site of inhibition could be detected as the glycerol concentration increased; in spite of the presence of glycerol and a large decrease of electron flow, the steady states of reduction of the carriers were remarkably constant, indicating that the effects of glycerol were not centered at a particular site but were distributed over a number of interaction sites in the chain. Nevertheless, it was considered important to repeat this result with a cell-free, nonphosphorylating system, and the data show very high inhibition in the oxidation of DPNH or succinate (11). Similar results have been observed when the electron transport system is suspended in deuterium oxide, and a similar interpretation has been proposed (12). It appears that glycerol and D_2O reach the phase in which the carriers interact, presumably in a water phase. From these results it appears that electron transport between hematins is due to their vibration and rotation about their

points of attachment to the cristae and, indeed, this appears to be consistent with one interpretation of the electron micrographic data (7).

Effect of glycerol upon the kinetics of light-induced cytochrome oxidation in Chromatium.

Encouraged by the striking effects of glycerol upon electron transport in the cytochrome chain, we have extended these experiments to studies of light-induced reactions in suspensions of photosynthetic bacteria. The experimental procedure is indicated in Fig. 2, which indicates the time course of light-induced cytochrome oxidation in suspensions of *Chromatium*. A measuring wavelength of 422 mμ and a reference wavelength of 460 mμ are employed (4). Anaerobiosis has previously been established in the bacterial suspension and illumination with infrared light (88 A filter, 400 Lux) causes an abrupt oxidation of the cytochrome component of the bacteria which is half complete in approximately 0.2 seconds. Independent controls indicate that the time for half-maximal illumination of the sample is approximately 0.03 seconds. The response time of the amplifier is also more rapid than the absorbancy changes. Thus, the upward slope of the trace is limited by the rate of chlorophyll activation and electron transfer between cytochrome and chlorophyll. As is characteristic of the light-induced phenomena, the rapid phase is followed by a slow phase and, after two seconds of illumination, the light is turned off in the same short time interval as it was turned on. The trace deflects downward in a half-time comparable to that observed upon turning on the light.

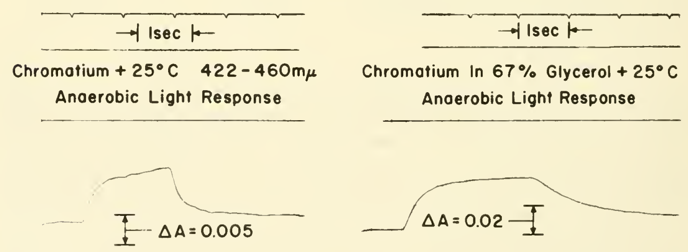


Fig. 2. An illustration of the experimental technique used in evaluating the rates of light-induced cytochrome oxidation in suspensions of *Chromatium*. The kinetics are measured by means of the double-beam spectrophotometer, employing infrared illumination of the sample. The record on the left represents the reaction kinetics in the absence of glycerol and, on the right, in the presence of 67 per cent glycerol. An absorbancy decrease at 422 mμ relative to 460 mμ is indicated by an upward deflection of the trace (HS-19).

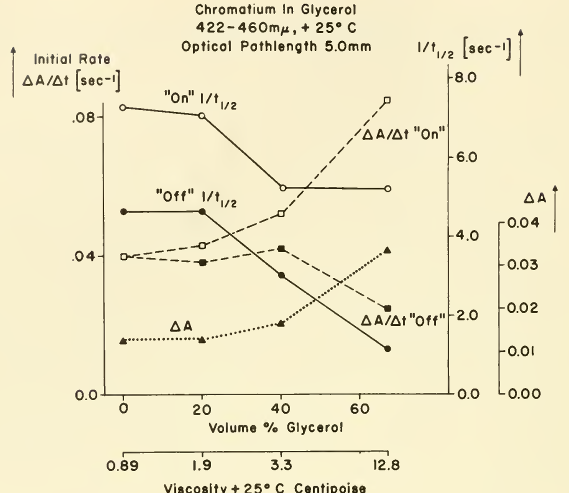
The reaction rates are evaluated by tangents to the linear portions of the cytochrome oxidation and reduction kinetics. Since relative rates are of interest here, slopes in millimeters of vertical deflection per millimeter horizontal deflection may be employed, although conversion to molar rates is employed in Fig. 3(b).

In Fig. 2b, the same technique is used in recording the light-induced reaction in the same *Chromatium* suspension which has been diluted with two parts of glycerol to a final concentration of 67 per cent. The kinetics of cytochrome oxidation on illuminating the suspension are rather similar to those in Fig. 2A. A remarkable difference in the two traces is the slow cytochrome reduction in the glycerol-treated cells; at least 0.4 second is required for half-maximal effect.

In Fig. 3, A and B, are plotted the results of two series of experiments on the effect of glycerol upon the light-induced response of cytochrome of type *c* in different suspensions of *Chromatium*. In both figures are represented the initial slopes of the light responses, together with the reciprocal of the time for half-maximal rise on illumination (rapid phase only) as well as the reciprocal of the time for half-maximal fall on cessation of illumination. Lastly, both graphs show the absorbancy increment on illumination. Fig. 3A covers the range to 67 per cent glycerol and Fig. 3B covers the range to 80 per cent glycerol. Considering Fig. 3A, we observe that the initial rate of absorbancy change on illumination is more rapid in the presence of glycerol and that observed on cessation of illumination is less rapid. Also the increment of absorbancy on illumination increases with increasing glycerol concentration. If, however, we measure the reciprocals of the times for half-maximal response, it is noted that the light-on response decreases 30 per cent at 40 per cent glycerol but remains constant thereafter. However, the reciprocal of the half-time "off" falls 87 per cent at 67 per cent glycerol. It should be pointed out that half-times for these reactions are sometimes difficult to measure because the traces are occasionally diphasic and more reliance is placed on the initial rates.

In Fig. 3B, it was possible to cover a wider range of glycerol concentration and it is seen that the initial slopes again diverge with the glycerol concentration; a 3:1 ratio of initial rates is obtained at 67 per cent glycerol. In this experiment the reciprocal of the time for half-maximal response to illumination is approximately independent of glycerol while the response to cessation of illumination drops precipitously at 80 per cent glycerol. Again the illuminated minus dark steady state change suggests an increased steady state oxidation of cytochrome component.

Both these results are consistent with a lack of inhibition of the light-induced response in *Chromatium* in the presence of a wide range of glycerol concentrations. The "initial slope" data further suggest that the light-induced oxidation of cytochrome proceeds more rapidly in the



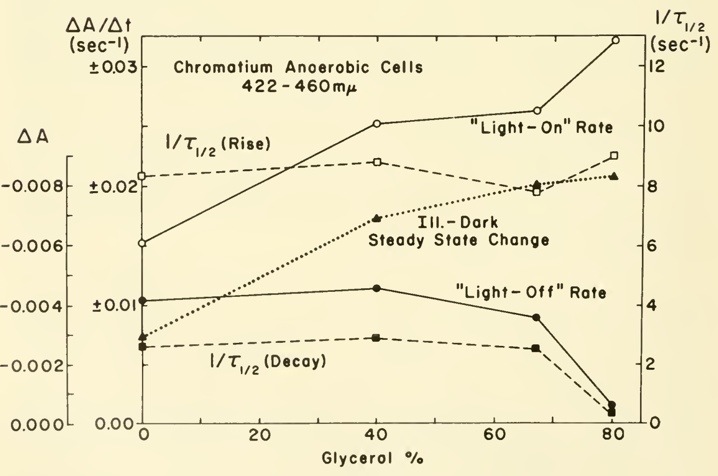


Fig. 3. Graphs of two experiments on the effect of glycerol upon the light-induced responses of suspensions of *Chromatium*. (Fig. 3A, HS-20; Fig. 3B, MN-20).

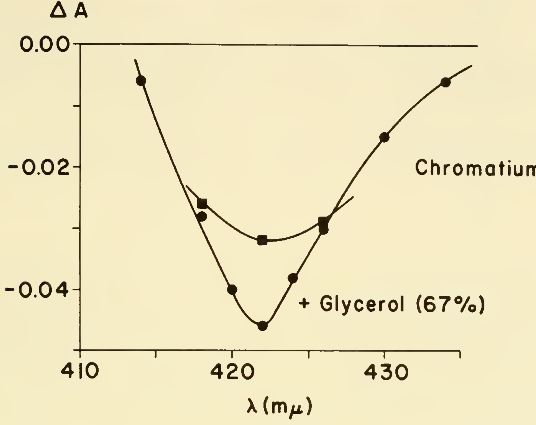


Fig. 4. The effect of glycerol upon the difference spectrum for cytochrome oxidation in *Chromatium*. (MN-16).

presence of glycerol than in its absence. Lastly, a consistent inhibition of the rate of the light-off reaction is observed.

An examination of the difference spectrum for the light effect in the presence of glycerol shows the peak to be somewhat intensified and shifted toward shorter wavelengths (Fig. 4). The maximum is approximately 422 mμ, the wavelength employed in Fig. 3.

Rhodospirillum rubrum.

A series of experiments illustrated in Fig. 5 indicate the effect of the aerobic-anaerobic transitions in *R. rubrum* (A), followed by illumination of the cells. The illumination interval is plotted on an enlarged scale in B; it is seen that the light reaction can readily be measured from the slopes of the traces. The effect of 40 per cent glycerol is indicated in Record C. First, it is seen that the respiratory activity is much slower, as evidenced by the prolonged interval required to reach the anaerobic steady state. Illumination gives approximately the same percentage change of steady states. In the enlarged view (D), one sees that the light-on reaction proceeds very rapidly in spite of the presence of 40 per cent glycerol but that the light-off reaction is considerably slowed. Thus, we see characteristics in *R. rubrum* similar to those in *Chromatium* and, on this basis, our data are consistent with the possibility that the cytochrome-bacteriochlorophyll electron transfer reaction in *R. rubrum* is of a similar mechanism to that of *Chromatium*.

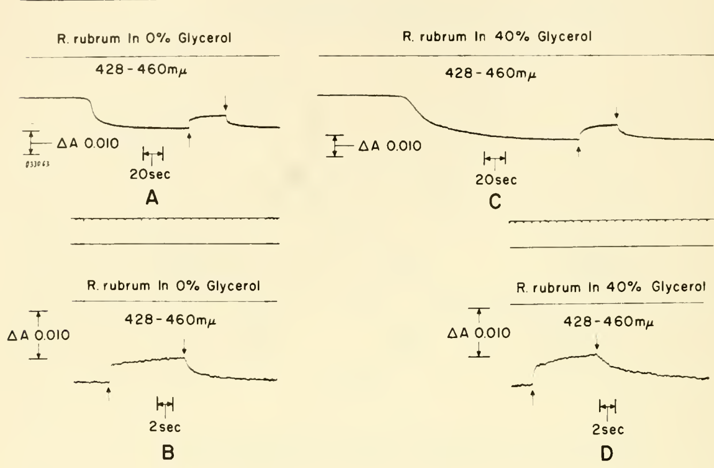


Fig. 5. An illustration of the light-induced kinetics of a suspension of *R. rubrum* in the presence and absence of 40 per cent glycerol (HS-18).

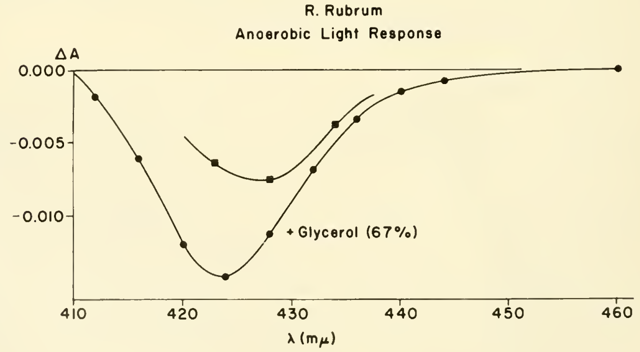


Fig. 6. Difference spectrum for light-induced responses of *R. rubrum* in the presence and absence of 67 per cent glycerol. (MN-17).

In Fig. 6 are light-induced difference spectra for *R. rubrum* suspensions in the presence and absence of 67 per cent glycerol. It is seen that there is a definite shift in the peak from 428 to 422 mμ. This is attributed to the observed decrease of the rate of oxidation of cytochrome *b* (or o-rhp) (13) measured at 428 mμ and to the increase in the rate of oxidation of cytochrome of type *c* in the presence of glycerol. The shift of the band position observed in *R. rubrum* makes it very unlikely that the effects of glycerol upon the reaction kinetics are an optical artifact.

Effects of glycerol upon absorption spectra.

Suspension of *R. rubrum* in 67 per cent glycerol causes a 9 per cent decrease of absorbancy in the prominent 549 mμ carotenoid band but no shifts of the peaks. In the region 750-900 mμ there is a decrease in absorbancy in *Chromatium* and *R. rubrum* of 34 per cent at 800 mμ and 35 per cent at 890 mμ , respectively. This decrease in absorbancy would be expected to decrease the rates of the light-induced reactions. The data of Fig. 3 may underestimate the increase of rate that actually occurs.

DISCUSSION

Mechanism of the electron transfer.

The lack of inhibition by increased viscosity of the light-induced oxidation of cytochrome of type *c* in *Chromatium* confirms the lack of temperature dependence of this reaction (5) and supports the idea that electron transfer between cytochrome of type *c* and bacterial chlorophyll is a collision-independent event. In *R. rubrum* similar responses to glycerol indicate a similar reaction between cytochrome and bacterial chlorophyll.

In confirmation of the temperature dependence of the light-off reaction (5), the light-off reaction is highly sensitive to effects of glycerol. We believe that glycerol increases the viscosity at the site of action of the enzymes transporting reducing equivalents to cytochromes of type *c* and interferes with collision mechanisms involved in electron transport.

Relation to the quantum requirement for cytochrome oxidation.

The apparent increase in the velocity of the light-induced oxidation of cytochrome of type *c* in *Chromatium* in the presence of glycerol suggests that an even higher quantum efficiency of the reaction might be obtainable under these circumstances and represents a factor that is worthy of consideration not only in purple bacteria (4,14,15) but also in green cells. If we accept, without further consideration of possible experimental artifacts, the twofold increase of initial velocity illus-

trated by Fig. 4, the quantum requirement of approximately two per electron can be converted very nearly to one per electron. It is possibly premature to make this calculation at the present time since the quantum requirement for the particular suspension of cells employed in this experiment was not measured and further experiments along these lines are needed. Nevertheless, a possible approach of the quantum requirement to one per electron is of considerable practical and theoretical interest.

SUMMARY

1. In 67 per cent glycerol, the rate of light-induced oxidation of cytochrome of type *c* is accelerated while the rate of reduction on interrupting illumination is decelerated.

2. These results are interpreted in terms of increased intracellular viscosity and suggest a viscosity-independent cytochrome-chlorophyll electron transfer in *Chromatium* and *R. rubrum* and a viscosity-dependent electron transfer to cytochrome of type *c* from electron donors of the cyclic or noncyclic system.

3. These results are consistent with the temperature independence of the light-on reaction and the temperature dependence of the light-off reaction in *Chromatium.*

4. The effects of both temperature and viscosity support the conclusion that electron transfer between cytochrome of type *c* and bacterial chlorophyll is an electron transfer reaction not requiring mobility of the cytochrome—whereas electron donation to cytochrome does require a collision mechanism.

5. The increased velocity of cytochrome oxidation in the presence of glycerol suggests a re-examination of quantum requirement and a possible revision of currently accepted values from two quanta per electron to approximately one quantum per electron.

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A KINETIC ANALYSIS OF THE LIGHT RESPONSES OF PHOTOSYNTHETIC BACTERIA AND PLANTS

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For some time, physical methods have been employed to record light-induced changes in photosynthetic bacteria (1,2), yet an interpretation of the reaction kinetics has only been made in the case of the "light-on" reaction in *Chromatium*. In this case, the cytochrome rate has been compared with the quantum rate to calculate the efficiency of the intracellular electron transfer reaction (3).

The basic assumption of such studies is that the initial rate of cytochrome oxidation is unimpeded by reducing reactions—an assumption that appears to be justified by the high quantum efficiencies that are computed on this basis.

This paper treats reaction kinetics of light-induced cytochrome oxidation in models for cyclic and noncyclic electron flow.

Cyclic electron flow.

As an example of cyclic electron flow, we shall use one of the current hypotheses of electron flow in *Rhodospirillum rubrum* and in their chromatophores (4) (Fig. 1).

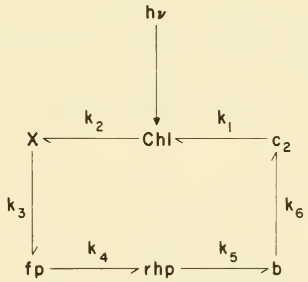

Fig. 1. Possible pathway of cyclic electron flow (ME-116).

Chlorophyll activated by light (hv) causes electron flow through the chain of electron carriers, cyt c_2 the primary electron donor, X the primary acceptor, fp, rhp, and cyt *b* being intermediate carriers.

X is not unambiguously identified at present although ubiquinone has been proposed as the primary electron acceptor. Flavoprotein has been isolated by Horio (4) and identified to be functional by Atebrin (quinacrine) inhibition of electron transport (5). Both quinone and flavoprotein may participate in one electron transfer reaction, but two electron reactions could easily be accommodated. Rhp participation in electron transport is suggested by Horio (4). This hemeprotein may react with oxygen as well. Cytochrome *b* and c_2 function in light-activated electron transfer was indicated by Smith and Ramirez (6). The reaction velocity constants $k_1 \ldots k_6$ are of the second order. The mechanism by which chlorophyll is activated directly by a photon or indirectly by resonance energy transfer is schematically represented:

$$\text{Chl} \xrightarrow{k_o} \text{Chl}^* \tag{1}$$

where $k_o = \epsilon \phi I$; ϵ is the extinction coefficient; ϕ the quantum efficiency; and I the light intensity.

The excited state of chlorophyll (Chl*) can accept an electron from cytochrome c_2 or donate an electron to X. Here we have chosen to represent the primary reaction as the acceptance of an electron from cytochrome for the simple reason that this reaction is found to occur at the same rate at 300 and 77°K (7).

$$c'' + \text{Chl}^* \xrightarrow{k_1} c''' + \text{Chl}^- \tag{2}$$

$$X + \text{Chl}^- \xrightarrow{k_2} X^- + \text{Chl} \tag{3}$$

Thus, activated chlorophyll is returned to the ground state and can be reactivated, as in Equation 1. The remainder of the electron transport reactions occur as for members of the cytochrome chain, in which one electron transfer reaction is assumed. The mechanisms described here are, of course, applicable to two electron transfer reactions as well.

$$X^- + \text{fp} \xrightarrow{k_3} \text{fp}^- + X \tag{4}$$

$$\text{fp}^- + \text{rhp} \xrightarrow{k_4} \text{rhp}^- + \text{fp} \tag{5}$$

$$\text{rhp}^- + b''' \xrightarrow{k_5} b'' + \text{rhp} \tag{6}$$

$$b'' + c''' \xrightarrow{k_6} b''' + c'' \quad (7)$$

Thus, the cycle is completed through the chain of carriers.

Differential equations.

The differential equations for the rates of electron transport are very similar to those for the respiratory chain (8) with two important exceptions. First, the activation of chlorophyll by light results in an average rate of conversion of chlorophyll to its activated state:

$$\frac{d\ Chl^*}{dt} = k_o\ Chl - k_1\ Chl^*\ c'' \quad (8)$$

Second, the rate of the primary electron transfer reaction consists of the rate of oxidation of reduced cytochrome c[1] by activated chlorophyll minus the rate of reduction of oxidized cytochrome c by reduced cytochrome b:

$$-\frac{dc''}{dt} = k_1\ Chl^*\ c'' - k_6\ b''\ c''' \quad (9)$$

Without a detailed study of the remaining equations, it is possible to draw the following conclusions. The observation that $-\frac{dc''}{dt}$ can be accelerated by addition of glycerol suggests that the term $k_6\ b''\ c'''$ in *Chromatium* is not negligible or that glycerol acts by affecting k_6 to a greater extent than k_1. From observations of the effect of phenyl mercuric acetate on the steady state level of cytochrome c (9), it is clear that cytochrome c is highly reduced. But very little is known about the steady state level of cytochrome b, and it cannot be assumed at present that $b'' = 0$. Inhibition of electron transfer by glycerol appears to cause the term $k_6\ b''\ c'''$ to diminish and under such ideal conditions we have the first useful expression:

$$-\frac{dc''}{dt} = k_1\ Chl^*\ c'' \quad (10)$$

Second, in the steady state, $\frac{dc''}{dt} = 0$ and $\frac{d\ Chl^*}{dt} = 0$

$$k_1\ Chl^*\ c'' = k_6\ b''\ c''' \quad (11)$$

and

$$k_o\ Chl = k_1\ Chl^*\ c'' \quad (12)$$

[1] In these equations we assume that c''' is zero in the dark.

Thus

$$k_o \text{ Chl} = k_6 \text{ b''} \text{ c'''} \qquad (13)$$

or

$$\epsilon\phi I \text{ Chl} = k_6 \text{ b''} \text{ c'''} \qquad (14)$$

Third, on cessation of illumination, Chl* falls to zero in a time that is probably much less than 10^{-4} sec. From Equation 9

$$\frac{dc''}{dt} = k_6 \text{ b''} \text{ c'''} \qquad (15)$$

In this way, the steady state flux is determined in moles/liter x sec of cytochrome *c*.

Also from Equation 14

$$\frac{dc''}{dt} = k_o \text{ Chl} = \epsilon\phi I \text{ Chl} \qquad (16)$$

i.e., the initial rate of cytochrome reduction on cessation of illumination should be equal to the rate of activation of chlorophyll.

EXPERIMENTAL RESULTS

In view of the mathematical analysis above, it is important to examine the ratio of on-off rates in photosynthetic bacteria. In cyclic electron flow, the initial rate of cytochrome reduction on interruption of the illumination may be equal to the rate of cytochrome oxidation (Equation 10) or, indeed, greater than the rate of cytochrome oxidation (Equation 9). We have, therefore, in Fig. 2 plotted the ratio of on-off velocities for an anaerobic suspension of *R. rubrum*, using a wavelength of 423 mμ appropriate to cytochrome of type *c* and of 428 mμ appropriate to cytochrome *b* or possibly rhp. It is seen over the range of light intensities (the abscissa are proportional to light intensity) that the on-off ratio at 423 mμ is relatively constant but that at 428 mμ is not. On this basis, cytochrome type *c* fulfills the requirements for a component of cyclic electron transfer while cytochrome *b* or rhp does not. A similar evaluation may be made of Fig. 3 of the accompanying paper (10) where, in the absence of glycerol, the on-off rates for cytochrome of type *c* are similar, but in the presence of high concentration of glycerol the on rate is much more rapid than the off

rate. A similar result may be inferred from the effects of low temperatures where the ratio of on to off rate approaches infinity at temperatures of liquid nitrogen (7).

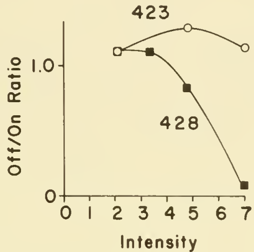

Fig. 2. Light-off/light-on rates for a component of type *c* (423 mμ) and *b* or rhp (428 mμ) in *R. rubrum* (900).

A striking example of a light-off reaction that is more rapid than the light-on reaction is afforded by the kinetics of cytochrome *f* of a pale green mutant of *Chlamydomonas*, in response to illumination (11). Here the ratio of the rates is about three (Fig. 3).

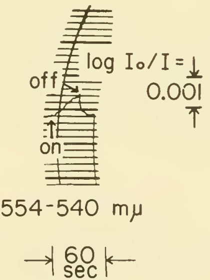

Fig. 3. Light-on/light-off kinetics for cytochrome *f* of a pale green mutant of *Chlamydomonas* (674-II). (Courtesy of *Plant Physiol.*, *32*, 557, 1957.)

It should be noted that Amesz (12, also see 13) employs the light-off reaction for the measurement of the quantum requirement for pyridine nucleotide reduction in purple bacteria. Interestingly enough, Amesz does not observe the need for establishing a steady state before cessa-

tion of illumination and thus a much more complicated equation than Equation 15 is required to evaluate his experimental data.

DISCUSSION

Based on the assumption that all electron flow observed in the photosynthetic bacteria is of the cyclic type, a simple mathematical derivation leads to the conclusion that the rate of cytochrome reduction on interruption of illumination may be equal to or greater than the rate of cytochrome oxidation on illumination. Preliminary observations on *R. rubrum* indicate that under a particular set of conditions the kinetics of cytochrome of type *c* meet this criterion, although it does not apply at low temperatures or in the presence of glycerol. In a pale green mutant of *Chlamydomonas*, the light-off rate for cytochrome *f* exceeds the light-on rate by three times.

This method may have possibilities for investigation of the nature of electron flow in a variety of photosynthetic systems.

SUMMARY

Under the assumptions made in our mathematical derivation, the rate of cytochrome reduction on interruption of illumination should be at least as great as the rate of cytochrome oxidation on illumination in systems involving cyclic electron flow. The rate of cytochrome reduction may be considerably less than that of the rate of cytochrome oxidation in noncyclic systems. For a particular set of conditions the light-induced kinetics of cytochrome of type *c* in *R. rubrum* are consistent with existence of cyclic electron flow, but those of cytochrome in a pale green mutant of *Chlamydomona's* are not.

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PHOTOCHEMICAL REACTION CENTERS IN PHOTOSYNTHETIC TISSUES[1]

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Current theories for the mechanism of photosynthesis are grounded in two major developments that began about thirty years ago. One of these was the discovery of the photosynthetic unit (1,2); the other was the emergence of the concept that photosynthesis begins with a photochemical separation of oxidizing and reducing power (3).

In modern terms the photosynthetic unit is a set of Chl[2] molecules that cooperate in harvesting the energy of light quanta and in channeling this energy to a photochemical reaction center. Its existence in photosynthetic tissues was established through Emerson and Arnold's studies of the photosynthetic yield of short flashes of light in *Chlorella* (1), and independently through Gaffron and Wohl's analysis of initial photosynthetic rates at the onset of illumination (2). The reaction center is regarded as a site where quanta of excitation energy bring about electron transfer events that lead to the storage of stable chemical potential. The stable chemical entities thus formed can serve as starting points for the many chemical reactions of photosynthesis.

In green plants and algae the characterization of photosynthetic units and reaction centers meets with a complication. Study of the Emerson red-drop and enhancement phenomena (4) has revealed the presence of two distinct photochemical systems (5-10). System I (the "far red" system) generates strong reductants such as $NADPH_2$; it receives electrons from the "short wave" System II and raises these electrons to a higher energy. System II mediates the evolution of oxygen and feeds electrons to System I. The partial reactions occurring in System II remain obscure, whereas the makeup of a reaction center in System I is beginning to emerge. This article will be restricted to a consideration of System I and its apparent counterpart in the photosynthetic bacteria.

It has not been settled whether the primary photochemical reactions (in green plants, algae, and photosynthetic bacteria) are of a "one

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1 Contribution No. 108 from the Charles F. Kettering Research Laboratory.
2 In addition to the standard abbreviations, the following is used in this article: BPh, bacteriopheophytin.

quantum-one electron transfer" sort, or whether they involve the concerted action of two light quanta. There are compelling arguments (11) favoring a two-quanta primary reaction. However, the most concrete spectrophotometric observations dealing with System I and the bacterial system (see later) suggest that the primary reactions involve single quanta driving single electron transfer acts. For the present the observable reactions will be discussed in "one quantum" terms.

On a "one quantum" basis, the flashing light experiments of Emerson and Arnold should be interpreted as follows: The photosynthetic unit in green plants and algae consists of a set of about 400 Chl molecules plus a reaction center. The energy of one light quantum, absorbed anywhere in the unit, is delivered to the reaction center. There it promotes a reaction in which one electron is transferred so as to produce a stable reductant separated from an oxidant. In photosynthetic bacteria the unit, as defined by flashing light experiments and interpreted on a one-quantum basis, consists of about 50 BChl molecules plus a reaction center (12).

A reaction center, functioning in a photosynthetic unit, can be delineated by identifying the primary photochemical events and any closely coupled "dark" reactions. Ideally the significance of observable reactions should be established by showing that these reactions are part of a sequence leading to complete photosynthesis. In most cases it has not been possible to apply this important test, and several less decisive criteria have been accepted by default. A primary photochemical reaction for photosynthesis should occur reversibly, in vivo, with high quantum efficiency. It should occur (not necessarily reversibly) under conditions, such as low temperature, that eliminate secondary enzymatic reactions. As for the dark reactions that follow the primary event, these should lead rationally into proven metabolic pathways. The light-induced formation of $NADPH_2$ (13), of oxidized Cyt (14), and of ATP (15) fall into this category.

A PHOTOCHEMICAL REACTION CENTER IN GREEN PLANTS AND ALGAE

In System I of green plants and algae the central position in a reaction center appears to be occupied by P700, a pigment having an absorption band at 700 to 705 mμ. The characterization of this pigment has been pursued principally in Kok's laboratory (6,16,17,18). P700 in chloroplasts and algae is bleached reversibly by light absorbed by Chl *a*. In this reaction fewer than five quanta suffice to bleach one P700 molecule (17). The bleaching results from oxidation of the pigment; chemical titration indicates that P700 is a one-electron transferring agent of oxidation potential 430 mv (16). In chloroplasts the

ratio of P700 to Chl *a* is about 1:400, in harmony with the size of a photosynthetic unit as determined by flashing light experiments. There is one molecule of Cyt *f* present for each molecule of P700 (approximately) in chloroplasts (16). The Cyt *f* (potential 365 mv) is also oxidized reversibly by illumination (19). Partially purified preparations of P700, obtained by extracting chloroplasts with acetone, retain the Cyt *f* in a 1:1 ratio to P700. In these preparations the light reaction of P700 persists, but that of Cyt *f* is absent (16). The reversible bleaching at 700 mμ, in partially purified P700 preparations, is accompanied by a bleaching at 432 mμ. This has led Kok (16) and others (7,20) to suggest that P700 is a chlorophyll-like pigment, perhaps Chl *a* in a specialized environment.

The light-induced oxidations of P700 and Cyt occur irreversibly at liquid nitrogen temperature (7). At room temperature a brief flash of light elicits a transitory oxidation of P700, the re-reduction requiring about 1 msec. Witt et al. (7) report that under these conditions the appearance of oxidized Cyt parallels the disappearance of oxidized

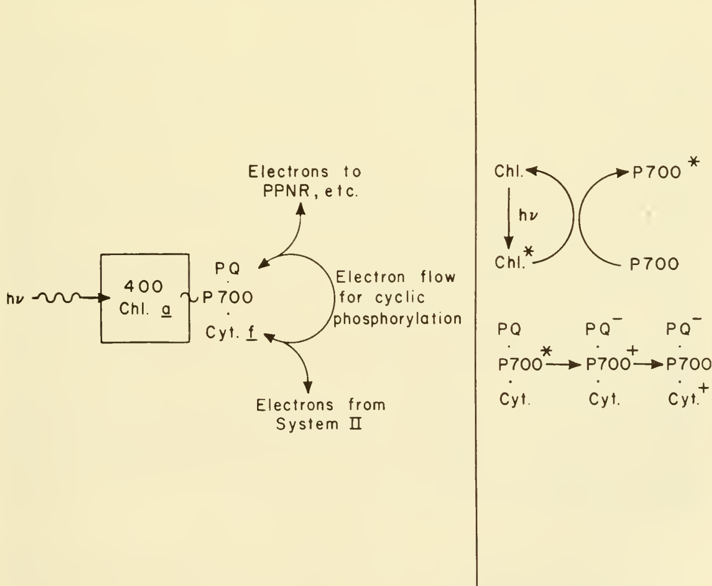

Fig. 1. Hypothetical mechanism for the operation of the far-red photochemical system (System I) in green plants and algae.

P700. Apparently the P700 is first oxidized and then receives electrons from a cytochrome, probably Cyt *f*.

The electron acceptor in the oxidation of P700 might be PQ. Klingenberg et al. (21) have described a light-induced bleaching in chloroplasts that is centered at 255 mμ (corresponding to the loss of oxidized PQ) and that has roughly the same kinetics as the oxidation of P700. However, the identification of PQ as a primary electron acceptor leads to a difficulty concerning its oxidation potential. The potential of PQ in vitro is about zero mv, but electrons from System I are able to reduce such strong reductants as PPNR and NADP. One way to deal with this problem is to assume that PQ operates in vivo at a potential of about -400 mv (23).

With reservations as to the role of PQ as primary electron acceptor, the foregoing evidence suggests the model shown in Fig. 1, for System I (the far red photochemical system) in chloroplasts.

LIGHT-INDUCED ABSORPTION SPECTRUM CHANGES IN PHOTOSYNTHETIC BACTERIA

Cells and chromatophores[3] of photosynthetic bacteria exhibit a variety of light-induced absorption spectrum changes (24-32). Many of these changes suggest reactions of known molecular types, as indicated in Table 1.[4] For convenience the various light-induced absorbancy changes will be referred to as "the P870 reaction," "the Cyt reaction," etc., even though these designations may be presumptive (as with the UQ reaction) or incomplete (e.g., where "Cyt reaction" refers to the oxidation of more than one type of cytochrome).

P870-890 is a pigment having an absorption band coincident with the long wave absorption maximum of BChl in vivo (27,33). This maximum is at 870 mμ in *Rhodopseudomonas spheroides* and at about 890 mμ in *Rhodospirillum rubrum* and *Chromatium*. As we shall see, P870-890 is probably BChl in a specialized environment. Its reversible photobleaching is a result of its oxidation (27); titration with ferricyanide establishes this pigment to be a single-electron donor of potential about 500 mv (25). The restoration of P870-890 after its light-induced bleaching is accelerated by a reducing environment (R. K. Clayton, unpublished). These properties indicate that P870-890 is the bacterial counterpart of P700.

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[3] The term "chromatophores" is used to denote a chromatophore fraction obtained by sonic disruption of cells followed by centrifugation for 90 min at 100,000 x g.

[4] Difference spectra for these light reactions can be found in references 27, 29, and 30.

TABLE 1.

Reactions suggested by light-induced absorption spectrum changes in photosynthetic bacteria.

Type	Substance	△ O. D.	Reaction
A	P870-P890*	Bleaching centered at the long-wave absorption band of BChl (870-890 mμ)	oxidation
B	one or more cytochromes	Blue-shift of soret band, bleaching of α and β bands.	oxidation
C	ubiquinone	Bleaching centered at 270-275 mμ.	reduction
D	carotenoids	Bleaching and red-shift of absorption bands.	
E	bacteriochlorophyll? P870-P890?	Bleaching at 605 and 380 mμ. Blue-shift of bands at 800, 590, and 375 mμ.	
F	oxidized P870-P890?	Bands appear at 435, 720, and 1000-1250 mμ.	oxidation

* P870 in *Rhodopseudomonas spheroides;* P890 in *Rhodospirillum rubrum* and *Chromatium* (see text).

The light reactions listed in Table 1 as Types E and F accompany the P870-890 reaction, in fixed proportions and with the same kinetics[5] (26,27). It is not clear to what extent these reactions are additional manifestations of the P870-890 reaction. The Type F reaction is produced by chemical oxidation as well as by light, in the same way as the bleaching at 870-890 mμ (27). Of the events listed as Type E, only the blue-shift at 800 mμ has been shown clearly to be duplicated by chemical oxidation. Moreover, this blue-shift can be produced chemically in varying proportion to the bleaching at 870-890 mμ, depending on the oxidation potential (25). The reactions of Types E and F are interpreted provisionally as follows. The bleaching at 380, 605, and 870-890 mμ reflects the bleaching of P870 or P890, this pigment being a specialized component of BChl in the chromatophore. The bands appearing at 435, 720, and 1000 to 1250 mμ (Type F) belong to oxidized P870 or P890. It should be noted that each of these bands represents an energy level about 0.4 electron volts below that of a BChl band.

[5] There is some confusion about the broad light-induced absorption band at 435 mμ; more than one substance may be involved (31,34). In washed or dried chromatophores this effect represents a single reaction showing the same kinetics as the P870-890 reaction (26,27).

Also, the intensities of the light-induced 435, 720, and 1250 mμ bands are in the same proportion as the heights of the BChl bands at 375, 590, and 870-890 mμ (27). No explanation is offered for the blue-shifts at 375, 590, and 800 mμ.

The carotenoid reaction cannot be essential for photosynthesis, because carotenoidless forms of purple bacteria exhibit normal photosynthetic metabolism and growth. The light-induced bleaching and red-shift of carotenoids might result from interactions with BChl in an excited triplet state, or from the diffusion of electrons or holes (electron vacancies) to the neighborhood of carotenoid molecules (29). The carotenoid reaction interferes with the observation of other light reactions; it can be eliminated selectively through the use of carotenoidless phenotypes or, in chromatophores, by treatment with deoxycholate (29).

The UQ reaction is identified as such on the basis of light-induced absorbancy changes in the region 240-340 mμ (30). These changes have the same form as the difference spectrum (reduced minus oxidized) of UQ. In chromatophores these absorbancy changes can be produced chemically by reductants, and not by oxidants.

A number of cytochromes are oxidized reversibly by light in cells of photosynthetic bacteria; perhaps four in *Chromatium* (32). In washed chromatophores the reaction of a single cytochrome appears to predominate in each species, and it becomes permissible to speak of "the Cyt reaction." The possibility of observing a Cyt reaction in chromatophores depends on the speed with which the Cyt is reduced in darkness, following its oxidation by light (29). If the reduction is extremely slow (as in dried chromatophores), the light-induced oxidation will be observed only in thoroughly dark-adapted preparations. The rate of reduction is influenced by the manner in which the chromatophores have been prepared, by the oxidation potential of their environment, and by prior illumination (light inactivates endogenous reducing systems reversibly). These factors are useful because they permit study of the P870-890 and UQ reactions with and without a concomitant Cyt reaction.

The maximum light reactions of Cyt, UQ, and P870 or P890, in washed chromatophores, correspond to the presence of one light-reacting molecule of each kind for every 30 to 50 BChl molecules (27-30,34). These figures are in satisfying agreement with the size of the bacterial photosynthetic unit as determined by flashing light experiments (see earlier).

The quantum requirement for the UQ reaction has not been measured. For the reactions of P870-890 and Cyt the requirement is less than three quanta (absorbed by BChl) per electron-transfer (28,33,35, 36).

KINETICS OF THE PRINCIPAL LIGHT REACTIONS IN BACTERIAL CHROMATOPHORES

For simplicity let us consider first the behavior of chromatophores in which the light-reacting cytochromes remain fully oxidized, so that the Cyt reaction is absent. This situation prevails in washed chromatophores under frequent illumination (29). In such preparations the P870-890 reaction, the UQ reaction, and the reactions of Types E and F (Table 1) occur reversibly with identical kinetics and in fixed proportions. These reversible events persist in dried chromatophores at 77°K. The P870-890 and Type E and F reactions occur reversibly at 1°K; the UQ reaction was not examined at this temperature. Recovery in the dark for all of these reactions requires several seconds at room temperature and about 20 msec at temperatures below 150°K (26). In intact cells these reactions are conspicuous when, through the use of inhibitors or aeration, the cytochrome components are kept in an oxidized state (27,37). These light reactions are essentially the same in *Rhodopseudomonas*, *Rhodospirillum*, and *Chromatium* (27). Their persistence under adverse conditions, with no temperature coefficient and with high quantum efficiency (at least for the P870-890 and Cyt reactions (28,36)), characterizes them as primary photochemical reactions. As a specific working hypothesis, the first observable photochemical act is held to be the transfer of an electron from P870 or P890 to UQ. This is the counterpart of a reaction involving P700 and (provisionally) PQ in chloroplasts.

A complete parallelism between the bacterial and green plant systems would require that the Cyt reaction in chromatophores is the transfer of an electron from a cytochrome to oxidized P870 or P890. There are circumstantial reasons for believing that this is the case. First, the Cyt reaction is closely coupled to a primary photochemical act. The light-induced oxidation of Cyt occurs reversibly (recovery requiring about 2 hours) in dried *Chromatium* chromatophores (29) and persists, although irreversibly, in *Chromatium* cells at 77°K (35). Under conditions in which the Cyt reaction is absent the P870-890 and UQ reactions still occur reversibly. A comparable situation is found in Kok's partially purified preparations of P700, where the light-induced bleaching of P700 occurs but the oxidation of Cyt *f* has been lost (16). These facts are consistent with the view that the oxidation of Cyt is an immediate sequel to a primary light-induced oxidation of P870 or P890. Other possibilities cannot be ruled out; among these is a primary reaction in which Cyt is oxidized and P870 or P890 is reduced (B. Chance, verbal communication). In that case the oxidation of P870-890 would be an alternative reaction that occurs only when the Cyt is oxidized (Fig. 2). Nothing that suggests the formation of reduced P870-890 (or BChl) has yet been observed in photosynthetic bacteria.

SCHEME A

$$\begin{array}{ccccccc} UQ & & UQ & & UQ^- & & UQ^- \\ \cdot & & \cdot & & \cdot & & \cdot \\ P870 & \xrightarrow{h\nu} & P870^* & \longrightarrow & P870^+ & \longrightarrow & P870 \\ \cdot & & \cdot & & \cdot & & \cdot \\ Cyt. & & Cyt. & & Cyt. & & Cyt.^+ \end{array}$$

SCHEME B

$$\begin{array}{ccccccc} UQ & & UQ & & UQ & & UQ^- \\ \cdot & & \cdot & & \cdot & & \cdot \\ P870 & \xrightarrow{h\nu} & P870^* & \longrightarrow & P870^- & \longrightarrow & P870 \\ \cdot & & \cdot & & \cdot & & \cdot \\ Cyt. & & Cyt. & & Cyt.^+ & & Cyt.^+ \end{array}$$

BOTH SCHEMES, IF CYT OXIDIZED
IN DARK STEADY STATE

$$\begin{array}{ccccc} UQ & & UQ & & UQ^- \\ \cdot & & \cdot & & \cdot \\ P870 & \xrightarrow{h\nu} & P870^* & \longrightarrow & P870^+ \\ \cdot & & \cdot & & \cdot \\ Cyt.^+ & & Cyt.^+ & & Cyt.^+ \end{array}$$

Fig. 2. Alternative schemes for the initial photochemical reaction and subsequent events in bacterial chromatophores.

An elucidation of the correct sequence of primary photochemical events should result from kinetic observations. Thus far the kinetic relationships between the reactions of P870-890 and Cyt have been made only with instruments that respond too slowly to measure rapid transient phenomena. What has been observed is that the P870-890 reaction, as well as the reactions of Types E and F, are maximal when the Cyt reaction is absent and become truncated when the light-induced oxidation of Cyt can occur (29). This observation is not sufficient to establish the reaction sequence. If oxidation of Cyt is a sequel to oxidation of P870-890, a brief flash of light should elicit an immediate oxidation of P870 or P890, and the appearance of oxidized Cyt should parallel the return of P870 or P890 to its reduced form. This important test, as applied by Witt et al. (7) for the reactions of P700 and Cyt *f*, has not been attempted with bacterial photosynthetic tissues.

A PHOTOCHEMICAL REACTION CENTER IN PHOTOSYNTHETIC BACTERIA

For the present, a working hypothesis will be entertained that the reaction center in photosynthetic bacteria parallels that of System I in

green plants as described in Fig. 1. For the bacterial system the following substitutions should be made:

400 Chl *a*	→	40 BChl
P700	→	P870 or P890
PQ	→	UQ
Cyt *f*	→	a bacterial Cyt
e^- from System II	→	e^- from substrate

If the photochemical reaction centers exist as organized entities it should be possible to separate them from the light-harvesting Chl or BChl and study them in isolation. Some progress has been made in this direction.

In chloroplasts Kok achieved a sevenfold enrichment of P700 relative to Chl *a*, through extraction with acetone (16). The P700 reaction remained intact in acetone-extracted chloroplasts, and Cyt *f* had not been removed. The light-induced oxidation of Cyt *f* no longer occurred.

In purple bacteria the P870-890 reaction appears as a slight bleaching of the long-wave absorption band of BChl. Until recently it could not be decided whether this reaction signals a slight change in most of the BChl molecules or the gross alteration of a few molecules. It has now been established that a distinct minor component (P870 or P890) is bleached completely. This conclusion was reached through a combination of techniques that eliminate the light-harvesting BChl without attenuating the light reactions of Table 1.[6]

In chromatophores of blue-green (carotenoidless) mutant *R. spheroides* suspended in water, the BChl is destroyed in the presence of light and oxygen. The light reaction of P870 is attenuated in proportion to the loss of total BChl. Addition of 1% Triton X-100 (a nonionic detergent) accelerates the photooxidative destruction of BChl, but not of the P870 reaction. In the presence of the detergent, 95% of the BChl can be destroyed without appreciable loss of the P870 reaction. Absorption spectra of blue-green mutant *R. spheroides* chromatophores, before and after photooxidation in the presence of Triton X-100, are shown in Fig. 3. In Fig. 3b ("after treatment") the bands at 535 and 760 mμ are due to the presence of a trace of bacteriopheophytin (BPh). Of the BChl remaining after photooxidation, a component absorbing at 800 mμ predominates. The dashed curves in Fig. 3 show absorption spectra of the same chromatophores under illumination and thus show the photobleaching of P870. In the BChl-depleted chromatophores (Fig. 3b), the 870 mμ band undergoes a 60% reversible bleaching. The ratio of P870 to BChl has been increased about twentyfold.

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[6] The ensuing material is reported in detail elsewhere (38).

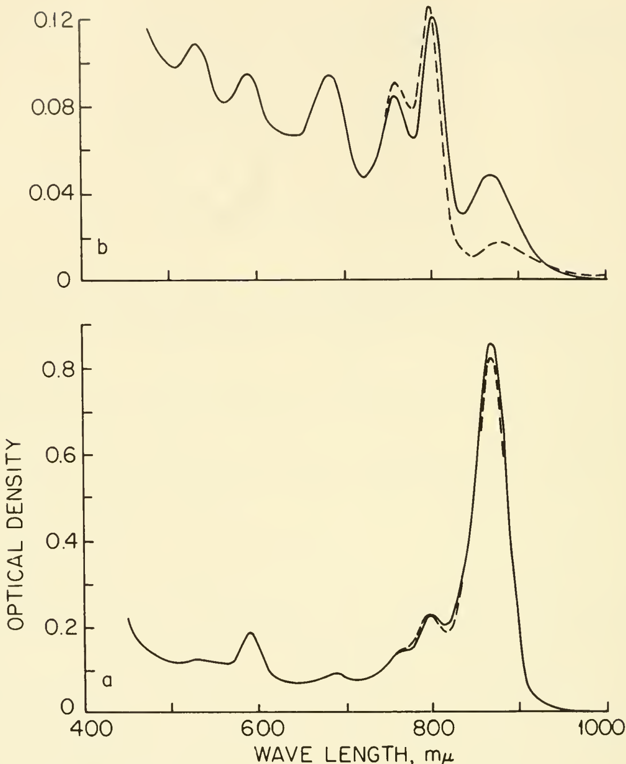

Fig. 3. Absorption spectra of chromatophores from blue-green mutant *Rhodopseudomonas spheroides*: (a) before treatment, (b) after photo-oxidation in the presence of 1% Triton X-100. The difference between the dashed curve and the solid curve shows the reversible light-induced absorbancy change.

When cells of blue-green mutant *R. spheroides* are incubated anaerobically in the light, their BChl is gradually converted in situ to BPh. This conversion is nearly complete after two weeks of incubation. Most of the BPh is retained in the chromatophore fraction in an aggregated state, while some (also aggregated) appears in the culture medium. This aggregated BPh has absorption maxima at 365, 535, and

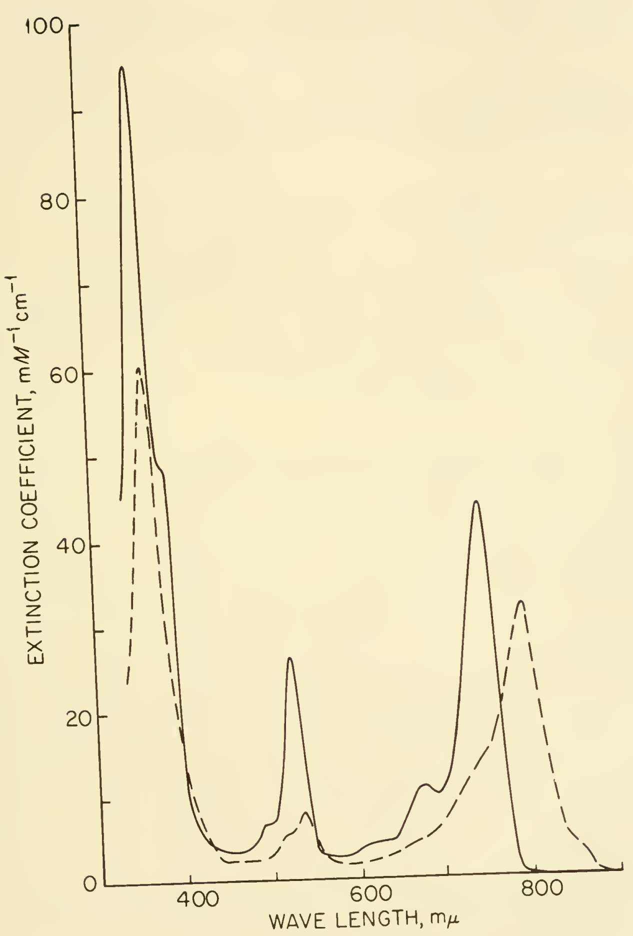

Fig. 4. Absorption spectra of bacteriopheophytin from blue-green mutant *R. spheroides*. Dashed curve, aggregated pigment recovered from culture supernatant. Solid curve, the same pigment dissolved in acetone-methanol.

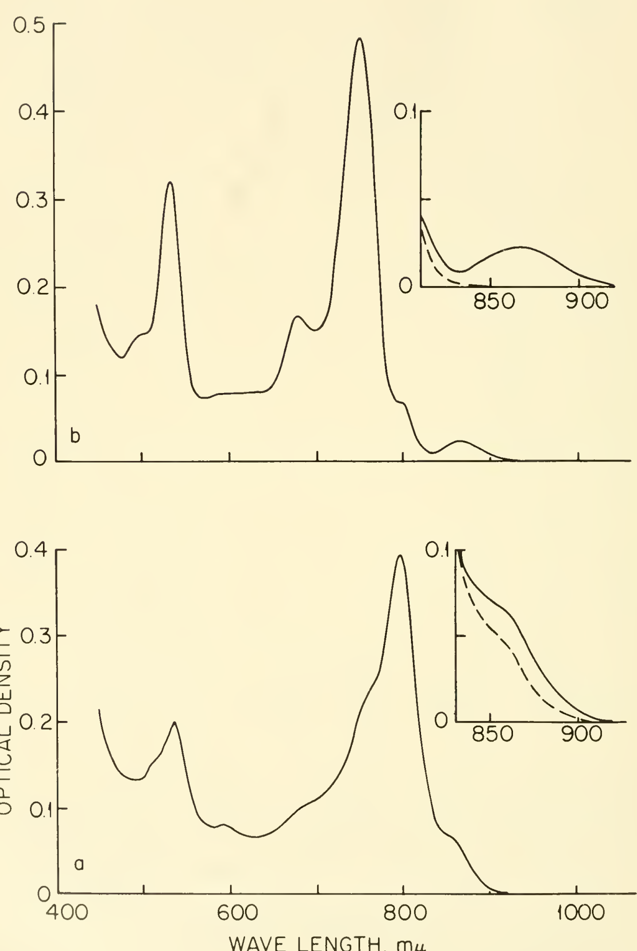

Fig. 5. Same as Fig. 3, except that the chromatophores were obtained from pheophytinized cells (see text).

800 mμ, with shoulders at 760 and 860 mμ (Fig. 4). Chromatophores from such "pheophytinized" cells contain very little BChl; their absorption spectrum (Fig. 5a) is predominantly that of the aggregated BPh.

Throughout the conversion of BChl to BPh, the light reactions of P870, UQ, Cyt, and Types E and F (Table 1) remain unimpaired. In pheophytinized material the light-induced bleaching at 870 mμ is a large fraction of the residual absorption at this wave length (inset, Fig. 5a).

When chromatophores from pheophytinized cells are treated with Triton X-100 the remaining trace of light-harveting BChl is destroyed. Also, the absorption spectrum is altered in a way that suggests dispersion of the aggregated BPh. The BPh bands at 860 and 800 mμ are shifted to 750 mμ, and the band at 535 mμ is intensified. In these chromatophores P870 has become isolated as a spectrophotometric entity (inset, Fig. 5b). The absorption band remaining at 870 mμ is bleached totally and reversibly by light. The P870, UQ, Cyt, Type E, and Type F reactions all survive the combination of pheophytinization and detergent treatment.

These experiments establish P870 as a distinct entity in *R. spheroides* chromatophores, and there is some evidence that P870 is simply BChl in a specialized environment. In extensively pheophytinized cells the amount of residual light-harvesting BChl is one-half to one-tenth the amount of P870. Extraction and chromatographic isolation of the pigments from such cells yield a pigment that appears to be BChl, having absorption bands at 375, 600, and 770 mμ in acetone-methanol. The amount of this pigment corresponds to the sum of the P870 and the smaller quantity of light-harvesting BChl.[7]

In *R. rubrum* the existence of P890 as a distinct substance has been inferred from the kinetics of BChl fluorescence. Vredenberg and Duysens (33) showed that the yield of BChl fluorescence increases during illumination in a way that parallels the bleaching of P890. It was assumed that P890 is nonfluorescent and that the transfer of energy from BChl to P890 competes effectively with the emission of fluorescence by BChl. When the P890 becomes bleached it can no longer accept excitation energy from BChl; the fluorescence emitted by BChl then increases. The expectations drawn from this model were corroborated quantitatively in Vredenberg and Duysens' experiments.

Experiments with *R. spheroides* chromatophores indicate that P870 (and, by extension, P890 of *R. rubrum*) is indeed nonfluorescent. In pheophytinized material the predominant fluorescence band, at 770 mμ, is due to BPh. A much smaller band at 890 mμ can be ascribed to the residual light-harvesting BChl or to P870. The yield of 770 mμ

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[7] In estimating the amounts of P870 and BChl in vivo it was assumed that the extinction coefficients of these pigments are equal at 870 mμ.

fluorescence increases during illumination in a way that parallels the bleaching of P870; energy transfer from BPh to P870 is thus indicated. In contrast, the yield of 900 mμ fluorescence is perfectly constant during illumination. If this fluorescence comes from P870, it should decline as the P870 becomes bleached. A reasonable interpretation is that, in this material, small amounts of P870 and BChl are isolated from each other in an ocean of BPh. The fluorescent BChl can receive excitation energy directly, or from BPh, but cannot transfer it to the nonfluorescent P870.

Excitation spectra have been measured for the fluorescence of BPh and BChl and for the light reactions of P870 and Cyt. For these experiments, cells and chromatophores were used in which about half of the BChl had been converted to BPh. For fluorescence and for the bleaching of P870, excitation spectra covered the region 450-650 mμ and thus encompassed the 535 mμ absorption band of BPh and the 590 mμ band of BChl. The excitation spectrum for Cyt oxidation, measured from 700 to 950 mμ, embraced the 760 and 800 mμ bands of BPh and the 870 mμ band of BChl. Comparison of these excitation spectra with absorption spectra showed that (a) no energy was transferred from BChl to BPh, (b) energy was transferred from BPh to BChl with approximately 25% efficiency, and (c) energy transfer from BPh to P870 proceeded with an efficiency of about 50%. These results suggest that energy can be transferred from BPh directly to P870, as well as by way of BChl.

In summary, P870 (or P890) is a distinct component of the chromatophore that functions as part of a photochemical reaction center. This reaction center is held provisionally to be the "bacterial" counterpart of the green plant reaction center as outlined in Fig. 1. P870 is probably BChl in a specialized environment that renders it nonfluorescent.

The occurrence of the reversible light reactions of P870, UQ, and Cyt, in chromatophores that contain almost no light-harvesting BChl, raises a question as to the obligatory role of chlorophyll in photosynthesis. Suppose that *R. spheroides* cells can be pheophytinized to the extent that *no* light-harvesting BChl (other than P870 itself) remains. If such cells can be made to perform complete photosynthesis it will follow that the requirement for BChl is restricted to its functioning in the capacity of P870.

MECHANISMS IN THE INITIATION OF PRIMARY PHOTOCHEMICAL REACTIONS

The functioning of a photosynthetic unit involves a distinct sequence of events. Energy, once absorbed by the light-harvesting system, must find its way to a reaction center. There it must be detained and utilized in a photochemical process.

The transfer of energy to a reaction center could occur by migration of electric charge or of excitation energy quanta. The first of these possibilities corresponds to the view that the photosynthetic unit functions as a semiconducting organic crystal, with light absorption leading directly to local photoionization. There is abundant evidence (26, 39-42) that photosynthetic tissues do behave like organic semiconductors. They exhibit such properties as semi-conductivity, photoconductivity, light-induced electric polarizability, thermoluminescence, and delayed luminescence. These effects are indicative of photoionization accompanied by the trapping and untrapping of electrons. The quantum efficiencies associated with these phenomena are extremely low or have not been measured, whereas the transfer of excitation energy in photosynthetic tissues (e.g., from accessory pigments to Chl) occurs with efficiencies approaching 100% (24). Accordingly, it is generally accepted that energy transfer in photosynthetic units proceeds through the migration of energy quanta, and not of charge.

Assuming that energy migrates in photosynthetic units as singlet excitation quanta, one may ask whether the migration occurs by a slow ("resonance transfer") or a fast ("delocalized exciton") transfer mechanism.[8] "Slow" and "fast" correspond roughly to $<10^{12}$ and $>10^{13}$ transfers per sec, respectively. Slow transfer should be temperature-dependent to the extent that temperature governs the amount of overlap between the absorption and fluorescence bands of the participating molecules. Fast transfer should be independent of temperature.

Slow transfer is favored, in chloroplasts and algae, by theoretical criteria involving the absorption spectra of Chl *a* in vivo and in vitro (45). However, a slow transfer mechanism should barely allow a photosynthetic unit of 400 Chl molecules to function. During its lifetime of 1.6×10^{-9} sec (46), a quantum of singlet excitation in Chl *a* will visit at most about 1600 molecules, in a "random walk," by the slow transfer mechanism. This difficulty suggests that the theoretical criteria ought to be relaxed to allow a faster rate of transfer.

In purple bacteria the photosynthetic unit is smaller and the foregoing restrictions do not become serious. Furthermore, there is evidence that a fast transfer mechanism prevails in chromatophores. The efficiency of BChl-absorbed light in mediating the bleaching of P870 is nearly as great at 1°K as at 300°K (28). Thus the efficiency of energy transfer in the BChl system is virtually independent of temperature; this result is probably incompatible with slow or resonance transfer. It remains to be seen whether these extremes of temperature have a significant effect on the amount of overlap between the absorption and fluorescence bands of BChl in vivo.

A quantum of energy migrating in a photosynthetic unit will not be available to a photochemical reaction center unless the center can

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[8] Discussions of these mechanisms can be found in references 43 and 44.

trap the quantum. It is easy to see how this trapping could occur in green plants and algae. P700, absorbing at a slightly longer wave length than Chl *a*, should act as an energy sink through the resonance transfer mechanism (6). Delocalized singlet energy in Chl *a* can thus be trapped as localized singlet energy in P700. In chromatophores the absorption maximum of P870 or P890 coincides with the long-wave band of BChl. P870 or P890 cannot be expected to act as an energy sink relative to BChl, and other mechanisms should be entertained for the trapping process. A singlet excited state, upon reaching P870 or P890, will be detained there if it is converted efficiently into a localized state of any kind. If the localized state is long lived, its utilization for photochemistry will be facilitated. A localized, long-lived state might be a triplet state, or a highly polarized state such as an $n\pi^*$ singlet, or a bimolecular charge-transfer state, or any sort of ionized state in which electrons or holes are trapped. Entry into a "forbidden" state such as a triplet is facilitated by factors producing asymmetry (47); a reaction center is automatically a point of asymmetry in a photosynthetic unit. Highly polarized states, including charge-transfer states and other ionized states, might be favored in P870-890 by the proximity of electron-acceptor or donor molecules such as UQ or Cyt.

These mechanisms for the trapping of excitation energy and the initiation of photochemistry in photosynthetic tissues are entirely speculative. Nevertheless it is gratifying that concrete mechanisms drawn from defined chemical models can be envisioned and, hopefully, tested.

CONCLUSION

Evidence has been presented that certain photochemical reaction centers exist in photosynthetic tissues. Light energy absorbed in a major Chl or BChl component is transferred to the reaction centers and mediates the reactions of specific molecules with high quantum efficiency. Among these specific molecules are cytochromes, P700 or P870-890, and perhaps also PQ or UQ. Lacking evidence to the contrary it is reasonable to conclude that these reactions reflect primary photochemical acts of photosynthesis.[9] This conclusion cannot be taken as proved until it has been shown that these reactions initiate the main stream of electron flow for the chemistry of photosynthesis.

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[9] The primary reactions of Photochemical System II (mediating oxygen evolution) in green plants and algae are a separate and poorly understood consideration.

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Note added in proof. Recent calculations by Z. Bay and R. M. Pearlstein (verbal communication) show that excitation energy transfer by the "slow" mechanism can account easily for the operation of a photosynthetic unit in chloroplasts.

PHOTOCHEMISTRY OF BACTERIOCHLOROPHYLL

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In the photosynthetic purple bacteria bacteriochlorophyll is the primary agent which transforms the incident electromagnetic radiation energy of about 1.4 ev (corresponding to a wavelength of about 880 mμ) into sufficient electron energy to perform various chemical reactions. We therefore will look at this pigment as a possible source of "high energy" electrons.

Bacteriochlorophyll (and in green plants chlorophyll *a*) may act in this respect in two fundamentally different ways:

1. According to the individual model.
2. According to the collective model.

In the first model a chlorophyll molecule donates, as a result of illumination, high energy electrons by its own chemical properties. At high pigment concentration, in this model radiation energy is transferred by the mechanism of inductive resonance.

In the second model the chlorophyll molecules are situated in a crystalline array. Only the properties of the "crystalline" configuration play a role in production of high energy electrons. The photochemical properties of the individual pigment molecules are of no importance. Energy is transferred by electron (or hole) migration within the crystal. The second model has received much attention in later years. It has the advantage of providing a mechanism for an effective electron-hole separation over a distance large as compared to the molecular dimensions. However, a separation between a) the high energy electrons expelled from chlorophyll molecules as a result of the absorption act and b) low energy electrons filling the empty space in the electron configuration of the pigment molecules may also be arrived at by the individual model.

Therefore, we will consider some photochemical and chemical properties of bacteriochlorophyll, to investigate whether this pigment indeed is able to act as a redox system and in this way provide electrons of sufficient energy content to perform photosynthesis.

REVERSIBLE CHEMICAL OXIDATION

Alcoholic bacteriochlorophyll solutions are "bleached" by addition of ferric chloride or various other oxidising agents. "Bleaching," however, refers only to the long wavelength absorption band (Fig. 1). The original spectrum is immediately restored by more than 90 per cent after addition of ferrous ions in excess, ascorbic acid or other reductants (*Goedheer,* 1). A high reversibility only occurs when a low concentration of oxidant is used ($\sim 10^{-5}$M) and the addition of reductant occurs within a few minutes after addition of the oxidant.

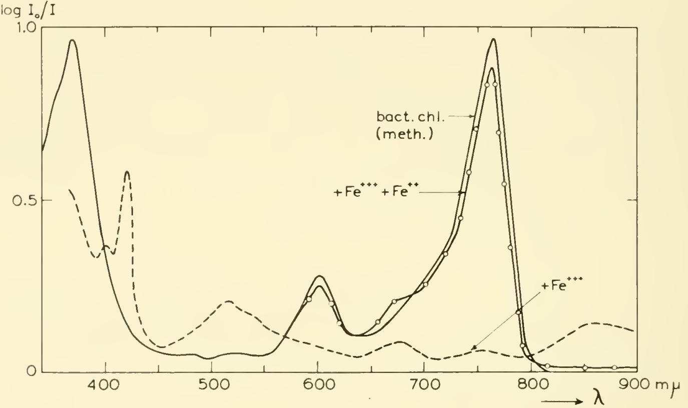

Fig. 1. Reversible chemical bleaching of bacteriochlorophyll in methanol or acetone. ——— before bleaching, - - - - after addition of Fe^{+++}, -·-·-·- after addition of Fe^{++} in excess, ascorbic acid or dithionite. The irreversible increase in absorption around 680 mμ is due to the formation of a green oxidised compound (chlorophyll *a* type of spectrum). Below 400 mμ the absorption spectrum of the bleached compound is uncertain due to high absorption of Fe^{+++}.

This reversible bleaching was interpreted—as was done by *Rabinowitch and Weiss* (2) for chlorophyll *a*—as a reversible oxidation:

$$BChl + Fe^{+++} \rightleftharpoons BChl^{+} + Fe^{++}$$

It was possible to detect the ferrous ions formed in this reaction by addition of potassium ferricyanide, giving rise to the formation of "prussian blue." No ferrous ions were detected when bacteriochlorophyll was bleached by light or when ferric chloride was added to bacteriopheophytin. In contrast to the behaviour of the red absorption band

of chlorophyll *a*, no slow restoration of the long wavelength band (770 mμ) occurs with "oxy"bacteriochlorophyll during prolonged storage in the dark. Instead, a different change in the absorption spectrum occurs (Fig. 2). The rate of change of the spectrum of the "oxy"form as given in Fig. 1 into the one as given in Fig. 2 depends upon the concentration of the oxidant (in about 30 min. with 10^{-5}M, within two minutes with 10^{-4}M). After the occurrence of this change the reversibility caused by addition of ferrous ions is lost for the greater part.

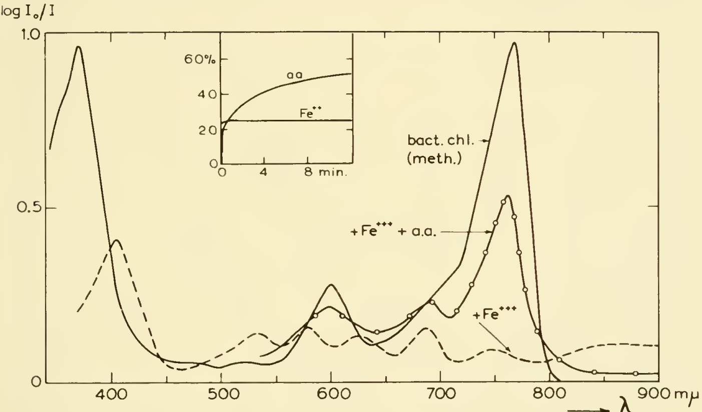

Fig. 2. Reversible chemical bleaching of bacteriochlorophyll in methanol after prolonged storage in the dark (25 minutes). The spectrum of the "bleached" compound is formed from the spectrum given in Fig. 1. The insert shows that addition of ferrous ions results in only a weak, immediate, restoration of the bacteriochlorophyll spectrum, while such a recovery is much higher and consists of a slow and an "immediate" reaction upon addition of ascorbic acid.

Also in contrast to the behaviour of chlorophyll *a*, reversible chemical oxidation of bacteriochlorophyll was found not only to occur in methanol, but also in acetone or ether. The difference in behaviour between the two pigments is most probably due to the lower redox potential of bacteriochlorophyll. Measured under the same experimental conditions, the redox value of the bacterial pigments (bacteriochlorophyll and chlorobium chlorophyll) is about 150 mV lower than that of chlorophyll *a* (cf. *Goedheer, de Haas and Schuller*, 3). In agreement with this it was found that also chlorophyll *a* dissolved in acetone can be oxidised with ceric ions (more strongly oxidising than ferric and

other ions used hitherto), while a partial (20 per cent) reversibility of the bleaching could be demonstrated by addition of ascorbic acid or sodium dithionite.

In solutions other than alcoholic ones a change analogous to the one represented by Fig. 1→Fig. 2 was not observed with bacteriochlorophyll. Dilution of a concentrated methanolic bacteriochlorophyll solution with water yields a colloidal nonfluorescent solution. The near-infrared spectrum of such a solution may show a complex structure (cf. e.g., *Komen,* 4) under favourable conditions (crude pigment solution, quick dilution). Addition of oxidants affects the two bands in a different way (Fig. 3).

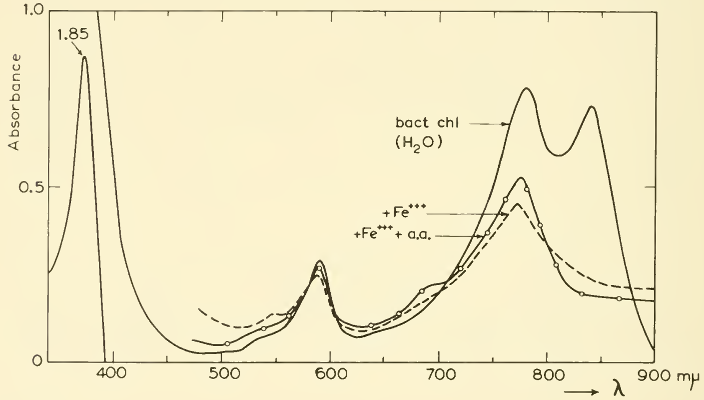

Fig. 3. Absorption spectrum and reversible bleaching of colloidal bacteriochlorophyll, formed by addition of water to a concentrated methanolic pigment solution. The absorption band with maximum around 785 mμ is bleached reversibly to some extent; the band around 845 mμ is very unstable. It also disappeared upon a slight heating or illumination.

Reversible oxidation by addition of oxidising and reducing ions can also be demonstrated with *in vivo* bacteriochlorophyll. In Fig. 4, reversible oxidation of *Rhodospirillum rubrum* chromatophores is shown. The weak absorption band at about 800 mμ is not bleached reversibly, but shows a marked stability against oxidation. Its position, however, is reversibly shifted about 5 mμ towards the blue (cf. *Goedheer,* 5). This band disappears completely at a pH below 4 (cf. *Thomas, Goedheer, Komen,* 6).

A prerequisite for the occurrence of reversible oxidation is the presence of the central magnesium atom. No reversible oxidation could be demonstrated with bacteriopheophytin.

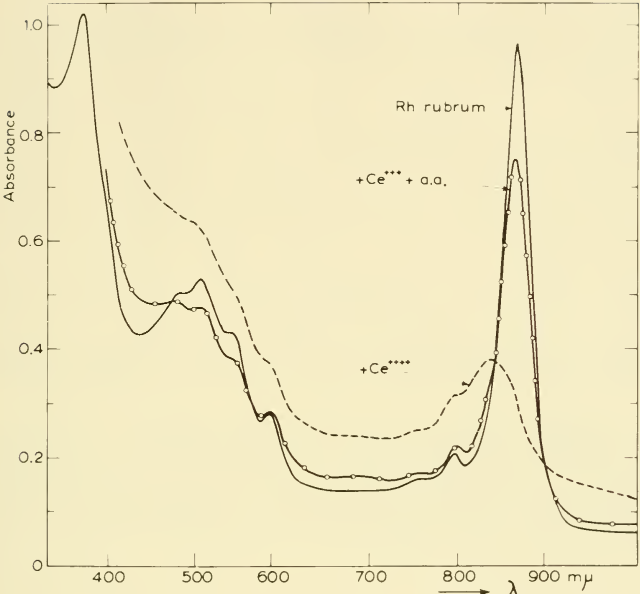

Fig. 4. Reversible chemical bleaching of the long wavelength band of chromatophores of *Rhodospirillum rubrum*. The remnant of the 880 mμ band in the oxidised state is shifted about 20 mμ towards shorter wavelength. The weak 800 mμ band is stable against oxidation. Although it appears that carotenoid bleaching may be partly reversible also, no definite conclusion can be made about this phenomenon.

REVERSIBLE PHOTOOXIDATION

Bacteriochlorophyll dissolved in methanol is very unstable in light. Under uptake of oxygen it "bleaches" within a minute into a compound with an absorption spectrum (Fig. 5) similar to that obtained after bleaching with ferric ions and prolonged standing (Fig. 2). The bacteriochlorophyll spectrum is restored in the dark by addition of ascorbic acid. This restoration is a slow, temperature-dependent reaction, in striking contrast to the immediate restoration of the spectrum immediately after chemical oxidation (Fig. 1). The restoration is not complete; about 10-20 per cent of the bleaching is irre-

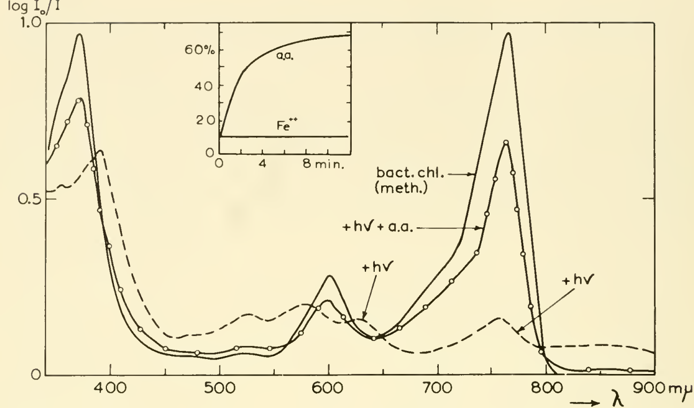

Fig. 5. Reversible photobleaching of bacteriochlorophyll in methanol (air not excluded). As shown in the insert, no reversibility is found after addition of ferrous ions, while addition of ascorbic acid results in a reversibility with a time response as observed with the slow component in chemical bleaching (Fig. 2). A change in temperature affects both curves in the same way.

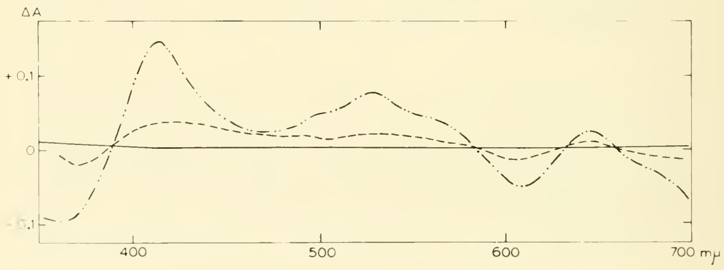

Fig. 6. Difference spectra between bacteriochlorophyll and slightly photo-bleached bacteriochlorophyll dissolved in methanol. -------- before bleaching, -..-..- after bleaching, - - - - - after addition of ascorbic acid and 15 minutes storage in the dark.

versible. A difference spectrum between nonbleached and slightly bleached bacteriochlorophyll is given in Fig. 6.

According to *Krasnovskii and Vonovskaja* (7), who first discovered this reaction, a peroxide test indicates that a peroxide is formed during bleaching.

The similarity of the absorption spectrum of the compound obtained by a) chemical oxidation and storage in air-saturated solution and b) photobleaching in light in air-saturated solution makes us suggest that the same compound is formed in both processes. This suggestion is supported by the following experiment.

Addition of ferrous sulfate to the compound mentioned under a) above results in only a weak immediate restoration of the far red absorption band (Fig. 2, insert), while addition of ascorbic acid—which restores the spectrum both in the chemically oxidised system and in the photochemically oxidised system—results in both an immediate and a slow restoration (Fig. 2, insert). In the latter case a much higher percentage of reversibility is obtained. The time course of the "slow component" obtained by chemical bleaching and the time course of reversibility of photobleaching (Fig. 5, insert) are similar; they both depend on temperature in the same way. These results thus strongly suggest that the secondary product obtained with chemical bleaching and the product obtained with photobleaching are the same.

From this result we would expect that, if oxygen is rigourously excluded, a compound with an absorption spectrum analogous to the one presented in Fig. 1 will occur. Due to the high sensitivity of bacteriochlorophyll to even traces of oxygen this has not yet been confirmed with this pigment. The reversible bleaching of chlorophyll in oxygen-free methanol and other solvents, observed by *Porret and Rabinowitch* (8), *Livingston* (9) and Linschitz and Rennert (10) might be explained in this way.

The sequence of events leading to chemical and photobleaching then may be written as follows:

Chemical bleaching:

$$\text{BChl} + \text{Fe}^{+++} \rightleftharpoons \text{BChl}^{+} + \text{Fe}^{++}$$

$$\text{BChl}^{+} \downarrow \text{spectrum Fig. 1}$$

$$\text{BChl}^{+} + \text{O}_2 \longrightarrow \text{BChlO}_2$$

$$\text{BChlO}_2 \downarrow \text{spectrum Fig. 2}$$

Photobleaching:

$$\text{BChl} \underset{\text{fluorescence}}{\overset{\text{absorption}}{\rightleftharpoons}} \text{BChl}'$$

$$\text{BChl}' \longrightarrow \text{BChl}^{+} + e^{-}$$

$$BChl^{+} + O_2 \longrightarrow BChlO_2$$

$$\downarrow$$

spectrum Fig. 2

The chemical back reaction with ascorbic acid:

$$BChl^{+} + a.a. + H^{+} \longrightarrow BChl' + a.a.^{+} + H_2O$$

$$BChl' \xrightarrow{\text{chemiluminescence}} BChl$$

A chemiluminescence in the spectral region of bacteriochlorophyll fluorescence indeed is measured upon addition of ascorbic acid to photobleached bacteriochlorophyll solutions (Fig. 7). This addition is successful up to 30 minutes after bleaching. If ascorbic acid is added before the onset of illumination, a photooxidation of this compound,

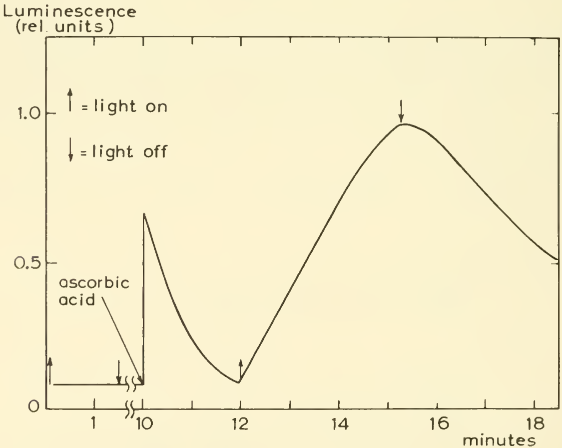

Fig. 7. Chemiluminescence due to addition of ascorbic acid to photobleached bacteriochlorophyll (added 10 minutes after bleaching) and chemiluminescence due to illumination of bacteriochlorophyll-ascorbic acid mixtures.

measured as oxygen uptake during illumination, as well as an emission of chemiluminescence, is observed. Part of the electron energy stored in ascorbic acid thus appears to be emitted as chemiluminescence.

A chemiluminescence also occurs if chemically bleached $BChl^+$ is reduced to chlorophyll by addition of excess ferrous ions or other reductants. Such a chemiluminescence may be thought to be brought about as follows. Removal of an electron from a chlorophyll molecule in its ground state due to the electron attracting force of a ferric ion occurs via the excited states. By a feeding of electrons back into $BChl^+$ from ferrous ions or ascorbic acid there is a certain probability that light emission from the first excited state will occur with the spectral distribution of bacteriochlorophyll fluorescence. The emitted light energy then is assumed to originate from the entropy difference existing between ferric and ferrous ions separated and mixed. The reversible chemical oxidation then might be written as

$$BChl + Fe^{+++} \xrightleftharpoons{\text{chemiluminescence}} BChl' + Fe^{+++} \rightleftharpoons BChl^{+} + Fe^{++}$$

An electron from bacteriochlorophyll may reach its first excited state by light absorption as well. If in this case an electron acceptor is present which does not possess sufficient electron affinity to oxidise bacteriochlorophyll in its ground state (and oxygen is rigourously excluded), bacteriochlorophyll will be oxidised as a result of light absorption and the electron acceptor reduced. Such experiments have not yet been done with bacteriochlorophyll, but results of *Linschitz and Rennert* (10) and *Tollin and Green* (11) with chlorophyll *a*-quinone mixtures at low temperatures yield evidence for the occurrence of such a mechanism in organic solution.

Chemiluminescence and reversible absorption changes due to oxidation of bacteriochlorophyll were not observed with the pheophytins. This indicates that most probably electrons do not leave excited bacteriochlorophyll and chlorophyll *a*—whether excited by light absorption or by the ferric ion—if the Mg ion is absent.

REVERSIBLE PHOTOREDUCTION

Reversible photoreduction, however, can be measured with the chlorophyllous pigments and as well as, or even better, with the pheophytins. *Krasnovskii and Vojnovskaja* (7) determined reversible absorption changes due to illumination of these pigments dissolved in air-free pyridine in the presence of ascorbic acid as an electron donor (cf. also *Krasnovskii*, 12). Such a reduction might be explained by the hypothesis that, as long as the excited electron lingers in some excited state (singlet or triplet), there is a certain probability for an "external" electron to enter the ground state at some site in the molecular configuration. As reversible reduction is independent of the

presence of Mg^{++}, this site should occur somewhere in the conjugated ring structure or its additives.

We thus see that some of the necessary conditions for bacteriochlorophyll to function as photochemically active molecules in photosynthesis, namely, the capacity to act as electron donor and electron acceptor at different sites in the molecule, may be deduced from the experiments with bacteriochlorophyll in organic solvents.

A highly schematic comparison of chemioxidation, photooxidation and photoreduction and their possible function *in vivo* is suggested in Fig. 8. In this figure an electron is assumed to be removed from the ground state of bacteriochlorophyll either by strong oxidants (Fig. 8: 1) or by light absorption (Fig. 8: 2-5). The ground state is filled by "high energy" electrons under emission of luminescence in Fig. 8: 2, 3 and 4.

In the natural state, the electron expelled by chlorophyll is used to reduce some compound of relatively high redox potential (e.g., cyt. *b*, DPN or other compound), while the ground state is filled with an electron from cytochrome *c*. A close connection is assumed to exist between bacteriochlorophyll and the electron donor at one site—*Chance and Nishimura* (13) found cytochrome *c* to be oxidised by bacteriochlorophyll even at liquid nitrogen temperature—and bacteriochlorophyll and the electron acceptor at the other site. If cytochrome *c* is in the oxidised state, it cannot provide electrons to the ground state of bacteriochlorophyll and during illumination a stationary concentration of $BChl^+$ will exist. After illumination, back reactions will restore the spectrum under emission of luminescence.

If reduced cytochrome *c* is present, it will donate electrons to the ground state of bacteriochlorophyll, resulting in a very low stationary concentration of $BChl^+$ during illumination, while back reactions are low in number; hence, afterglow is low. Emission of luminescence of photosynthetic bacteria indeed was found to be manyfold enhanced by the addition of quinone, ferricyanide or hydrogen peroxide, which addition is assumed to result in oxidation of cytochrome *c*.

A continuous conversion of radiation energy into electron energy by oxidation-reduction is unlikely in organic solvents. To obtain such a storage, a solvent should be chosen which facilitates both the leaving of an electron from the excited state at the Mg-site and an entering of the electron from an electron donor of lower energy into the ground state at the site of the ring structure. However, even in such a hypothetical solvent the random movement of molecules in solution results in a reentry by predilection of the electrons of higher energy, and thus in a "short-circuit" of the pigment molecule. This "short-circuit" will be prevented only if the pigment is attached to some structure of highly specialised biological organisation. There are various indications that the bacteriochlorophyll molecules *in vivo* are attached to proteins (cf. e.g., *Bril*, 14). In the bacteria, nearly all

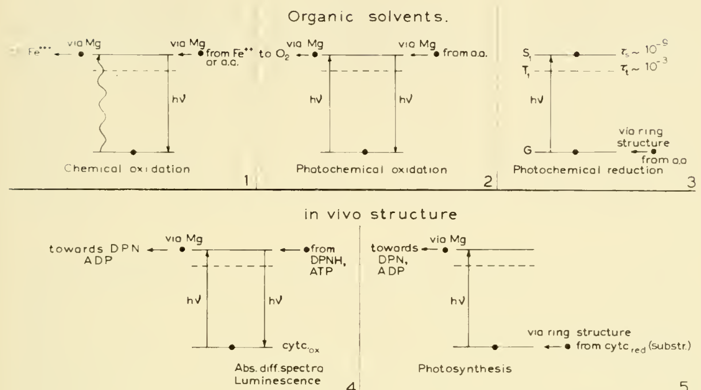

Fig. 8. Hypothetical energy scheme of oxidations and reductions of bacteriochlorophyll *in vitro* and *in vivo*.

1. An electron is removed from the ground state of the pigment by a strongly oxidising agent, leaving oxidised bacteriochlorophyll. The bacteriochlorophyll spectrum is restored by a feeding of electrons into the ground state from a reductant, added in excess. This restoration coincides with emission of chemiluminescence. A similar picture holds for bacteriochlorophyll in chromatophores. As this reaction does not proceed with pheophytin, it is assumed to be mediated by the Mg ion.
2. An electron is removed from the ground state by light absorption. The excited bacteriochlorophyll molecule reacts with oxygen. Addition of ascorbic acid results in oxidation of this compound and reformation of excited bacteriochlorophyll, resulting in luminescence.
3. The electron removed from the ground state by light absorption is assumed not to be able to leave the bacteriochlorophyll molecule in the solvent used (pyridine) in the absence of oxygen. It thus lingers some time in the first excited triplet or singlet state. During this time an electron from ascorbic acid is assumed to be able to enter the ground state, resulting in a reduced pigment molecule. As this reaction occurs also with pheophytin, the site of entry is assumed to be somewhere in the ring structure.
4. An electron excited by light may leave the pigment molecule to an electron acceptor situated close to the molecule, and from there to DPN. Oxidised bacteriochlorophyll is left over if the empty space in the ground state is not immediately filled by a closely adjacent cytochrome molecule, for example, if cyt. *c* is oxidised. The spectrum is restored under emission of luminescence by back reaction from the high energy compound.
5. Under photosynthesising conditions, when cyt. *c* is kept reduced by the substrate, the stationary concentration of $BChl^+$ is very low, coinciding with a very low luminescence.

absorbed light is transferred to the absorption band of longest wavelength (*Duysens*, 15). The absorption band of longest wavelength thus is expected to be reversibly oxidised and reduced upon illumination. In the chromatophores, the energy transfer can be reversibly disturbed, without a marked altering of the absorption spectrum, by affecting the carrier complex configuration with detergents (*Bril*, 16). As mentioned before, reversible chemical bleaching could be measured with bacteriochlorophyll in chromatophores as well as in organic solvents. Reversibility, however, was only prominent for the long wavelength band or shoulder (around 880 mμ) with chromatophores of *Rhodospirillum rubrum*, *Rhodopseudomonas spheroides* and *Chromatium*.

Duysens (15) measured reversible changes in absorption of the long wavelength bacteriochlorophyll bands in photosynthetic bacteria, which he suggested to be due to photooxidation. The changes were only measurable when the cells were aerobic and devoid of substrate. In the presence of substrate the changes occurred only at high light intensities (*Duysens*, 17). Later it was observed (*Duysens*, 18) that these spectral changes occurred only when bacterial cytochrome *c* was in its oxidised state, a situation which is schematically indicated in Fig. 8: 4.

Difference spectra similar to those obtained upon illumination can be obtained by addition of ferri-ferrocyanide mixtures of about 0.5 V to bacterial chromatophores (*Duysens*, 19; *Goedheer*, 20). With stronger oxidising mixtures the reversible bleaching of the long wavelength band exceeds the *in vivo* one (cf. Fig. 4).

The difference spectra obtained upon addition of ferri-ferrocyanide mixtures or illumination appear to consist of two different parts: 1) a reversible bleaching around 880 mμ, similar to the reversible bleaching in organic solvents, and 2) a reversible shift of the small absorption band at 800 mμ.

The reversible absorption difference spectra in the far red region are analogous for *Rhodospirillum rubrum*, *Chromatium* and *Rhodopseudomonas spheroides*, although the absolute absorption spectra are markedly different.

Phenomenon 1) listed above is explained as a light-induced reversible oxidation of bacteriochlorophyll absorbing around 880 mμ. Reversible bleaching in intact bacteria around 880 mμ is saturated when only a few percent of the pigment molecules are in the oxidised state. From fluorescence measurements *Vredenberg and Duysens* (21) concluded that the major fraction of bacteriochlorophyll molecules at 880 mμ in *R. rubrum* transfer their absorbed light energy to a small fraction called P890. From here the energy is assumed to enter the photosynthetic system. *Clayton* (22) comes to a similar conclusion. He states that this fraction (called $BChl_2$) comprises 2-5 per cent of the total BChl.

In crude bacterial chromatophore suspensions a reversible bleaching of a relatively large fraction of the 880 mμ band can be demonstrated in some cases (Fig. 9), a small part of which is irreversible. In the visible part of the spectrum, reversible cytochrome changes may obscure changes due to reversibly bleached bacteriochlorophyll. Re-

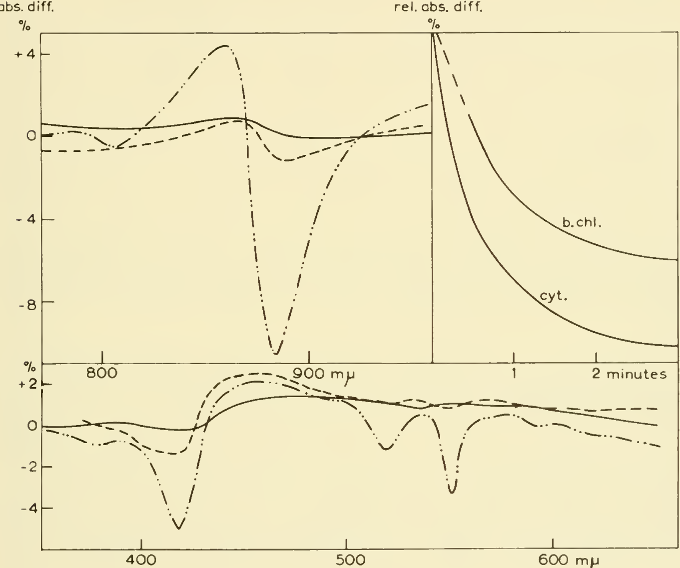

Fig. 9. Difference spectra between illuminated and nonilluminated *R. rubrum* chromatophores, measured with a standard Beckman recording spectrophotometer. The spectra are disturbed due to the fact that the scanning time of the machine is not short as compared to the spectral restoration times. In the visible part of the spectrum a marked reversible cytochrome change is observed, while the long wavelength part shows a reversible decrease at 880 mμ, while a smaller increase around 860 mμ is visible. ------ before experiment, -..-..- after 5 minutes bleaching, - - - - - - after 20 minutes storage in the dark.

versible bleaching at 880 mμ (phenomenon 1) here by far exceeds the reversible shift (phenomenon 2) at 800 mμ. It could be suggested that we here have a reversible bleaching analogous to the one in Fig. 5, time curves of spectral restoration being similar (Fig. 10).

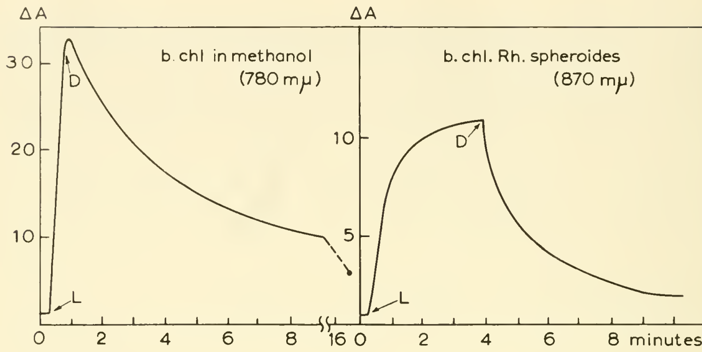

Fig. 10. Restoration curve of photobleaching of bacteriochlorophyll in methanol, with ascorbic acid) and in chromatophores of *Rps. spheroides*. In this sample about 20% of the absorption around 880 mμ was bleached reversibly, Usually, however, the percentage was much smaller but the time curve similar.

Phenomenon 2) is not shown with any of the *in vitro* experiments of bacteriochlorophyll. This, of course, does not mean that a reversible oxidation resulting in an absorption shift of about 5 mμ is not possible *in vitro*. In such a case the reversible shift at 800 mμ might represent a second pigment system in photosynthetic bacteria; one might think of, e.g., a system taking the place of the water-splitting system in green plants (cf. eg., *Goedheer,* 23). However, up till now there are no indications whatsoever that such a second pigment system exists (enhancement effect, luminescence "quenching," action spectra). Using a circulating flow system, we found that the shift at 800 mμ occurs in bacteria also if irradiated at 880 mμ, where the bacteriochlorophyll molecules responsible for the 800 mμ band are assumed to have no absorption at all. The time curves of 800 mμ shift and 880 mμ reversible bleaching were found to be, in first approximation, equal and independent of wavelength.

As, therefore, a close correlation between both phenomena is apparent *in vivo,* whereas *in vitro* no reversible shift is found, we will consider a possible explanation of the reversible 800 mμ shift in a way different from a direct participation in some photochemical act. The difference in position of absorption bands *in vivo* and in diluted organic solvents is due to electronic interaction with neighbouring molecules, e.g., proteins, other pigment molecules or cytochromes. If there is a change in electronic properties of these molecules as a result of illumination, such as oxidation of a bacteriochlorophyll

molecule, the band position of another closely adjacent pigment molecule may be influenced. In this way a reversible shift of the small 800 mμ band in *R. rubrum* may be directly coupled with the oxidation of the reactive part of the long wavelength band (P890). As both the small 800 mμ band and the reactive part of the long wavelength band cover a few per cent of the total number of bacteriochlorophyll molecules, the hypothesis seems plausible that one 800 mμ bacteriochlorophyll molecule is intimately connected with one P890 molecule. As the light-induced absorption difference spectra in the near infrared are similar for various types of purple bacteria, notwithstanding their different absolute absorption spectra, such a combination might be essential for bacterial photosynthesis. It seems tempting to see bacteriochlorophyll 800 mμ (in *R. rubrum*) as an "accessory pigment" to P890, similar e.g. to chlorophyll *b* or phycocyanin. In our scheme the function of such "accessory" pigments then might be seen as an aid in effective separation of electrons leaving and electrons entering bacteriochlorophyll and chlorophyll *a*. To function as such, the accessory pigment should be located close to the main pigment molecule, and therefore show an effective energy transfer, while the redox potential of the accessory pigment should be more negative than that of the main pigment. For the mentioned bacteriochlorophyll 800 mμ type and for chlorophyll *b* these conditions seem to be fulfilled.

It does not seem impossible that more reversible absorption changes (e.g., the reversible shift in position of carotenoid absorption bands, cf. *Clayton*, 24), might be due to change in electronic interaction.

The foregoing may demonstrate that it is possible to devise a mechanism based upon the photochemical properties of the individual pigment molecules. Such a mechanism enables a continuous conversion of radiation energy into electronic energy, without introducing the assumption of solid state phenomena.

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ENERGY TRANSFER AND CYTOCHROME OXIDATION IN GREEN BACTERIA

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The photosynthetic bacteria consist of two main groups: the purple bacteria and the green bacteria. The pathways of energy transfer in purple bacteria have been elucidated mainly through the efforts of Duysens (1). The light-induced oxidation of cytochrome(s) in purple bacteria is well documented (2-7), and it is now quite well established that energy absorbed by or transferred to bacteriochlorophyll drives photosynthetic electron transfer systems composed of *b*- and *c*-type cytochromes (as well as cytochromoids in some instances). The characterization of *c*-type cytochromes from green bacteria has been carried out by Gibson (8), and a new chlorophyll, which is probably bacteriochlorophyll, has been isolated as a protein-chlorophyll complex (9).

In this paper we present evidence that the terminal acceptor of electronic excitation energy in green bacteria is the bacteriochlorophyll-like chlorophyll-770, and that chlorobium chlorophyll serves as an accessory pigment for efficient collection of light energy. Light-induced oxidation of *c*-type cytochrome(s) is demonstrated, and the quantum efficiency shown to be comparable to the efficiency of cytochrome oxidation in purple bacteria.

METHODS AND MATERIALS

Chloropseudomonas ethylicum, strain 2K, and *Chlorobium thiosulfatophilum*, strain L, were grown on 0.1 per cent ethanol and 0.1 per cent $Na_2S_2O_3 \cdot 5H_2O$ respectively (See Appendix). *Cps. ethylicum* 2K contains chlorobium chlorophyll-660 (10) and *C. thiosulfatophilum* L contains chlorobium chlorophyll-650 (11). Both also contain a relatively small amount of chlorophyll-770. Absorption spectra of whole cell suspensions are given in Appendix, Fig. 5, p. 497.

Fluorescence emission spectra ($dQ/d\bar{\nu}$) were determined as described previously (12) and are expressed in quanta cm^{-2} sec^{-1} $sterad^{-1}$ μ. Total quantum emission was determined from the area under the emission spectrum.

Light-induced absorbancy changes were measured with a double-beam spectrophotometer as described elsewhere in this volume (7). Monochromatic actinic light with a half-maximal band width of 13 mμ or less was obtained with interference filters.

Transmission spectra of whole cell suspensions were obtained by the opal glass method in the Cary 14R spectrophotometer.

RESULTS

Fluorescence.

Emission spectra from *Cps. ethylicum* excited by 436 mμ light are shown in Fig. 1. Essentially all of the excitation light is absorbed by chlorobium chlorophyll and γ-carotene. At room temperature (294°K) the main fluorescence emission at 814 mμ comes from chlorophyll-770 (9) and indicates the transfer of electronic excitation energy from

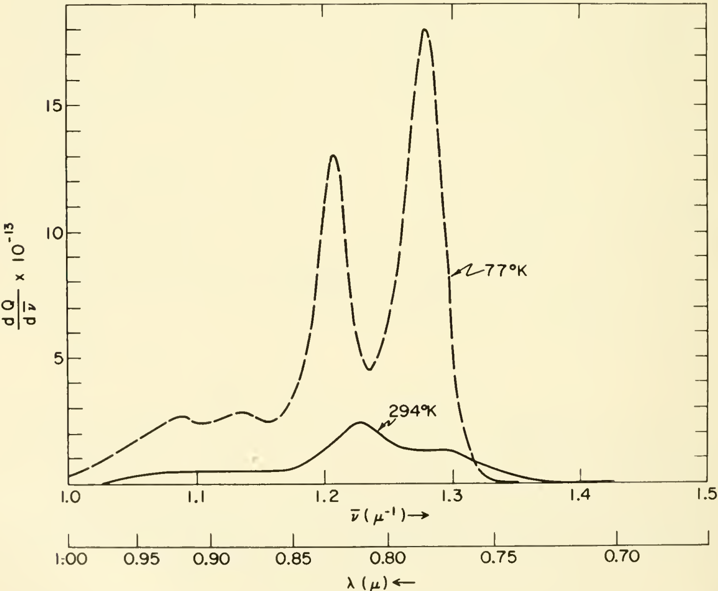

Fig. 1. Fluorescence emission spectra (dQ/d$\bar{\nu}$) from *Cps. ethylicum* at 77°K and 294°K caused by excitation at 436 mμ. The cell-suspension was taken from a one-day-old culture.

chlorobium chlorophyll and carotenoid to chlorophyll-770. The lesser emission band at 769 mμ belongs to chlorobium chlorophyll. At liquid nitrogen temperature (77°K) the chlorophyll-770 emission sharpens and shifts to 827 mμ, and two minor bands at 880 mμ and 917 mμ can now be resolved. The chlorobium chlorophyll emission also sharpens and shifts to 781 mμ at 77°K. Similar observations have also been made with *C. thiosulfatophilum* L and another strain of *Chlorobium* (13).

Two estimates for the efficiency of energy transfer to chlorophyll-770 in *Cps. ethylicum* are given in Fig. 2. The intensity of the 436 mμ exciting light (10^{18} quanta cm^{-2} sec^{-1}) determined from the emission

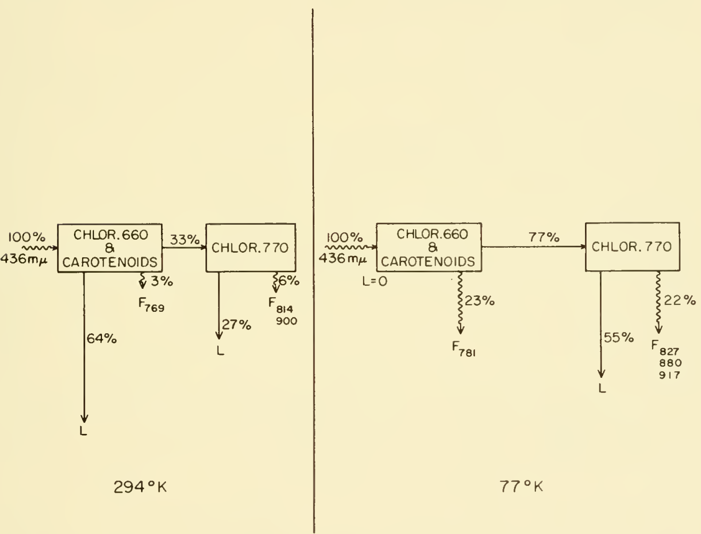

Fig. 2. Pathways for excitation energy in *Cps. ethylicum* Symbols *L* and *F* denote nonradiative losses and fluorescence respectively. The percentages shown are based on the data in Fig. 1 and assumptions stated in the text.

of fluorescence standards (12) was well above the intensity required for saturating the cytochrome oxidation reaction. The quantum emission from each chlorophyll at each temperature was determined from Fig. 2. The nonradiative losses L from chlorophyll-770 *in vivo* were calculated from the fluorescence yields at 77° and 294°K for the protein-chlorophyll-770 complex *in vitro* (9). The calculated transfer efficiency

of 33 per cent at 294°K is a minimum estimate, since the fluorescence yield of chlorophyll-770 *in vivo* may be lower than assumed.

Evidence that photochemistry can compete with fluorescence emission from chlorophyll-770 in *Cps. ethylicum* is given in Fig. 3. When the intensity of the 436 mμ exciting light is decreased from 10^{18} to 10^{15} quanta sec^{-1} cm^{-2}, the intensity of the chlorophyll-770 emission relative to the chlorobium chlorophyll emission drops about 30 per cent. The fluorescence yield of the chlorobium chlorophyll appears to remain about the same over this range of intensities. These observations, extending to very low levels of excitation, suggest that chlorophyll-770 is involved in some photochemical reaction, but that chlorobium chlorophyll is not.

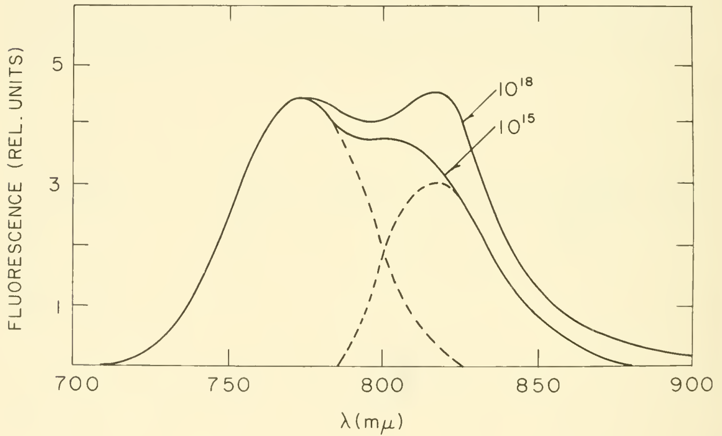

Fig. 3. Uncorrected fluorescence emission spectra of *Cps. ethylicum* at 294°K caused by 436 mμ excitation at intensities of 10^{18} and 10^{15} quanta cm^{-2} sec^{-1}. The spectra have been made to coincide at 769 mμ. The dashed curves represent the two components assumed to account for the overall emission spectrum at the lower intensity of excitation.

Light-induced absorbancy changes.

In the spectrophotometer the most easily discernible chemical reaction caused by light is the oxidation of cytochrome. Irradiation of either *C. thiosulfatophilum* L or *Cps. ethylicum* with red light under anaerobic conditions causes the oxidation of mainly *c*-type cytochrome(s). Fig. 4 shows the disappearance of bands at 423 mμ and 553 mμ. The asymmetry of the α trough suggests the oxidation of some *b*-type cytochrome(s) as well.

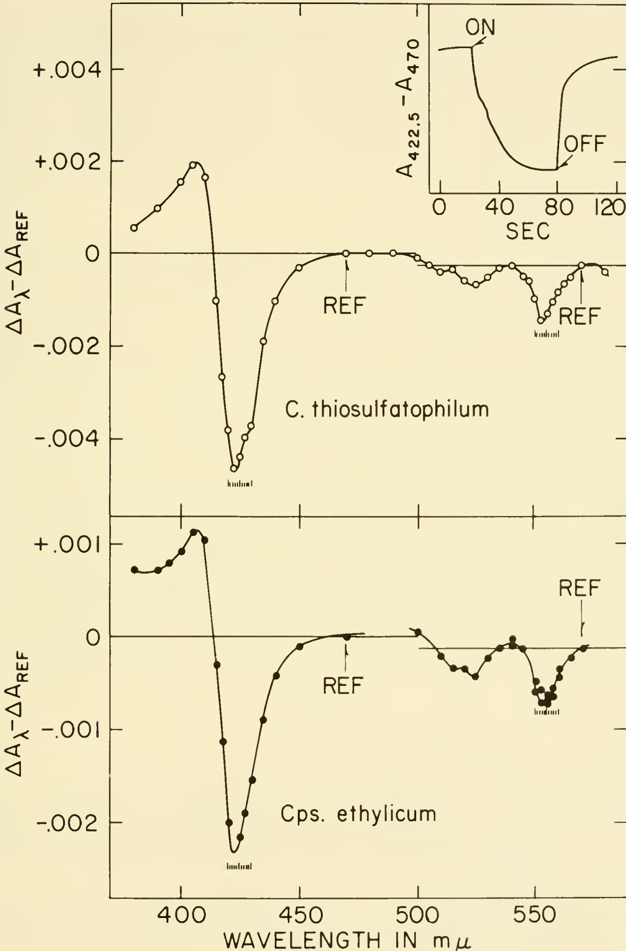

Fig. 4. Light-induced absorbancy changes in anaerobic green bacteria. Upper panel: changes in *C. thiosulfatophilum* L caused by 0.80 μ light of "high" intensity (7×10^{-9} einstein cm^{-2} sec^{-1}). Lower panel: changes in *Cps. ethylicum* caused by 0.80 μ light of "low" intensity (1×10^{-9} einstein cm^{-2} sec^{-1}).

The oxidation of cytochrome can be induced with light absorbed by either chlorobium chlorophyll or chlorophyll-770. No significant differences in the absorption spectrum changes or the kinetics of the light reaction have been noted when the wavelength of the actinic light is varied from 0.68μ to 0.85μ. At the wavelength of maximum absorption in the red for *Cps. ethylicum* ($\sim 0.75\ \mu$) the intensity for half-maximum cytochrome oxidation in the steady-state at room temperature is about 10^{-9} einstein $cm^{-2}\ sec^{-1}$ (6×10^{14} quanta $cm^{-2}\ sec^{-1}$).

Rate of cytochrome oxidation.

Initial rates of cytochrome C-553 (8) oxidation in *Cps. ethylicum* were measured with known quantum intensities of monochromatic red light in order to determine the quantum efficiency and action spectrum of cytochrome oxidation. The measurements were carried out at 2°C to lower the rate of cytochrome reduction in the dark. (At this temperature very little *b*-type cytochrome is oxidized in the light, and the troughs in the light-minus-dark difference spectrum appear at 422 $m\mu$ and 553 $m\mu$.) The results for light of 0.81μ are given in Fig. 5, where initial rates of absorbancy change are plotted versus intensity of actinic light. It appears that the initial rate of reaction is not strictly proportional to light intensity, but that the slope actually increases slightly as the intensity increases. This phenomenon is much more pronounced at room temperature (24°C). The apparent quantum efficiency (ϕ) may be calculated for any intensity from Fig. 5 and the percentage of actinic light absorbed by the bacteria (14). This calculation has been made for intensities approaching zero and for 10^{-9} einstein $cm^{-2}\ sec^{-1}$ on the assumption that for cytochrome C-553 $\Delta\epsilon_{422} - \Delta\epsilon_{470} = 6.8 \times 10^4\ M^{-1}\ cm^{-1}$. Table 1 gives the apparent quantum requirements

TABLE 1.

Apparent quantum requirement of cytochrome oxidation in Cps. ethylicum

Figures within parentheses are calculated on the basis of no additional correction for light-scattering beyond the use of opal glass (see text).

$1 - T_{812}$	Intensity range	
	"low"	"high"
.22	3.2	2.4
(.32)	(4.8)	(3.6)
.12	2.6	2.0
(.17)	(3.5)	(2.9)

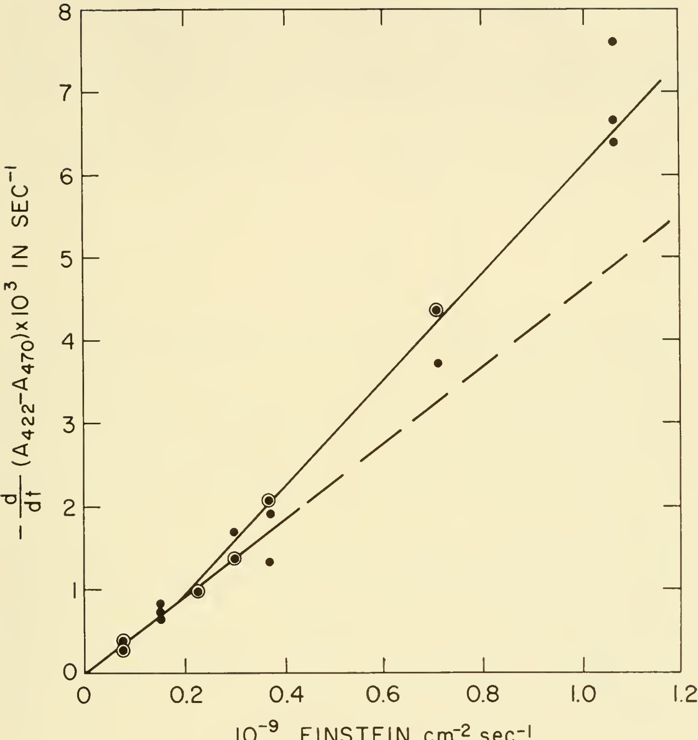

Fig. 5. Initial rate of absorbancy change at 422 mμ as a function of intensity of irradiation at 812 mμ. The suspension of *Cps. ethylicum* absorbed 22% of the actinic light. The dashed line indicates the slope of the curve at very low intensities.

($1/\phi$) for cytochrome oxidation at 2°C for two concentrations of bacteria. The minimum requirement appears to be two quanta per electron.

The action spectrum of cytochrome oxidation is compared with the absorption (1 - T) spectrum of the bacteria in Fig. 6. The absorption spectrum given by the opal glass method has been further corrected for light-scattering by the assumption of a slight scattering error, $T_{error} = k\ (\lambda - 900)$, calculated from the slope of the Cary transmission curve between 900 and 850 mμ. Without the correction the

estimated absorption of the sample would rise more steeply than shown in Fig. 6 as the wavelength decreases from 850 to 750 mμ. Even with the correction, it is clear that the efficiency of light at 812, 825, 837, and 850 mμ is higher than the efficiency of light at 774, 786, and 798 mμ.

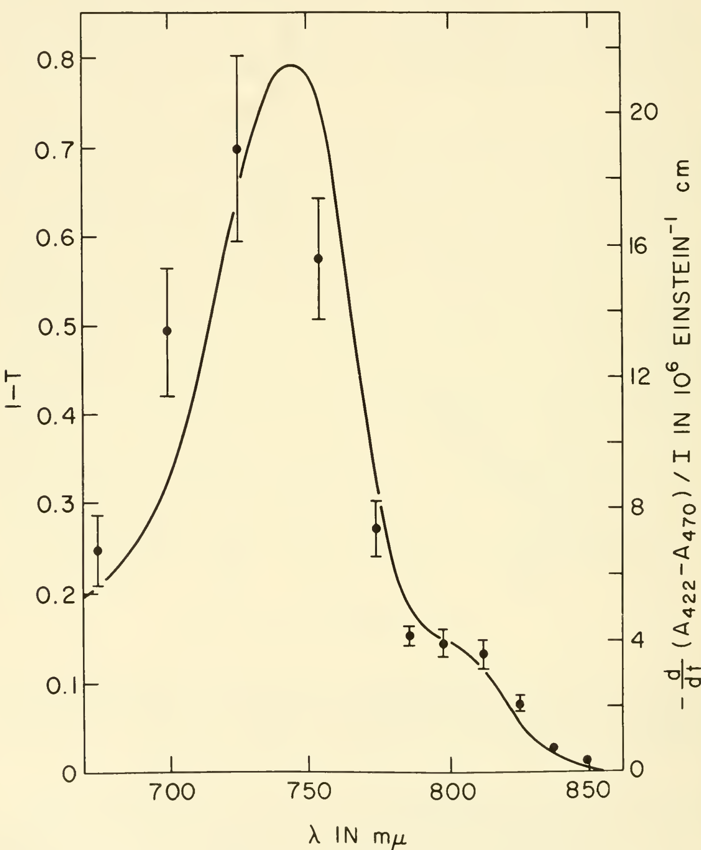

Fig. 6. Absorption (1 − T) spectrum (solid curve) and action spectrum of cytochrome oxidation (closed circles) for a suspension of *Cps. ethylicum*. The absorption spectrum has been corrected for light scattering as described in the text. The points of the action spectrum are ratios of initial rate of absorbancy change at 422 mμ to quantum intensity of actinic light.

(At wavelengths below 774 mμ both the absorption curve and the initial rate measurements should be taken with some reservation.) The ratio of action to absorption at 812, 825, and 837 mμ is about 50 per cent higher than the ratio at 774 and 786 mμ. This indicates that light absorbed directly by chlorophyll-770 is about 50 per cent more efficient than light absorbed by chlorobium chlorophyll in sensitizing cytochrome oxidation and corresponds to an efficiency of 60 to 70 per cent for transfer of excitation energy from chlorobium chlorophyll to chlorophyll-770.

DISCUSSION

These results show that chlorophyll-770 can sensitize the oxidation of *c*-type cytochrome(s) in green bacteria with good efficiency. Energy absorbed by chlorobium chlorophyll is transferred to chlorophyll-770 with an efficiency of at least 30 per cent in *Cps. ethylicum*. The true value is probably between 50 and 80 per cent. Although the possibility that chlorobium chlorophyll may participate directly in some photochemical reactions is not completely ruled out, the evidence favors its function solely as a light-gathering pigment which transfers energy to chlorophyll-770.

Energy transfer by inductive resonance (15) is probably as good a mechanism for green bacteria as for other photosynthetic organisms (1). Based on the fluorescence and absorption characteristics of *Cps. ethylicum*, the critical distance R_0 for 50 per cent probability of energy transfer either between chlorobium chlorophyll-660 molecules or from chlorobium chlorophyll-660 to chlorophyll-770 was estimated to be about 35 Å (16). If the efficiency of energy transfer from chlorophyll-660 to chlorophyll-770 is assumed to be between 50 and 80 per cent, the ratio of chlorophyll-660 to chlorophyll-770 should be between 80 and 20 according to an approximate equation given by Duysens (Ref. 1, p. 88). This is not too far from Fuller's (p. 71, this vol.) estimate of 100, in view of the uncertainties involved in each estimation.

The light-induced cytochrome reactions in the two green bacteria studied are similar to those observed in purple bacteria, especially *Chromatium* (5). The close relationship of chlorophyll-770 to bacteriochlorophyll further implies a basically similar type of chlorophyll-cytochrome interaction in the green and purple bacteria. From a phylogenetic point of view the similarities between these two groups of photosynthetic bacteria are seen to be more numerous and fundamental than previously realized.

ACKNOWLEDGMENTS

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Note added in proof. Recent experiments (Sybesma, C., and Vredenberg, W. J., *Biochim. Biophys. Acta,* in press) indicate that excitation energy is further transferred from chlorophyll-770 to a reaction center having an absorption maximum at 840 mμ.

THE PROTEIN-CHLOROPHYLL-770 COMPLEX FROM GREEN BACTERIA

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Last year a protein-chlorophyll complex was extracted from two species of green bacteria and separated from the predominant pigment, chlorobium chlorophyll (1). The chlorophyll of the complex was named chlorophyll-770 because of the position of the main absorption band in ether. The spectral resemblance to bacteriochlorophyll was noteworthy. The general occurrence of chlorophyll-770 in all green bacteria is suggested by the absorption spectra of cell suspensions and extracts obtained by various workers (1-3) with different strains. The function of chlorophyll-770 as an acceptor of electronic excitation energy from chlorobium chlorophyll has recently been demonstrated (4), and its ability to sensitize the light-driven oxidation of cytochrome *in vivo* is described elsewhere in this volume (5). In the present paper the following properties of the protein-chlorophyll-770 complex from *Chloropseudomonas ethylicum*, strain 2K, are given: absorption spectra, fluorescence emission spectra, sedimentation coefficient, molecular weight, magnesium content, and nitrogen content.

PREPARATION OF MATERIAL

Protein-chlorophyll preparations were obtained from both *Cps. ethylicum* 2K and *Chlorobium thiosulfatophilum*, strain L, by the procedure shown in Fig. 1. Bacteria were ruptured either by freezing and thawing or by means of a French pressure cell. The eluate from the DEAE-cellulose could be further purified by readsorption onto DEAE-cellulose followed by gradient elution with NaCl in 0.02 *M* phosphate buffer, pH = 7.8. Preparations sufficiently pure for quantitative characterization have so far been obtained from *Cps. ethylicum* only.

Extraction of the chlorophyll-770 from the complex was accomplished by precipitating the complex with $(NH_4)_2SO_4$, 30 per cent (W/V), and homogenizing the precipitate in 80 per cent methanol at 40°C. The chlorophyll-770 was transferred to ether (1) and chroma-

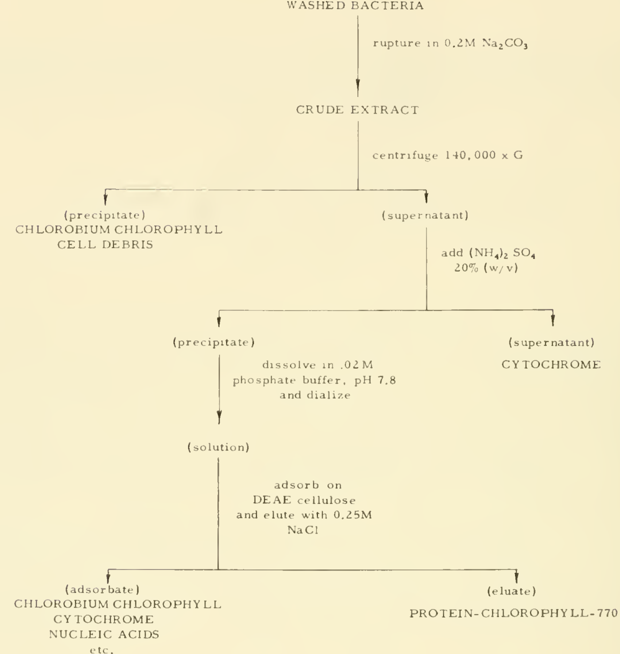

Fig. 1. Flow sheet for preparation and purification of the protein-chlorophyll-770 complex from green bacteria.

tographed (6) on powdered sugar (Jack Frost 10X) using a mixture of 2 per cent tetrahydrofuran and 0.75 per cent *n*-propanol in petroleum ether (Skellysolve B). After development of the sugar column, the blue band at the bottom was dug out and eluted with ether.

ABSORPTION AND FLUORESCENCE SPECTRA

The absorption spectrum of the protein-chlorophyll complex is given by the solid curve in Fig. 2. Most of the spectrum is due to the chlorophyll, but the slight shoulder at about 280 mμ is probably due to protein. The absorption spectrum of a typical preparation from *C. thiosulfatophilum* differs from the solid curve in Fig. 2 mainly in

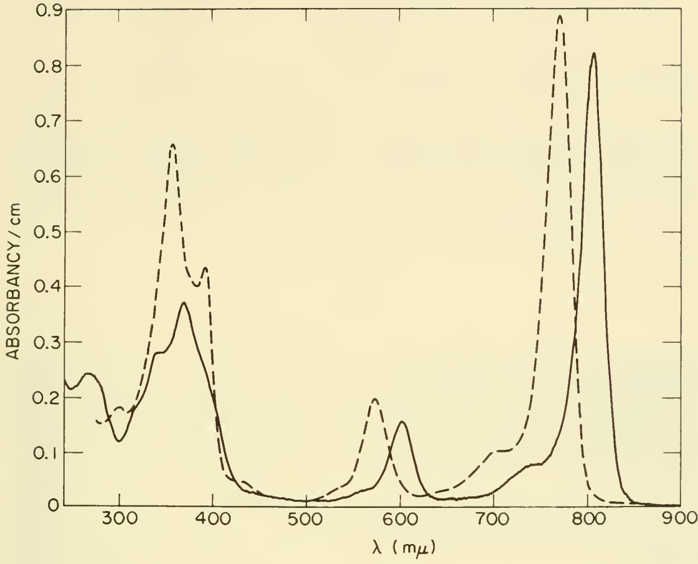

Fig. 2. Absorption spectrum of the protein-chlorophyll-770 complex from *Cps. ethylicum* in 0.25 *M* NaCl and 0.02 *M* phosphate buffer pH 7.8 after elution from diethylaminoethyl cellulose (————) and absorption spectrum of chlorophyll-770 in ether after sugar chromatography (----------). The chlorophyll concentrations for the two curves are *not* equivalent.

having a very high peak at 275 mμ and a very pronounced shoulder at 405 mμ, which are presumed to indicate contamination by cytochrome and other proteins.

The absorption spectrum of chlorophyll-770 in ether is given by the dashed curve in Fig. 2. This spectrum, which is virtually the same as that obtained for bacteriochlorophyll from *Chromatium* after sugar chromatography, agrees with the observations based on other species of green bacteria (7,8). The spectral characteristics are presented in Table 1.

Fluorescence emission spectra for the complex are shown in Fig. 3. At 293°K the main emission band appears at 818 mμ with a broad shoulder on the red side of the main peak. At 77°K the main peak sharpens and shifts to 827 mμ, and the shoulder is resolved into two minor bands at 880 mμ and 917 mμ. The fluorescence yields for 366 mμ excitation are 0.19 and 0.29 at 293°K and 77°K respectively (4).

TABLE 1.

Characteristics of absorption spectra

Spectrum I is for the protein-chlorophyll-770 complex from *Cps. ethylicum* dissolved in 0.2 *M* NaCl and .01 *M* Tris, pH 8.0; spectrum II is for chlorophyll-770 dissolved in ether after purification by sugar chromatography; and spectrum III is for bacteriochlorophyll from *Chromatium* dissolved in ether after sugar chromatography. Peak positions (λ_n) are given in mμ, and the peak height ratios (r_n) are relative to the maximum red absorbance.

Spectrum	λ_1	r_1	λ_2	λ_3	r_3	λ_4	r_4	λ_5	r_5	λ_6	r_6	λ_7
I	267	.28	315	343	.34	371	.45	603	.19	~740	.09	809
II	~260*	-	301	358	.75	392	.50	575	.23	~710	.12	770.6
III	-	-	302	358	.77	392	.50	575	.23	~710	.12	771.2

* This absorption band was observed before sugar chromatography, but could not be seen after sugar chromatography because of the UV absorbing material in Skellysolve B which is also eluted from the sugar by ether.

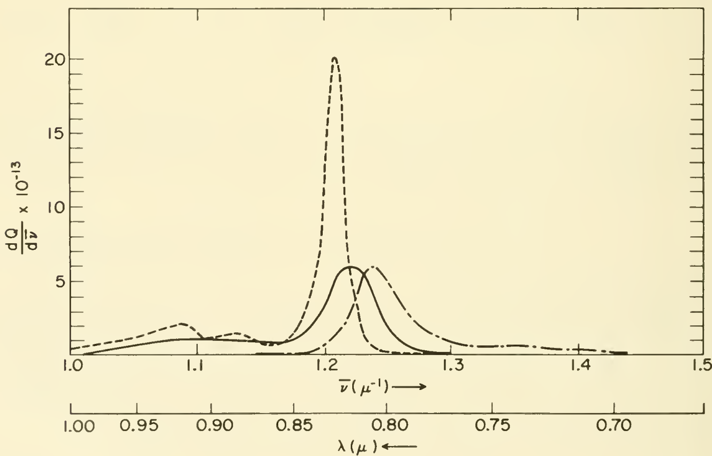

Fig. 3. Fluorescence emission spectra (dQ/dν) at 294°K (————) and 77°K (---------) of the protein-chlorophyll-770 complex from *Cps. ethylicum* caused by excitation at 366 mμ, and the far red absorption (–·–·–·–) normalized to the main fluorescence peak at 294°K.

SEDIMENTATION COEFFICIENT AND MOLECULAR WEIGHT

The sedimentation coefficients at four different protein concentrations were measured from the sedimentation velocity in the Spinco analytical ultracentrifuge, Model E. The chlorophyll-protein was dialyzed against 0.2 *N* NaCl in 0.01 *M* Tris, pH 8.0, and sedimented at 59,780 rpm in a double-sector cell. The Schlieren pattern (see Fig. 4) was photographed at 8-minute intervals, and the distance of the peak

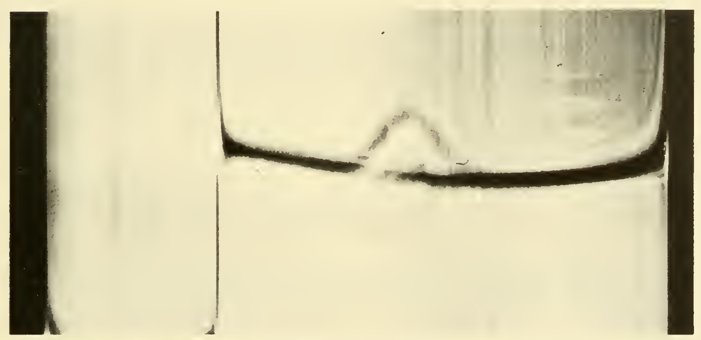

Fig. 4. Schlieren pattern of the descending boundary in the double-sector cell after approximately 30 minutes of centrifugation at 59,780 rpm. A_{809} = 31.4 cm^{-1}, t = 25.6°C.

from the center of rotation was plotted versus time on a semilog scale. The sedimentation coefficient was calculated from the slope of the best straight line fitted to the data according to the usual equation and then corrected for solvent viscosity and temperature (9). The corrected values for $s_{20,w}$ at 4 concentrations of material are shown in Fig. 5. The linear extrapolation to zero concentration was fitted to the experimental points by the method of least squares. In spite of the

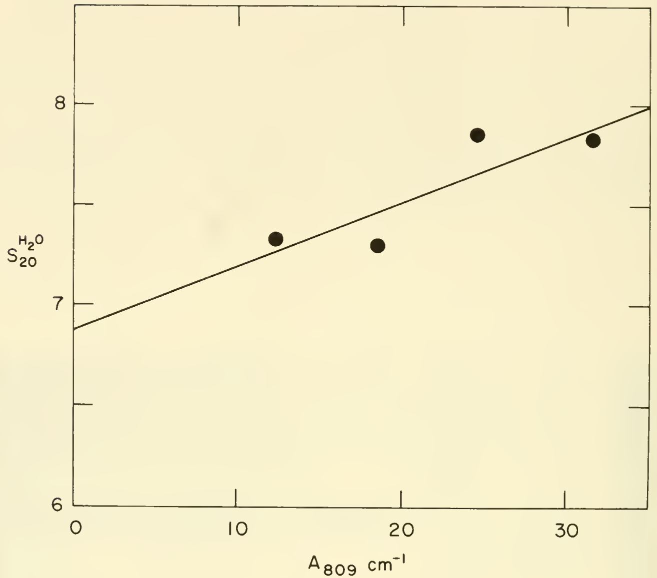

Fig. 5. Sedimentation coefficients $s_{20,w}$ calculated from four measurements of sedimentation velocity at different starting concentrations. The straight line was fitted to the data by the method of least squares.

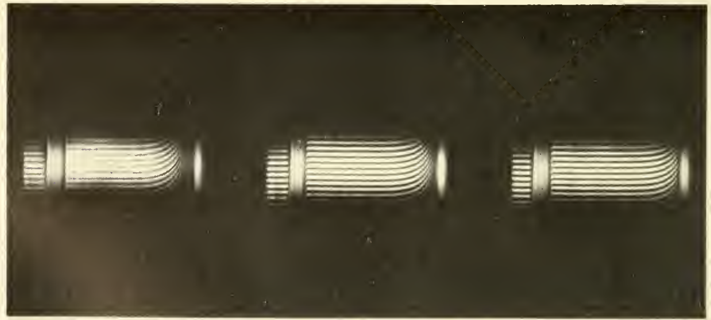

Fig. 6. Rayleigh interference patterns at equilibrium for relative initial concentrations of 1.0, 0.5, and 0.25 (left to right). Rotor speed 20,410 rpm, t = 17.4°C, A_{809} = 6.0 cm^{-1} for highest initial concentration.

rather poor fit of the data to any monotonic function of concentration, it appears that the sedimentation coefficient at infinite dilution and 20°C is about 7 Svedberg units.

The molecular weight was determined by the method of equilibrium sedimentation in the Spinco Model E equipped with Rayleigh interference optics (10) and a special double-channel cell developed by Yphantis (11), in which each channel is divided into three separate compartments (~2.5 mm column height). The three compartments of one channel were filled with three concentrations of the protein-chlorophyll complex, and the compartments of the other channel were

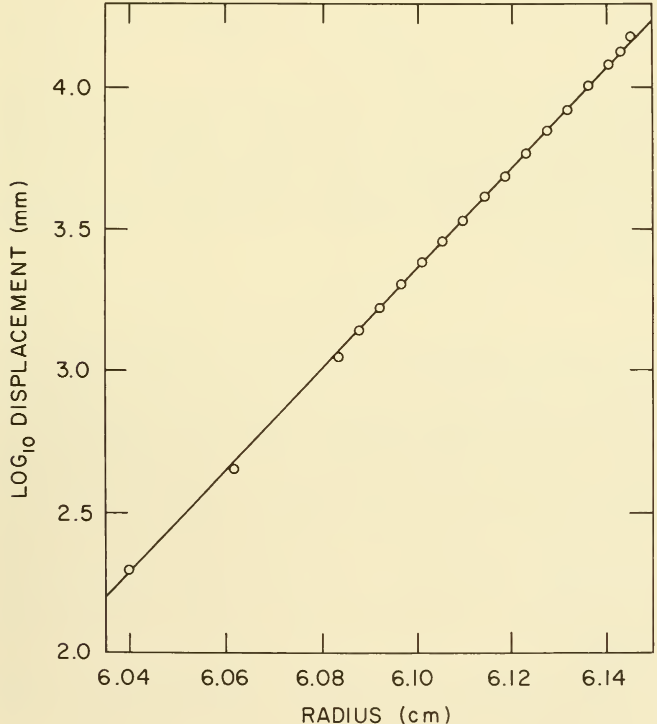

Fig. 7. Logarithm of fringe displacement versus distance from center of rotation from left hand interference pattern in Fig. 6. The data shown are taken from the "bottom" 1 mm portion of the ~2.5 mm column.

filled with solvent. The loaded cell was centrifuged for 24 hours at 20,410 rpm, and the equilibrium distribution of material in each sample compartment determined by photographing the interference patterns resulting from the index of refraction gradients in each compartment (see Fig. 6). The fringe shift at any point is directly proportional to protein concentration. The magnitude of the fringe shift (and thus, the relative concentration) was measured at regular intervals in each compartment, and the logarithm of the displacement was plotted versus the distance from the center of rotation as in Fig. 7. The molecular weight was calculated from the slope of the best straight line fitting the experimental points according to the equation

$$M = \frac{RT}{\omega^2 r\,(1 - \overline{V}\rho)} \; \frac{d\,(\log c)}{dr}.$$

$\overline{V}$ was assumed to have a value of 0.74. The calculated values of molecular weight from the three compartments ranged from 1.31 to 1.42 x 10^{-5} and gave an average value of (1.37 $\pm$ .05) x 10^5.

The diffusion coefficient at infinite dilution and 20°C, calculated from the sedimentation coefficient and molecular weight, is about 5 x 10^{-7} cm^2/sec.

CHLOROPHYLL-TO-PROTEIN RATIO

A preliminary estimate of the magnesium content of the protein-chlorophyll complex has been made by emission spectrography. The intensity of the Mg emission line(s) from a solution having an absorbancy of 3.0 cm^{-1} at 809 mμ indicated a Mg concentration of 8.8 x 10^{-6} *M* after correction for the Mg in the buffer against which the sample had been dialyzed. This level of magnesium corresponds to an absorbancy index of 3.4 x 10^5 M^{-1} cm^{-1} at 809 mμ based on chlorophyll, if the Mg-to-chlorophyll ratio is assumed to be unity.

A preliminary estimate of the nitrogen content has been made by micro-Kjeldahl analysis (12). A solution having an absorbancy of 4.6 cm^{-1} at 809 mμ had a nitrogen content of 0.045 g/l. Protein concentration was estimated to be 0.28 g/l on the assumption of a nitrogen content of 16%.

From the magnesium and nitrogen determinations, the minimum value of the chlorophyll-to-protein ratio is estimated to be 4.8 x 10^{-5} mole chlorophyll per gram protein. For a unit of 1.4 x 10^5 molecular weight, this means a minimum of six bound chlorophyll groups.

ACKNOWLEDGMENTS

The magnesium determination was done under the direction of Mr. S. J. Tassinari in the Department of Nuclear Engineering. The nitrogen determination was carried out by Miss Frieda Englberger under the direction of Dr. M. E. Koshland. Research was carried out at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

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LIGHT-INDUCED ABSORBANCY CHANGES IN *RHODOMICROBIUM VANNIELII*

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In both purple and green photosynthetic bacteria, light absorbed by chlorophyll(s) causes the oxidation of cytochrome(s) under appropriate conditions (1-5). In several strains of purple bacteria the cytochrome changes are masked by a pronounced red shift of the carotenoid absorption peaks (6,7). These general observations have been found to apply also to the obligate, anaerobic photoheterotroph, *Rhodomicrobium vannielii*, which is similar to the purple bacteria in its complement of photosynthetic pigments.

METHODS

The bacteria were grown as described previously (8) and harvested by centrifugation. The cells were resuspended in 0.05 *M* phosphate buffer, pH 7.4, for observation in a Chance-type double-beam spectrophotometer (9) in which the measuring beams were deflected by a half-silvered mirror vertically down through the sample cuvette to minimize changes in light transmission due to the settling of the filamentous mass of bacteria in the cuvette. Monochromatic actinic light (0.80μ) was projected through the half-silvered mirror down onto the sample cuvette from a slide projector with appropriate filters. Actinic light intensity was monitored by a calibrated photodiode. A blue-green filter between sample cuvette and photomultiplier transmitted the measuring light while attenuating the actinic light to a negligible level.

In most experiments the sample cuvette was completely filled with bacterial suspension. However, in experiments in which the transition from anaerobiosis to aerobiosis was observed, a thin layer (2-3 mm) of a dense cell suspension covered the bottom of the cuvette while the remainder (7-8 mm) was flushed with either wet N_2 or O_2.

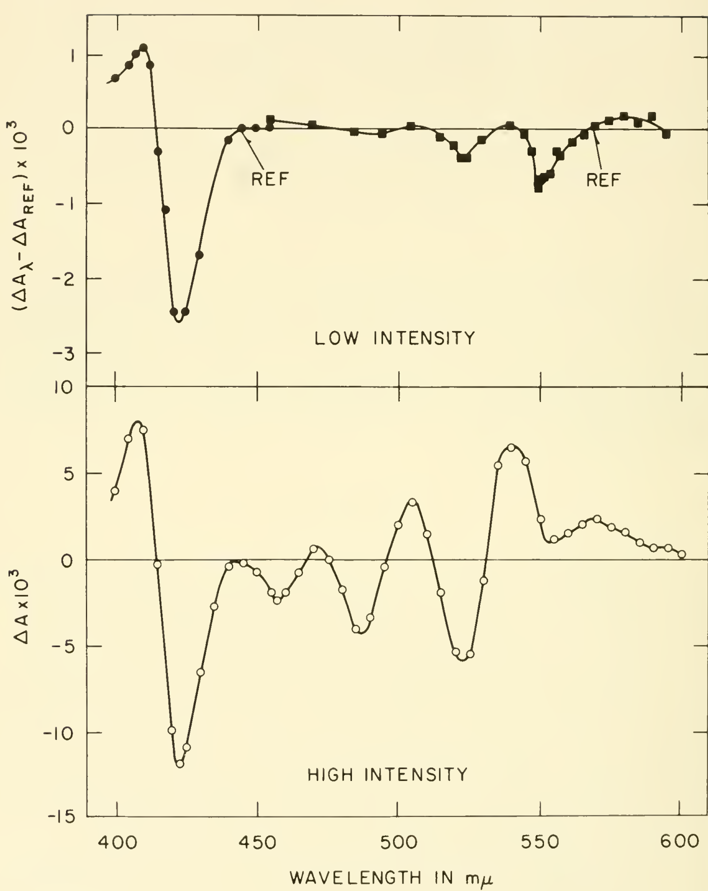

Fig. 1. Absorption spectrum changes in anaerobic bacteria induced by 0.80 μ light. Low intensity = 5×10^{-11} einstein cm^{-2} sec^{-1}. High intensity = 4×10^{-8} einstein cm^{-2} sec^{-1}.

RESULTS

Spectral changes.

The absorption spectrum changes in the region 400 to 600 mμ caused by high intensity far red light are shown in the lower portion of Fig. 1. The trough at 423 mμ and the broad dip around 552 to 555 mμ indicate cytochrome oxidation. Our tentative interpretation is that all three cytochromes found in *Rhodomicrobium* (8) are involved: C-550, C-553, and B-563. Overshadowing the cytochrome absorption changes in the region from 450 to 600 mμ are a series of three troughs and three peaks which indicate a shift in the carotenoid absorption bands toward the red. The decrease in carotenoid absorbancy at the usual maxima is about 1 to 2 per cent. The light-induced spectral changes are quite similar in either aerobic or anaerobic suspensions at high intensities.

Low intensity irradiation gives the results shown in the upper portion of Fig. 1. The absorbancy changes due to cytochrome oxidation are an order of magnitude smaller than at high intensity, but the shifts in the carotenoid peaks are not even detectable. The asymmetrical alpha trough at 550 mμ implicates mainly cytochromes C-550 and C-553 with some contribution from B-563.

Variations with light intensity.

The marked contrast between the qualitative features of the spectra shown in Fig. 1 indicate the importance of light intensity. In Fig. 2 the

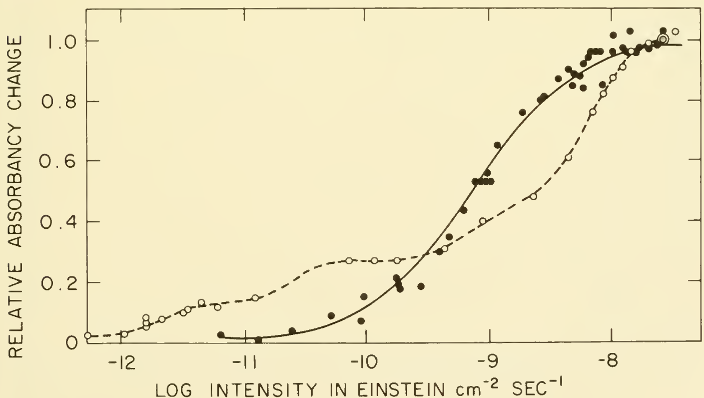

Fig. 2. Light-induced absorbancy changes in anaerobic bacteria versus logarithm of light intensity at 0.80 μ. Change in steady-state cytochrome oxidation in terms of $\Delta(A_{422.5} - A_{445})/\Delta_{max}$ (------). Carotenoid change in terms of $\Delta(A_{540} - A_{530})/\Delta_{max}$ (———).

difference between the "cytochrome response" and the "carotenoid response" is clearly demonstrated. The steady-state carotenoid shifts (solid curve) plotted versus logarithm of intensity fit the symmetrical sigmoid curve for a rectangular hyberbola, whereas the steady-state cytochrome change (dashed curve) shows four inflections suggesting separate electron transfer systems having distinctly different sensitivities to light. The two inflection points for the most sensitive cytochrome reactions occur at intensities well below the inflection point for the carotenoid reaction and are consistent with the observation in Fig. 1 that the light-induced carotenoid changes disappear before the light-induced cytochrome oxidation.

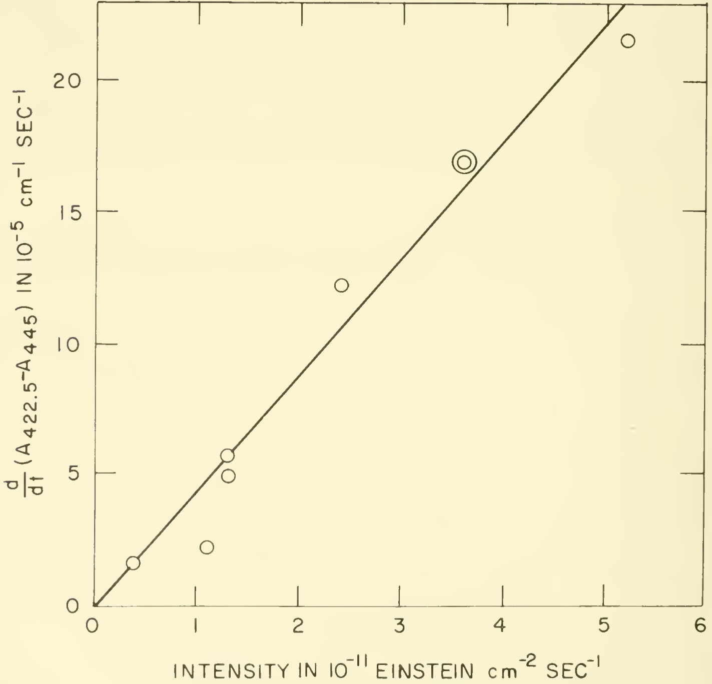

Fig. 3. Initial rate of absorbancy decrease at cytochrome Soret peak versus quantum intensity of actinic light (0.80 μ). Anaerobic bacteria suspended in 0.1 *M* succinate.

For purposes of analysis it is convenient to number the inflection points in the cytochrome curve (Fig. 2) I, II, III and IV in order of increasing light intensity and to assume that each inflection is associated with a cytochrome reaction which is more or less independent of the others. By this approach it is possible in principle to elucidate kinetic and spectral differences between the various reactions.

From Fig. 2 it is clear that the steady-state level of cytochrome oxidation is proportional to intensity up to the first inflection point approximately. The initial rate of cytochrome oxidation upon illumination, however, is linear with intensity beyond the third inflection point. From the dependence of the initial rate on intensity, one can estimate the quantum efficiency of cytochrome oxidation for the most sensitive electron transfer system (10). From the slope in Fig. 3 the maximum quantum efficiency estimate is 0.1 mole/einstein based on the following assumptions:

$$\Delta\epsilon_{422.5} - \Delta\epsilon_{445} = 60 \text{ m}M^{-1} \text{ cm}^{-1}, \; A_{800} = 0.5.$$

Effect of oxygen.

Oxygenation by itself causes absorption changes in whole cells, and in addition modifies the light effects to some extent, as shown in Fig. 4. The absorption spectrum changes in the region 540 to 570 mμ indicate the oxidation of *c*-type cytochromes upon the addition of oxygen.

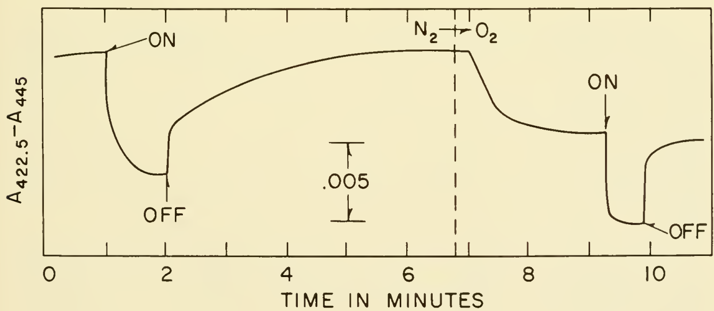

Fig. 4. Kinetic trace of absorbancy changes caused by 0.80 μ light and oxygen. Cytochrome oxidation denoted by a decrease in $A_{422.5} - A_{445}$.

The absorbancy changes induced by high intensity light reach completion more rapidly and also decay more rapidly in the presence of oxygen, while the extent of change is slightly diminished. Nevertheless,

the light-minus-dark difference spectrum for aerobic cells is very similar to that for anaerobic cells (Fig. 1) and indicates the involvement of both cytochromes and carotenoids.

An important effect of oxygen on the cytochrome response to light is the abolition of cytochrome reaction I, found in anaerobic cells (see Fig. 2). This observation along with the oxygen-induced cytochrome oxidation in illuminated cells indicates that oxygen affects both light-sensitive and light-insensitive cytochrome systems, even though most of the light-sensitive cytochrome remains reduced in the presence of oxygen alone.

Effects of electron transfer inhibitors.

The effect of n-heptyl-hydroxyquinoline-N-oxide (HOQNO) was to cause a concentration-dependent inhibition (half maximal at 2×10^{-6} *M*) of cytochrome and carotenoid reactions induced by high light intensity. Antimycin A (50 μg/ml), on the other hand, showed relatively little effect on the light-induced reactions except to maintain the typical kinetics for aerobic cells in contrast to the typical kinetics for anaerobic cells exhibited by the control. It appeared that this concentration of antimycin A inhibited respiration and thus prevented the suspension from becoming anaerobic without otherwise affecting the light-induced reactions.

DISCUSSION

These observations of *Rhodomicrobium* are comparable in many respects with the results obtained with other photosynthetic bacteria. The light-induced carotenoid shifts are strikingly similar to those observed by Smith and Ramirez (3) and Clayton (7) in *Rhodopseudomonas spheroides*. The absorbancy changes due to light-induced cytochrome oxidation are very similar to those observed in *Chromatium* (4), *Chlorobium* and *Chloropseudomonas* (5) in being attributable in the main to *c*-type cytochrome(s). The cytochrome and carotenoid reactions appear to be independent of one another as in *Rps. spheroides*.

The presence of multiple inflection points in the curve of steady-state cytochrome oxidation versus logarithm of light intensity is similar to the results previously observed in *Chromatium* where three inflection points were noted. The abolition of the most sensitive cytochrome light reaction by oxygen is also common to both species. In *Chromatium* this is effected through the oxidation of the most sensitive cytochrome in the dark; in *Rhodomicrobium* this happens also, but in addition other cytochromes not affected by light appear to be oxidized at the same time. *Rhodomicrobium* appears to have three types of cytochromes: (1) those which react to both light and oxygen, (2) those

which react only to light, and (3) those which react only to oxygen. It appears to be relatively deficient in the first type, and appears to have roughly twice as much of the second type as of the third type (see Fig. 4).

The apparent quantum requirement of 10 for cytochrome oxidation in *Rhodomicrobium* is rather high in comparison with reported requirements of 1 for *Chromatium* (10) and 3 to 4 for *Rhodospirillum rubrum* (11). The quantum requirement for CO_2 fixation in photosynthetic bacteria ranges from 9 to 12 (12,13). This corresponds roughly to a maximum quantum requirement of 3 to 4 for cytochrome oxidation. It is possible that the few experiments upon which the quantum efficiency estimate for *Rhodomicrobium* are based were not carried out under optimum conditions.

ACKNOWLEDGMENTS

Our thanks go to Dr. Sam Conti for stimulating interest and helpful discussion.

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Upper left: W. Arnold; *upper right:* D. L. Keister, G. Hind, A. Jagendorf; *lower left:* J. W. Newton, R. Y. Stainer; *lower right:* C. B. van Niel, L. P. Vernon.

SUMMARY

EXTEMPORANEOUS REMARKS BY WAY OF SUMMARY

MARTIN D. KAMEN

As I attempt this summary, I am reminded of an old vaudeville act which featured a very famous comedian named Bert Williams. His particular forte was a routine in which he would get up and say: "Ladies and Gentlemen, I am going to do something positively fantastic. With my left hand I am going to play Swannee River, with my right hand Yankee Doodle, and at the same time, I will sing the Star Spangled Banner." Then, in mock self-admiration, he would exclaim, "God, whew!"

If I were to summarize adequately what has transpired here in the last few days, I would be doing something as fantastic—and with no preparation. It is difficult enough to produce, extemporaneously, simple declarative sentences for at least half an hour! So let us see what can be done.

First, let me recall to you the admirable slide displayed by Dr. Geller in his lecture yesterday.

The Hon. R. Rubrum.

I was much impressed with the expression on the face of this creature. Since it has been the sole support of many of us for years, it deserves some examination. You will notice the sly, calculating look which is depicted so skillfully by Mrs. Geller's hand. Most of us who have been involved with this creature will testify that it is the nearest thing to the Shmoo we have in biology. It has an infinite capacity for surprises; and

it can outsmart any investigator. This it has been doing consistently for decades, ever since Esmarch first saw it. Now, its cousins and brothers and uncles and aunts have not been tested so earnestly, but I'm sure that if they are, they will all show the same delinquent tendencies.

First things first! This is a symposium in honor of Professor van Niel—Kees, that is—and it is very much in the nature of an offering from most of us to him, to show him what we have been doing in the last twenty years since he taught us. For many of us had our experience with him over twenty years ago. If we take his 1944 paper as a point of departure for this seminar, you would pretty much have today a synopsis of what has occurred since that paper was published. You may recall that in this classic paper he finished with a plea for biochemists to pay some attention to the Athiorhodaceae because they would certainly prove fruitful for further investigation. I forget the exact wording, but that certainly was the sense of it, and a more prophetic statement is hard to imagine.

Now to get to what has happened in the last twenty years, viewed through the activities of the last three days. I think the easiest thing to do is to begin with the program itself, to take it in large chunks, and to get a bird's-eye view of what has happened. The first three papers of the first day formed a coherent group—which is remarkable even though it was planned that way! These were concerned mainly with chemical aspects of photsynthesis. The first paper, a beautiful presentation by Dr. Jensen on carotenoid biosynthesis, a subject in which Dr. van Niel was interested early, seemed to me definitive, and I believe there is no point in summarizing it because a very adequate summary has been given in her paper in the form of a diagram. What we do derive is the incentive for some biochemistry. I don't think many of the conversions postulated have been placed on a biochemical basis, as far as enzyme content is concerned. It seems to me Dr. Jensen's findings afford a prolific, fertile field for many future Ph.D.'s and for the biochemists hard up for ideas who have graduate students to nurture, and who have somebody in their vicinity who knows carotenoid chemistry. Perhaps we can build a library of these carotenoids for use as substrates so we can fish out the enzymes. It is about time this was done, even though the carotenoids apparently enjoy less noble status in the field than some other pigments I might mention.

Now the presentation on tetrapyrroles by Dr. Lascelles and the following one on heme proteins, which were displaced from each other by two days, had been intended to make a logical, consistent unity, and I think they do inasmuch as the cytochromologists had no results which indicated porphyrin types other than those expected. It does appear that there is very little, if any, unusual porphyrin either in the cytochromes, or in the unusual heme proteins that are contained in photosynthetic bacteria, or in green plants. What is unusual are the proteins, and that is where the problem sits now. The capacity of the heme

proteins to change their catalytic properties by the mere substitution of one or another ligand in the five or six position is wholly unexampled in the chemical literature; there are no models to account for it. There are some brave people who, starting from models, have attempted to derive a series of generalizations for the aid and comfort of the investigator who seeks to imagine what the ligand positions might be that correspond to the potentials observed, and to the catalytic property observed. Papers bearing on this are available in a bibliography which I gave in my paper—particularly the considerations of J. P. R. Williams, which were published about six months ago in the *Biochemical Journal*.

After a break for refreshments (which, I suspect, were sorely needed!), we had the structure group. The question was: What is the photochemical apparatus in bacteria? Now this never has been a question in the green plants, even though I have a feeling that if you take a similar collection of experts in the chloroplast field, you will find divergences of opinion about what constitutes the photosynthetic apparatus. In the case of the green plant, the question is how dirty must it be before it satisfies the plant physiologist, and on the other hand, how clean must it be before it satisfies the biochemist? The same problem arises with the chromatophores, only at a level where you can't see them in the microscope. At least the chloroplast people can see what they are looking at and don't have to depend on fixation procedures. So they have arrived at their present state of disgrace, you might call it, perhaps some twenty years before we are going to get there.

If I may make a small extrapolation, I suspect that in about ten years we will have enough data to be arguing at the level that the chloroplast people argue at now. This may be a dim prospect. But in connection with it, in a rump session held last night (one of the two rump sessions held and which lasted for the longest seven minutes on record) there was a statement produced by Roger Stanier, speaking for the assembled group, which I will read *in extenso:* "In every purple bacterium"—I don't know why the green bacteria were left out, but anyway—"In every purple bacterium, the photosynthetic pigments and photochemical electron transport system are associated with the unit membranes of the cell. The unit membrane, comprised of cytoplasmic membrane, together with internal membrane, may take either vesicular or lamellate form. In some cases, the internal vesicles are lamellate and are physically continuous with the cytoplasmic membrane, but it is not known how complete the continuity is between the cytoplasmic and internal membrane systems. Photosynthetic pigments certainly are present in the internal membrane, but it is not known whether they are also present in the cytoplasmic membrane. The form and extent of the internal membrane system, in any one species, can change in response to environmental conditions during growth of a cell, so that

in addition to the uncertainties which attend investigation at any particular instant, there is also an uncertainty in time. Breakage of the cell by mechanical means leads to the formation of membrane fragments, heterogeneous in size, which bear the pigments in a photochemical electron transport system. When comminutive treatment, such as sonic oscillation, is used to disrupt a cell, the average size of membrane fragments obtained decreases with the duration of treatment. By differential centrifugation of bacterial extracts, the membrane fragments that are homogeneous in size and density may be separated; and these homogeneous preparations and particles have usually been found to be from 300 to 600 angstroms in diameter. These particles were originally designated as 'chromatophores.' *However, this term is now undesirable because it implies that such independent structures exist in the cell before destruction. There is no evidence to support this assumption.* [Italics mine.] Nevertheless, some descriptive term applicable to the photochemically active particulate preparation that can be obtained after cell disruption is needed. We propose that any such preparation should be designated as a 'chromatophore fragment.' Irrespective of the particle size or homogeneity, any such fraction should be defined operationally in terms of the methods used for its preparation." So that is the statement.

From now on, we have "chromatophore fragments," regardless of size or homogeneity, but there has to be somewhere in any paper dealing with them an accurate description of how they were obtained and preferably, maybe some pictures. Well, perhaps we won't ask for the pictures, but at least there should be some description of the culture conditions of the organism, its age, the media used, the number of times it was washed, and even perhaps a designation of the strain (if you can obtain this from some local expert) and how many years you've had this strain. It might even be a good idea to have occasional comparisons or recalibrations of the strain, and finally, the nature of the equipment used to break up the cell, in detail. This brings us to the end of the first day.

The second day brought us to a more vexatious point. The question of "cyclic" versus "noncyclic" photophosphorylation, and also, of course, the metabolic aspects in general of bacterial photosynthesis, with some digressions on the nature of the present character, or present status, of the unitary theory of photosynthesis, whatever it may be. The unitary theory has taken many forms since the Thirties, and there have been a number of suggestions as to what the primary chemistry should be to define a unitary theory. The primary chemical process back in 1935 was the "splitting of water," and everything was classified in terms of what happened in reactions involving the products of splitting of water. This is still, for purposes of classification, a perfectly fine scheme. It is not intended to imply a mechanism, and it is necessary that we should be looking for definitions which will

specify more accurately the nature of the process in terms of which we speak about a unitary theory. We have, I think, in bacterial photosynthesis, already a certain amount of information. It seems safe to say that one process which unifies all photosynthesis is the photooxidation of a *c*-type cytochrome. I haven't heard anything in these last three days which seems to indicate that anybody has doubts about this; of course, it is still a question for future research as to whether the kinetics of these reactions accord with a primary process, but they certainly do not have to accord with a primary process to be considered a feature of the unitary theory. Whether there is any other reaction that is common to all photosynthesis or not, this is one that certainly is. There is some question as to whether the splitting of water might be considered common to all photosynthesis, but everything that has been looked at so far does show an oxidation of cytochrome. In the case of the splitting of water, the objection has been you couldn't see it, you only could see the products. In the case of the oxidation of cytochromes, you can see it in the test tube, in the spectroscope, and you can see it *in vivo*; so perhaps this is progress.

Now the other thing that is important about this definition is the fact that it leads to experiments. I hold, wholly out of prejudice, perhaps, that the notion of a heme-protein magnesium tetrapyrrole complex, which was first enunciated by Robin Hill, on the basis of comparative biochemistry and structure, is a fruitful hypothesis, however true it may be. The work which has been done with flash spectrophotometry by Witt and others, and with dynamic spectrophotometry by Chance, Nishimura, Olson, and others, also indicates the possibility of interpreting optical density changes in a rational, consistent way. And these same studies dovetail beautifully with the sort of thing that goes on in the kind of research which has been described by Vernon, by Horio, and by others, on the enzymatic activities in photooxidation and photoreduction processes of model systems. I don't think I have to summarize these things further, but what I must do is get to the vexing question of the terms cyclic and noncyclic.

From the beginning it was my impression that there was only one kind of phosphorylation, the esterification of the secondary phosphate in ADP to ATP. What happened after could be specified in terms of some reservation or qualification about the nature of the process accompanying this phosphorylation. It seemed to help some people to attach an appendix to the word phosphorylation—other than "photo," that is.

Now we should, I think, take our lead on these matters from the people working in oxidative phosphorylation; it's not necessary, but it may be helpful. They have been at it a much longer time than we have and they have some possibility, apparently, of understanding this process in the near future. Those of you who have followed the literature in oxidative phosphorylation in the last two years will probably

know what I mean when I say it's amazing what has happened after a long dry spell of something like twenty years. All you have to do is look at the literature in 1935, 1940, 1945, 1950, and so on, to see what little was done back then, despite enormous efforts, and note what has happened lately.

Since we now have coupling enzymes and even active sites in the mitochondria (which are incidently much more difficult systems to work with than chromatophores, whatever they may be), I think it is helpful to consider whether the sites in photophosphorylation have any relation to the established sites in oxidative phosphorylation. Here I think we must be guided by the concept of a certain unity in biochemistry and suppose there is probably a coenzyme Q—pardon me, a ubiquinone—mediated site, a flavin mediated site, a pyridine nucleotide mediated site, and a cytochrome mediated site possible. Now it may be that the photosynthetic apparatus has short-circuited a few of these, but it is still possible that Pinchot's work on bacteria will tell us about one site, and the work on the cytochrome site in Green's laboratory and in Boyer's laboratory will tell us about a second site. Two sites is all we want right now apparently, so it will be fruitful to operate on this assumption. As provided by Baltscheffsky, the data so far for two sites in photophosphorylation are quite good, but not convincing.

Now having gotten over this, then, the picture one derives for the photophosphorylation is that of an electron transport chain, in which the usual oxidase is missing and which has the chlorophyll in a photoactivated form as the oxidase. This idea is not new; it's quite old. I don't know how far back it goes, but I know the first I heard of it was from Hill. It doesn't make much sense of course, teleologically, for an organism that is interested in throwing away oxygen to have an oxidase; it doesn't make much sense for a strict anaerobe to have one either, and yet both of them do in photosynthesis. So on this basis, one would desire not to see an oxidase. It would create great difficulty also, at least in my mind, to see how to avoid oxidation in chloroplasts and chromatophores if there were an oxidase present. But there are some difficulties about the chlorophyll as an oxidase on the basis of potentials; I will speak of this a little bit later. Now to the cyclic and noncyclic controversy.

It seems to me this is a very much unneeded tempest. The question of cyclic and noncyclic phosphorylation arises mostly out of the desire to have a scheme which includes both the closed and open systems which are possible in photosynthesis, and which can be manifested by suitable experimental conditions. The crux of the matter is, what is the stoichiometry? I don't think we went into this very much at this meeting. If you have strict stoichiometry, then you have noncyclic without any question, that is, if you can get nothing but 1.0 or 0.95 or 1.05 for the ATP:DPN ratios. It is obvious that such values are not

found invariably and that none of the data at hand force the use of the nomenclature championed by adherents of the cyclic and noncyclic schemes. The majority opinion at our discussion last night was that there was no definitive experiment now, or in prospect, that would force adoption or abandonment of either the hypotheses of a unidirectional electron flow, on the one hand, or of a coupled backward flow on the other. At the moment, neither hypothesis can be dignified by the term "mechanism." Of course, all those who find it convenient or comfortable to use the terminology of cyclic and noncyclic should by all means be allowed to do so.

Now we come to a very important phase—the presentation of data bearing on the primary photochemical act. Here we have a great burgeoning of activity.

Nowadays, symposia on photosynthesis are dominated by characters who show us difference spectra, flash photometer traces, and so on, and we have to learn their language, I suppose. Elsewhere I have mentioned that photosynthesis falls into a number of categories, each of which is dominated by a certain discipline, and between which communications do not exist. The first discipline is radiation physics, which would include people like James Franck, and the next discipline is photochemistry, of which there are few representatives here, actually few card-carrying photochemists, because most such will never be seen dead with a thing as complicated as chlorophyll. If they do have it, they have it in a form which hardly has anything to do at all with biologically interacting photochemical systems—and for a good reason! Then there are the biochemists, who exist in a world by themselves, and whose researches begin about the time the primary act has been over for several decades, so to speak. Then there come the botanists and plant physiologists, who have seen with regret their field disappear from under their hands. Then we have the ecologists—and occasionally phenomena like Billy Sol Estes.

Some of us have spent time in a number of these disciplines and carry over certain rigid attitudes about the use of terms which have well-defined meanings in one area of research and appear to be misapplied in another. There are also divergences about what areas are critically in need of development. For myself, I feel that enzymology is not a critical phase of photosynthesis research at this time. There are lots of good enzymes around and it seems that hard work, rather than fresh ideas, is needed. The trouble comes, I think, in that shadowy region between photochemistry and biochemistry. I won't mention the trouble between the physicist and the photochemist, between whom communication is minimal. But there is lots of trouble at the point where we have people like Witt talking to people like Warburg, to take two extreme examples.

Now let me try to summarize the situation with regard to the photochemical act. The primary photochemistry is electron chemistry; it is

not the chemistry of atomic nuclei. Atoms don't move, there is not atomic chemistry, in the the sense of diffusion-limited processes. The processes which precede the photochemistry involve only photon interactions. All of this is over in $\sim 10^{-9}$ seconds, and what the photochemical apparatus has to be set up to do is to quickly grab the products of a reaction which is over in 10^{-9} seconds, and which will dissipate itself if nothing is done before the products back-react. So what we have to find out is how this is arranged.

So far we know what the primary electronic redistribution is. It involves a movement of electrons from a region of low potential to a region of high potential. The low potential is something near the oxygen electrode; the high potential is something near the hydrogen electrode. This is the simple primary reaction, and this electron drift is stabilized by the formation of products which live for as long at least as 10^{-3} seconds. They may live even longer, because we now seem to have, at least in green plant photosynthesis, two processes which can be combined over periods as long as 10 to 15 seconds, to give a high yield, or to give an enhancement in yield. It is not clear to me that it is necessary there be a two-quantum action for green plant photosynthesis; nobody's shown us *no* photosynthesis for just one quantum at one wavelength, but there is no question that there is an enhancement. Now in bacterial photosynthesis, the pressing problem which has arisen, and for which we are still awaiting the answer, is: Is there a two-quantum or one-quantum process in bacterial photosynthesis? I've heard opinions or sentiments expressed that there was a two-quantum act in bacterial photosynthesis, and I've also heard other sentiments expressed that there should be only a one-quantum act. Today, for instance, we heard numerous examples of the camp which holds that bacterial photosynthesis is merely green plant photosynthesis with the one half knocked off. This remains to be established; it is certainly simplest, however, to think of it that way. The data we have on cytochrome-bacteriochlorophyll interactions was very well summarized by Clayton. There is nothing from EPR work yet which helps us in this respect. (See "Note added in proof," page 455.)

As far as the photochemistry of bacteriochlorophyll is concerned, a topic which was taken up by Goedheer, there is very interesting work here, showing the necessity to reinvestigate the Krasnovsky reaction. The data are in extreme confusion. Livingston has been looking into this for the last two or three years and he is not certain just what the phenomena are which are being seen. I won't go into some of the strange spectra which show up in flash work, where you look at the first steps of the reaction, but there is no question that in the reduction of the dyes, especially in the reduction of chlorophyll in the Krasnovsky reaction, there appear four or five transient intermediates which live for very short periods of time before the final pink product shows up. These can be shown to have very little to do with what is finally seen

as the pink intermediate known as the Krasnovsky product, which is reduced chlorophyll.

Not much is known about the photochemistry of chlorophyll itself. It is an extremely reactive molecule even when not activated by light, and I think it would not profit us to attempt a review of the chemistry of chlorophyll now, but it would profit all of us to take a look at what is known about it when we go back home.

There is a question about the potential that is assigned to bacteriochlorophyll in the experiments of Goedheer, Kok, and others. The potential given is 0.46 volts. Now if the chlorophyll which is receiving this energy transfers an electron by a process of this type and we're going down from chlorophyll to quinone (which everybody by popular acclaim wants to have as a first resting place for the first electron) at about 0.05 volts, then 0.4 volts is all we get out of this first quantum, which has 1.3 volts in the infra-red band. So we throw away 0.9 volts! This seems very wasteful. It seems more logical to assume that the final resting place of the first electron is in a system which is at a potential much more reducing then quinone. This is what worries me about assuming an oxidation of chlorophyll, followed by a reduction of quinone, as the sole result of the first photochemical act. And perhaps, in private conversation, after this survey is over, I will hear why this is no trouble.

The observations of Goedheer appear to bear out that chlorophyll is oxidized in the course of the photochemical act; but note, there are many ways of getting oxidized chlorophyll besides charge transfer to quinone. Just to give you one example, you could have two chlorophylls in a complex which on absorption of light gives a practically complete conversion of all the energy in a quantum, with production of oxidized and reduced chlorophyll simultaneously. Then you have a double-sided sandwich, a "Dagwood," with quinone on one side, and cytochrome on the other. This seems to me to give a better looking picture, but I don't hold it up as an example of anything beautiful, even though it looks prettier than some others I've seen.

Another thing I should like to emphasize again, as I have done before, is that there is another aspect to this reaction producing a reduced chlorophyll with a potential of minus one volt, or close to it. Nobody has ever measured this reaction in chlorophyll, but they have measured it in comparable chlorins and porphyrins. Let me remind you again, the tetrapyrrole structure is symmetrical, around half-reduced and half-oxidized semiquinone structures. You can get either one in photochemistry, because photochemistry supplies the energy for both; and what happens after the excitation depends entirely on the environment in which the tetrapyrrole then finds itself. If, as in the case of the Krasnovsky reaction, for instance, the solvent is a basic solvent, an electron donor, then there will be a reduction. If the solvent is an acidic solvent, there'll be an electron acceptor there, and there will be

oxidation. This has been shown *ad nauseam* in photochemistry and in radiochemistry. You can get any product you want by manipulation of the environment.

Now the question is, what is the environment of the chlorophyll in the photosynthetic apparatus? It is my contention that the proteins, or the fats in which the chlorophyll is imbedded, are more likely to be electron donors than electron acceptors. Most of the potentials one finds in these organic substances are down in the negative reducing region. Of course, there are double bonds around, which could be acceptors, too. So some bright fellow has got to sit down and find out what is the compound which sits next to chlorophyll, and in what kind of an environment it is. This is the major problem in photosynthesis today. If we could only know this much, then it would follow quite obligatorily what the reaction chemistry was, and that would help the EPR people too. All we have heard today assures us that there is some photochemistry, and that we have plenty of reactants for the chlorophyll to react with; which they may be depends entirely on what final structural analysis shows.

In the case of bacteriochlorophyll, there is a burning question of what those bumps in the bacteriochlorophyll spectrum could mean. Nothing like them is seen in the green plant chlorophyll, which apparently differs from the bacteriochlorophyll only in that there are a few minor changes in the hydrogenation level. There is a shift of over a thousand angstroms, going from the native form to the monomer in organic solvent. Moreover, as far as I know, nobody has been wholly successful in reconstituting bacteriochlorophyll in its native form. Thus, there is a question which has been left wide open as to what the significance of these different absorption maxima are, in what is presumed to be a single tetrapyrrole structure. The very interesting experiments of Newton on conformational changes evoked by variations in ionic strengths may open avenues for elucidation of this problem.

Well, I think I have tried to summarize—and to introduce, of course, some propaganda of my own—on the status of bacterial photosynthesis. I feel we know as much about it as we do about green plant photosynthesis, and this, I say chauvinistically, with much less effort put into it in terms of man-hours, or woman-hours, than has been done with green plant photosynthesis. I believe this is because Professor van Niel, years ago, set the pattern with the comparative biochemical approach. It brought into the field people who wouldn't ordinarily look at an organism, as they wouldn't in the case of green plant photosynthesis, which was supposed to be the domain of plant physiologists, more or less! And besides, the bacteria have a much better biochemical apparatus to play with, and are much more flexible. Even though they make no oxygen, which renders them no use whatever to mammals, still the chances are we have found out more about photosynthesis by study of bacterial systems. Most of the quantitative work on

primary reactions has come out of the bacterial systems which can be manipulated in such a way as to make interpretations easier.

Well, I think last night I tipped my hand a little, by telling you all the final moral for this speech, but let me repeat it: Don't let your theories interfere with your practice.

Thank you.

Note added in proof (page 452). Recently, L. R. Blinks and C. B. van Niel have shown that in *R. rubrum* there is no enhancement either of photosynthetic rates or of photosynthetic anaerobic growth when cultures are exposed to simultaneous light beams of different wavelengths. *(vide* pp. 297-309, *Microalgae and Photosynthetic Bacteria,* ed. Jap. Soc. Plant Physiology. Univ. of Tokyo Press, 1963.)

Left, top to bottom: R. Hill, H. Baltscheffsky, L. Smith (back of head);
M. Gibbs, J. E. Carnahan; H. Gest, R. C. Fuller, L. P. Vernon;
right: scenes of the closing banquet.

APPENDIX

A BRIEF SURVEY OF THE PHOTOSYNTHETIC BACTERIA

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INTRODUCTION

The photosynthetic bacteria known at present comprise three major groups. These are:

(1) The green sulfur bacteria, or Chlorobacteriaceae, represented by the genera *Chlorobium* and *Chloropseudomonas*.
(2) The purple sulfur bacteria, or Thiorhodaceae, among which the best known are species of *Chromatium* and *Thiospirillum*.
(3) The nonsulfur purple bacteria, or Athiorhodaceae, with the genera *Rhodopseudomonas*, *Rhodospirillum*, and *Rhodomicrobium*.

Members of the first two groups are regularly found in nature in environments that contain H_2S and are exposed to light; here they often develop in such profusion that they are readily visible to the naked eye ("blooms"). Owing to their ability to use near infrared radiation for photosynthesis, the green and purple sulfur bacteria can grow at the expense of radiant energy that is not absorbed by the plant chlorophylls. This accounts for the fact that so often mass developments of the colored sulfur bacteria can be observed underneath a layer of algae and that for their cultivation in the laboratory incandescent bulbs are far more effective as a source of radiant energy than fluorescent lights, which do not emit sufficient radiation in the infrared region.

The green bacteria have a greater tolerance for H_2S than do the purple sulfur bacteria. The former are therefore apt to occur closer to the source of H_2S, which is usually generated by biological activity (sulfate reduction) in bottom sediments. Hence mass developments are frequently stratified, with the purple above the green sulfur bacteria.

Comparable blooms of nonsulfur purple bacteria have not been reported so far; but these organisms are regularly found in stagnant water, mud, and soil, from which they can be selectively cultivated in the laboratory.

As the names indicate, the Chlorobacteriaceae are green, while the Thio- and Athiorhodaceae appear in various shades of purple, red, and brown. All of the photosynthetic bacteria contain chlorophyllous as well as carotenoid pigments, and the differences in color of cultures of various types are mainly attributable to variations in the nature and proportions of the carotenoid components. Besides, the color may be influenced by environmental conditions. For example, cultures of green and purple sulfur bacteria growing in the presence of H_2S are usually opaque and pastel-colored, due to the presence of highly refractile sulfur globules. In the Thiorhodaceae these globules are found within the cells, in the much smaller Chlorobacteriaceae, outside. After the H_2S supply has been depleted and the sulfur further oxidized to sulfate, the cultures become more transparent, and the color changes to a deeper hue.

The chlorophyll of the green sulfur bacteria has been designated as "chlorobium chlorophyll." It accounts for the strong *in vivo* absorption in the near infrared region, around 750 mμ. The pigment can be extracted from these bacteria by organic solvents, such as methanol, ethanol, acetone, and diethyl ether; but such solutions do not exhibit the marked infrared absorption band. The maximum is shifted towards shorter wavelengths, and the *in vitro* absorption spectra of extracts from various strains of *Chlorobium* have revealed that there exist at least two different chlorobium chlorophylls (1). Furthermore, the recent studies of Olson and Romano (2) and of Sybesma and Olson (3) have shown that both *Chlorobium* and *Chloropseudomonas* also contain a chlorophyll that is spectroscopically indistinguishable from the major chlorophyllous component of the Thio- and Athiorhodaceae: bacteriochlorophyll *a* (see below).

The green pigment of the Thio- and Athiorhodaceae, designated as bacteriochlorophyll, has long been considered to represent a single entity, even though the *in vivo* absorption spectrum of various purple bacteria may show clear-cut differences. The absorption maxima in the infrared region are situated between 800 and 900 mμ. But some species exhibit up to three well-defined maxima, usually at 800, 850, and 890 mμ, while in others only one absorption peak, at 870 mμ, may be evident. Nevertheless, the *in vitro* absorption spectra of the bacteriochlorophyll extracted from different strains—which is shifted toward the shorter wavelength side, as in the case of chlorobium chlorophyll, and corresponds to about 770 mμ—has often been reported as identical; and this holds equally for the absorption spectra of the pheophytin solutions prepared by treatment of bacteriochlorophyll with acid. These observations have been interpreted to mean that the *in vivo* differences should be attributed (a) to the presence of a single bacteriochlorophyll which, in the native state, can be bound to different proteins; and (b) to a close spatial association of the green pigment with other substances, in particular with carotenoids (4,5). However,

the recent report of Eimhjellen et al. (6) implies that this interpretation may be inadequate; they have presented evidence for the existence of two distinct bacteriochlorophylls, which they have designated as bacteriochlorophyll *a* and *b*, respectively, in a strain of *Rhodopseudomonas*.

While the separation of the green from the purple bacteria rests on the color of the respective organisms, the subdivision of the purple bacteria into Thio- and Athiorhodaceae is based on the fact that the former are predominantly autotrophic, the latter heterotrophic. The distinction is not, however, as straightforward as was once believed. It is true that in nature the Thiobacteriaceae, as typical sulfur bacteria, are always associated with the presence of H_2S, which they oxidize by way of sulfur to sulfate, while the nonsulfur purple bacteria do not require H_2S and depend on the presence of organic substances. But some strains of small purple sulfur bacteria—and until recently these were the only Thiorhodaceae that had been obtained in pure culture—can also grow in media devoid of H_2S but supplied instead with any one of a number of organic compounds; they then behave as Athiorhodaceae in this respect. Conversely, some typical nonsulfur purple bacteria can use inorganic oxidizable sulfur compounds or molecular hydrogen as oxidation substrates, and thus mimic the behavior of the Thiorhodaceae.

For some time a distinction between the two groups could still be defended on the ground that such Athiorhodaceae cannot be cultivated in strictly inorganic media, because all members of this assemblage require one or more B-vitamins for growth. But now even this criterion can no longer be considered valid. On the one hand, *Rhodomicrobium*, allocated to the Athiorhodaceae, does not appear to require any vitamins for growth, while on the other hand it has recently been found that the growth of several Thiorhodaceae, such as the large *Chromatium* and *Thiospirillum* species, is dependent on an external supply of vitamin B_{12}, so that the designation "autotrophic" cannot be applied to these organisms in its strict sense.

The position of *Rhodomicrobium* is rather intriguing in this connection. The organism is occasionally encountered in elective cultures for Thiorhodaceae, and it has been cultivated in purely mineral media containing H_2S (unpublished results). This suggests that it might more properly have to be regarded as a member of the purple sulfur bacteria.

But apart from these difficulties it is still feasible to maintain a fairly clear distinction between the Thio- and Athiorhodaceae, partly on the basis of ecological and physiological behavior, partly also because of the striking morphological differences between the representatives of the two groups. This consideration has further raised the question whether there might not exist green bacteria that are physiologically counterparts of the Athiorhodaceae, exhibiting an essentially heterotrophic type of metabolism.

The cultures of *Chlorobium* species that have thus far been studied appear to be obligate sulfur bacteria. Even though they can grow at the expense of acetate, such growth is strictly dependent upon the simultaneous utilization of H_2S; in the absence of the latter, acetate is not used for growth (7). And Pfennig (personal communication) has failed to obtain growth of several *Chlorobium* strains in ethanol-containing media over and above the amount due to the H_2S present.

In this context the green bacterium, *Chloropseudomonas ethylicum,* discovered by Shaposhnikov et al. (8,9), is of special interest. It can grow in strictly mineral media or at the expense of ethanol, acetate, pyruvate, sugars, and some amino acids, even in the absence of H_2S, and thus displays heterotrophic tendencies. To be sure, it is also a typical sulfur bacterium, so that its physiological properties resemble those of some small *Chromatium* species rather than those of *Rhodopseudomonas* and *Rhodospirillum,* the most characteristic representatives of the Athiorhodaceae. Nevertheless, the very fact that *Chloropseudomonas* is the first green bacterium for which true heterotrophic growth has been established suggests that a concerted search for other heterotrophic green bacteria may be rewarding.

DESCRIPTION OF SOME GENERA OF PHOTOSYNTHETIC BACTERIA

The green bacteria: Chlorobacteriaceae.

Microscopic examinations of samples from natural habitats of sulfur bacteria have suggested that there exist several types of green bacteria, and these have frequently been described and named. Consequently no less than eight different genera have been proposed, each with one or two species. But nearly all the descriptions are so fragmentary that an unambiguous identification of green bacteria found in nature with any of these species is practically impossible.

Up till now, representatives of only two genera, *Chlorobium* and *Chloropseudomonas,* have been isolated and studied in pure culture, and in sufficient detail to permit a reasonably satisfactory delineation of these entities. In addition, the availability of such cultures enables students of the green bacteria to use them for comparative studies, involving new isolates as well, so that the classification of this group can be gradually improved as a result of accumulating experience.

It is therefore reasonable to limit the present account to a description of the properties of the above-mentioned genera.

Chlorobium.

This genus comprises the small, bead-shaped, nonmotile, nonsporeforming, Gram-negative, green bacteria. They often grow in chains resembling streptococci; under unfavorable conditions they tend

to form irregular, elongated cells (rods and filaments). Occasionally they appear as tightly wound coils, but the conditions under which these are formed are not clearly defined.

All *Chlorobium* strains thus far tested are strict anaerobes, and appear to be obligate sulfur bacteria.

The two hitherto recognized species, *Chl. limicola* and *Chl. thiosulfatophilum,* can be most readily distinguished by their differential behavior towards thiosulfate, which the former does not oxidize to sulfate, while the latter can do so (10).

Pfennig (personal communication) has recently isolated strains of nonmotile green bacteria that are uniformly rod-shaped. Except for the absence of flagella, they morphologically resemble *Chloropseudomonas*. Physiologically they also differ from the latter by their inability to oxidize organic substrates. They have not yet been studied in sufficient detail to determine whether it would be advisable to establish a separate taxon for these organisms.

Chloropseudomonas.

The cells of representatives of this genus are small, straight rods, nonsporeforming, Gram-negative, and motile by means of polar flagella.

They are anaerobes that can grow autotrophically in media containing H_2S, or heterotrophically at the expense of ethanol, acetate, pyruvate, sugars, and some amino acids. This feature, together with the cell shape and motility, distinguishes these green bacteria from *Chlorobium* species.

Only a single species, *Cps. ethylicum,* has so far been described (8,9).

The purple sulfur bacteria: Thiorhodaceae.

Like the green bacteria, the purple sulfur bacteria found in natural habitats represent an assortment of morphological types, which differ in the shape, size, color, and aggregation patterns of the individual cells. But they are generally much larger than the green bacteria, and can therefore be differentiated more satisfactorily on the basis of morphology alone.

Winogradsky (11) proposed a subdivision of the group into thirteen genera, under the names *Chromatium, Thiocapsa, Thiocystis, Thiodictyon, Thiopedia, Thiopolycoccus, Thiosarcina, Thiothece, Amoebobacter, Lamprocystis, Lampropedia, Thiospirillum,* and *Rhabdomonas.* The description of these genera was based entirely on observations of material collected from natural sources and of slide cultures prepared therefrom; pure cultures were not obtained.

The subsequent introduction of simple, defined media for the cultivation of Thiorhodaceae in the laboratory permitted the isolation of a number of pure cultures, representing morphologically distinguishable types (12). But studies with such cultures in media of different com-

position revealed that the morphology of each individual isolate varied considerably with the environmental conditions, so that an adequate description and classification of the strains could not be accomplished without taking this morphological variability into account. This was further complicated by the fact that the cultures of any single strain often suggested that they consisted of mixtures of cell types more or less resembling several of Winogradsky's genera. The most serious drawback was that none of the truly distinctive forms found in natural habitats could be cultivated in the laboratory under controlled conditions. This precluded an evaluation of Winogradsky's classification on the basis of comparative studies with pure cultures.

Fortunately, this situation has been drastically changed as a result of the notable advances scored by the recent work of Schlegel and Pfennig (13,14) and of Pfennig (15, and unpublished results communicated to the author). It is now clear that the previously used media are unfit for the cultivation of most species of purple sulfur bacteria and permit only a few of the small representatives to grow; these are obviously the types that were obtained in the earlier work. By successive modifications of the culture solution, involving the addition of sewage sludge, subsequently replaced by ascorbic acid and vitamin B_{12}, the use of low H_2S concentrations, repeated "feeding" and adjustment of the pH to various levels, and by introducing special regimes of incubation in light and darkness at specified temperatures, it has now been possible to prepare elective cultures of a number of the large and distinctive species of Thiorhodaceae and to isolate these in pure culture. The morphological uniformity of such cultures is impressive and supports the view that Winogradsky's concepts of speciation in this group are likely to be upheld to a large extent by future comparative studies. It is hoped that this important program will be diligently pursued and adequately supported; it may be confidently expected that the availability of the many kinds of purple bacteria that exist in nature will be of great benefit to those who are engaged in studies of the photosynthetic bacteria.

A brief description of the two best known genera follows.

Chromatium.

The only pure cultures of purple sulfur bacteria that have so far been used extensively are small species of *Chromatium*. This genus can be defined as composed of Thiorhodaceae whose cells are ovoid to vibrioid in shape, and motile by means of a single polar flagellum. When grown in media containing H_2S, the cells are stuffed with sulfur globules; these disappear after the sulfide has been used up.

It is probable that the currently used cultures, including Roelofsen's "strain D," should be assigned to the species group that includes *Chr. minus*, *Chr. gracilis*, and *Chr. minutissimum*. These species have been established on the basis of differences in the size of the cells as

found in natural environments. But because the sizes reported by different investigators range from 1.7 to 3.5 by 3.5 to 7 μ, 1 to 1.5 by 2 to 6 μ, and 1 to 3 by 2 to 5 μ, respectively, for the individual species, it is clear that at present a distinction between these species can only be arbitrary.

The available pure cultures are anaerobic and can be grown in vitamin-free mineral media with H_2S, thiosulfate, H_2, or simple organic compounds as the oxidation substrates. In sulfide media the cells may vary considerably in shape and size, depending on the sulfide concentration and pH of the medium. It now seems probable that far better media for their cultivation can be devised along the lines explored by Pfennig and that cultures in such media will provide information on which a more up-to-date description and classification can be based.

Pfennig (personal communication) has now isolated pure cultures of several large *Chromatium* species, among which *Chr. okenii* (5 to 6.5 by 10 to 20 μ) and *Chr. warmingii* (3.5 to 4.5 by 5 to 10 μ), the latter with its characteristically bipolarly arranged sulfur globules, have been definitively identified.

Thiospirillum.

One of the most conspicuous among the Thiorhodaceae is *Thiospirillum jenense,* whose spiral cells measure 3.5 to 4.5 by 30 to 40 μ. It is sometimes encountered as the nearly exclusive component of natural blooms, and a number of strains have recently been isolated in pure culture by Pfennig (personal communication).

Other *Thiospirillum* species have been described, but not yet isolated in pure culture.

The nonsulfur purple bacteria: Athiorhodaceae.

In contrast to the Thiorhodaceae, the representatives of this group are small and tend to a heterotrophic metabolism; most of them are capable of aerobic growth. They can be cultivated at the expense of any one of a variety of simple organic compounds, such as alcohols, fatty acids, hydroxy acids, di- and polybasic acids, and amino acids. All except *Rhodomicrobium* require one or more B-vitamins for growth.

Three genera can be recognized: *Rhodopseudomonas, Rhodospirillum,* and *Rhodomicrobium.* These are differentiated on the basis of cell morphology; the first two have been treated in detail elsewhere (16).

Rhodopseudomonas.

This genus is defined as composed of small, rod-shaped, polarly flagellated, nonsporeforming, Gram-negative bacteria, containing bacteriochlorophyll and a variety of carotenoids. The nature of the

latter determines the color of the cultures, which range from purplish to numerous shades of red and brown.

The four currently accepted species, *Rps. palustris*, *Rps. spheroides*, *Rps. capsulatus*, and *Rps. gelatinosa*, are distinguished on the basis of minor morphological and physiological differences, including specific B-vitamin requirements.

All strains thus far examined can grow anaerobically in the light as well as aerobically in darkness; in dark-grown cultures the production of pigments is inhibited. The presence of oxygen has little or no effect on growth in light, except in the case of certain carotenoidless mutants, which are blue-green in color. These are rapidly killed, by a bacteriochlorophyll-sensitized photoxidation, when they are exposed to light and oxygen simultaneously (17).

Rhodospirillum.

The spiral-shaped nonsulfur purple bacteria are classified as *Rhodospirillum*, with the species *R. rubrum*, *R. fulvum*, *R. molischianum*, and *R. photometricum*. Species differentiation rests on cell size and pigmentation.

The species of *Rhodospirillum* are distinct from those of *Thiospirillum* primarily by the absence of sulfur globules in the cells of the former, even if they are exposed to H_2S.

R. rubrum has a specific requirement for biotin as the only vitamin; the other species also require growth factors, but it has not yet been determined what they are.

Like the *Rhodopseudomonas* species, *R. rubrum* can grow both anaerobically in the light and aerobically in darkness. But the other three *Rhodospirillum* species appear to be strict anaerobes, and hence can be cultivated only in the light.

Rhodomicrobium.

Some years ago, Duchow and Douglas (18) described a morphologically unique species of purple bacteria. Because its mode of development bears a strong resemblance to that of the colorless *Hyphomicrobium*, it was given the name of *Rhodomicrobium*.[1]

It grows in the form of branched colonies, in which the pear-shaped cells are connected by slender tubes. These are produced by the pear-shaped cells in the course of their development, and represent a sort of germ tube, at the end of which a bud is formed. The latter grows

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[1] In the seventh edition of Bergey's Manual of Determinative Bacteriology (1957) this genus is not included among the Athiorhodaceae (p. 53-61), which, as polarly flagellated bacteria that multiply by fission, are placed in the order Pseudomonadales. Instead, because of its unusual morphology, it is grouped with *Hyphomicrobium* in the order Hyphomicrobiales, family Hyphomicrobiaceae (p. 276-78).

into a new pear-shaped cell; it becomes separated from the mother cell by the formation of a septum in the connecting tube.

Originally *Rhodomicrobium* was described as a nonmotile organism, but since then it has been found that in young liquid cultures motile cells, singly or in small groups of three or four, are present (19). The single swarm cells arise from terminal elements of the colony, and are peritrichously flagellated, in contrast to the swarm cells in *Hyphomicrobium* cultures, which are polarly flagellated.

Rhodomicrobium can be grown photosynthetically in mineral media supplied with an organic compound, lactate and ethanol being the preferred substrates, or with H_2S. It does not require vitamins, and appears to be obligatorily anaerobic. Thus it has not been cultivated in the dark.

The ability to grow at the expense of H_2S, even though development is slow under such conditions, suggests that *Rhodomicrobium* may actually be a purple sulfur bacterium.

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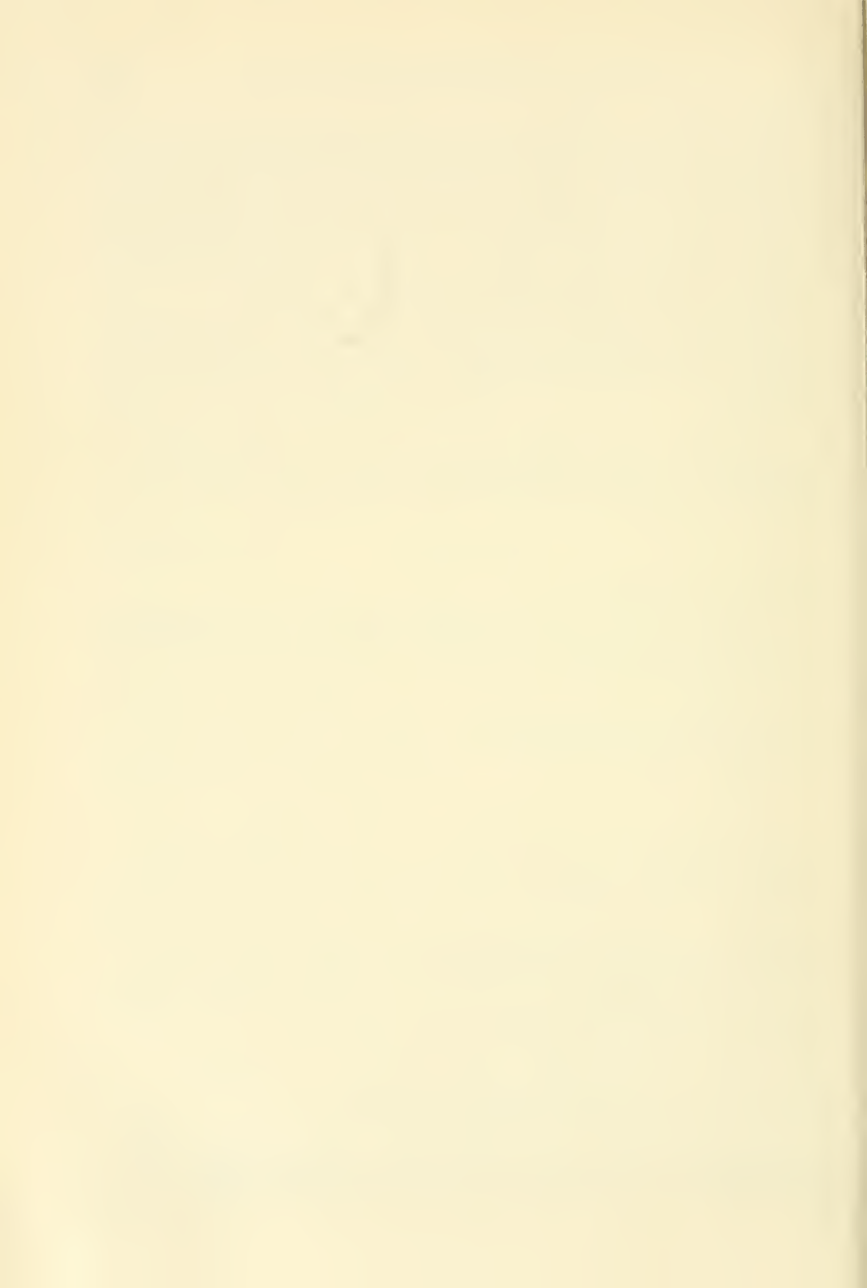

COMPOSITION OF BACTERIAL CHROMATOPHORES

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An understanding of the function of chromatophore material must ultimately depend upon knowledge of what is present in association with the pigment complex. In addition there is the feature, unique perhaps to photosynthesis as a problem, of the primary process being separated in time from subsequent biochemical events by a large order of magnitude. Hence, as frequently pointed out by Franck, a need arises for appreciable concentrations of biochemically useful reagents to be present in the chromatophore if these reagents are to be coupled effectively to the photochemical process. Possibly the photosynthetic cell has solved this problem in a way that is still obscure, but for the moment one assumes that coupling of the photophysical and photobiochemical events is reasonably direct and that a knowledge of the concentration of the various chromatophore components is very relevant to the problem.

Gross Composition.

The variable cultural conditions under which photosynthetic bacteria can be grown (1) and the changes which take place within these cells make it extremely difficult to identify essential chromatophore components by anything other than functional criteria. If one limits chromatophore function to the primary photochemical act, then by definition the only unambiguous component is bacteriochlorophyll. The problem thus becomes one of finding components always associated with the pigment complex in order to obtain clues to functional relationships. Further complicating this situation is the fact that the internal structure of the cells of photosynthetic bacteria varies under different growth conditions (2); hence attempts at isolating the photosynthetic apparatus in anything resembling its "native" state are at the moment complicated, although some progress has been made along these lines (3).

Bearing in mind the potential compositional variation in chromatophore material, one can take as a first approximation to gross chromatophore composition some early data on *Chromatium* chromatophore fractions illustrated in Table 1 (4). Such data led to the recognition

TABLE 1.

Gross Composition of Chromatium *Chromatophore Fractions* (4)

Component	Fraction sedimented at	
	25,000 X G	100,000 X G
	mg/mg protein	
Carbohydrate	0.410	0.116
P-lipid	0.53	1.03
Chlorophyll	0.020	0.040

that chromatophore material was not readily separated from the large amount of cell wall carbohydrate present in bacterial extracts. Furthermore, the pigmented fractions always contained relatively large amounts of phospholipid, which suggested a membranous origin. The enrichment of phospholipid with pigment increase in derived fractions and the simultaneous loss of polysaccharide from these fractions suggest that the pigmented material, although attached to the cell wall in some manner in the intact cell, can be divorced from it by mechanical means on cell rupture (4,5). That the cell wall and cytoplasmic membrane of gram-negative bacteria, of which photosynthetic bacteria are examples, are not readily separable has been known for some time. Probably, purified chromatophore material will ultimately be shown to contain little more than protein, pigment and phospholipid as major constituents. The bacterial analog of starch formation in chloroplasts appears to be synthesis of the cell wall polysaccharides and slime layers; though glycogen deposits can be observed in the bacterial cells, the deposits are not structurally associated with the photosynthetic apparatus (6).

Compositional Variation.

The pigment composition of cells of photosynthetic bacteria can vary extensively with growth conditions as was shown by Cohen-Bazire *et al.* (1). Regulating factors are light intensity, aerobiosis and the electron donor supplied for growth. A control point for this regulation may be the redox level of a cellular respiratory carrier. The chlorophyll-carotenoid ratio of the cells can also be varied although the two pigment classes respond as a group to similar factors (Table 2). This response indicates no rigid control over the introduction of the pigments into chromatophore material as a stoichiometric combination. Furthermore, chromatophore formation does not require simultaneous introduction into the structure of colored carotenoids, as indicated by work with carotenoidless mutants, which yield photosynthetically active chromatophore material containing chlorophyll but devoid of carotenoids.

TABLE 2.

Chlorophyll-Carotenoid Ratios of R. rubrum *Cells Grown Under Different Conditions* (7)

Electron donor	Light intensity, ft.-c.	Chlorophyll / Carotenoids
Acetate	1100	1.62
Succinate	1100	4.25
Malate	860	3.90
Malate	200	5.35

Measurements on intact cells provide little information about the variability of chromatophore composition and about possible means of chromatophore replication. Although chromatophore material constitutes as much as 40% of the total cell protein, it is desirable to study compositional variation with isolated cell fractions. Cohen-Bazire and Kunisawa (8) made a start in this direction by isolating chromatophore fractions by differential centrifugation in sucrose gradients and examining the chlorophyll-protein ratios in the isolated fractions derived from cells undergoing rapid pigment synthesis. Table 3 contains some of their results, which show that under these conditions the percentage of cell protein in the chromatophore fraction does not change appreciably even though the chlorophyll-protein ratio in the chromatophore fraction is changing greatly. These data suggest that during pigment

TABLE 3.

Specific Chlorophyll Contents of R. rubrum *Cells and Chromatophores During Induction of Pigment Synthesis* (8)

Hours growth	Chlorophyll, μg/mg protein		Total cell protein in chromatophores, %
	Extract	Chromatophores	
1	1.55	3.28	47.3
2	2.68	7.37	36.4
4	9	22.6	40
6	11.8	31.5	37.5

synthesis there is not *de novo* synthesis of the entire photosynthetic apparatus. A similar conclusion is suggested by the finding that dark-grown cells of *Rhodospirillum rubrum* contain large amounts of antigenic material which cross-reacts serologically with specific chromatophore antibody (9). These data also suggest that studies of the dark phosphorylation system in *R. rubrum* may ultimately aid in under-

standing the light-activated system, which could be a variant form of the respiratory enzyme complex (10).

On the other hand, studies by Sistrom (11,12) show clearly that pigment formation cannot take place in the absence of protein synthesis in *Rps. spheroides*, even in cells which were forming pigments before protein synthesis was inhibited and, hence, contain the requisite enzymes for bacteriochlorophyll formation. To reconcile this observation with the other data indicating that chromatophore formation does not take place *de novo*, Sistrom suggests that chromatophore material once formed cannot be modified and that subsequent material formed under different growth conditions has a different composition. Consequently the average composition will vary, but this average represents a variety of chromatophore material of greatly different composition. If his suggestion is correct, the different species of particles might be physically separable because they do vary greatly in composition.

None of the present models of chromatophore formation satisfactorily account for the observed fact that chromatophore material contains unique antigens not present in the cell grown in the dark (9). If the chromatophore is not formed *de novo*, then some unique structural feature must be added to it during chlorophyll insertion. Similarly, any hypothesis concerning chromatophore compositional variability must take into account the fact that the basic structure does contain a colorless component not present in dark-grown cells. Conceivably a chlorophyll bearer moiety is in the chromatophore; it is the unique chromatophore antigen and its formation is required coincident with chlorophyll deposition in the photochemical apparatus; hence the requirement for protein synthesis during chlorophyll formation. If this antigen represents a small fraction of the total protein of chromatophore material, then its addition to the structure would not be readily detectable by gross compositional analysis.

Electron Transport Components.

Table 4 lists the relative concentrations of chlorophyll and various electron transport constituents frequently associated with chromatophore fractions. Except for quinone content, all the data were obtained on the same preparation and should not be subject to errors of extrapolation from one set of growth conditions to another. Apparently the preparation contains the minimum requirements of a "conventional" electron transport system. In early experience with such analyses, the cytochrome *c* component proved the most tenaciously bound constituent of the particles and was the only one consistently enriched by increasing chlorophyll content of the fraction.

These early data indicated the presence of large amounts of nonheme iron in the chromatophore preparations. This simple approach had forecast the possible involvement of nonheme iron components

TABLE 4.

Pigments and Electron Transport Components in Chromatium *Particles* (4,13)

	mμmoles/mg protein
Chlorophyll	40
Cytochrome (total)	3
Flavin	0.5
Pyridine nucleotide	1.0
Coenzyme Q	10
Total Fe	29

in photosynthetic electron transport, though the nature of the nonheme iron was obscure at the time. This fact points out the potential value of studying chromatophore composition: the nature of possible unrecognized functional chromatophore components can be suggested on purely chemical grounds. With the sophisticated methods of analysis currently available, this area of research should continue to be fruitful to biochemists with the patience required to pursue structural work in the absence of any immediate and obvious functional relationships.

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SPECTROSCOPIC PROPERTIES OF PURIFIED CYTOCHROMES OF PHOTOSYNTHETIC BACTERIA[1]

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The primary reason for studying pure cytochromes isolated from photosynthetic bacteria is to correlate chemical and physical properties of an isolated cytochrome with the functional role of that cytochrome in light-induced or dark electron transfer processes of the cell. In addition, as emphasized by Kamen in this volume, p. 61, the cytochromes that have been isolated from photosynthetic bacteria possess distinctive properties which give them intrinsic interest as heme proteins. Here is summarized the available information on spectroscopic properties of various types of pure cytochromes isolated from photosynthetic bacteria.

METHODS

When investigating absorption properties of the Soret band, cytochrome concentration is chosen to give ferro-Soret bands of nearly 1 absorbancy. For the visible region 3-fold greater concentration is used. The original Cary model-14 spectrophotometer tracings are reproduced to facilitate interpolation of wavelength or absorbancy data and to preserve as faithfully as possible minor inflections in the absorption curves which seem always to be obliterated in ink tracings of the original spectra.

Before dilution, cytochrome *c* samples are oxidized by adding excess ferricyanide to buffered stock solutions, after which the mixture is passed through a G-25 Sephadex column to remove salts. Two successive treatments with ferricyanide are necessary to completely oxidize the cytochromes. The cytochromoid C (RHP)[2] samples and

[1] The work reported here was performed in the laboratory of Professor M. D. Kamen with support from the National Institutes of Health (Grant No. C-5992), National Science Foundation (Grant No. G-19642), and the C. F. Kettering Foundation.

[2] Cytochromoid C is the name suggested by the International Union of Biochemistry Commission on Enzymes (1) for the heme protein previously named RHP.

the *Chromatium* cytochrome 553 become completely autoxidized during purification and require no further treatment. Sodium dithionite is used to reduce the cytochromes. For spectra of carbon monoxide complexes, the reduced cytochrome solution is equilibrated with pure carbon monoxide at 1 atmos. pressure in an anaerobic cuvette. The cuvette contents are first degassed by evacuating with a vacuum line and then flushing with high-purity argon.

Dr. S. Taniguchi supplied *R. rubrum* cytochromes and H. de Klerk supplied the *Rps. palustris* cytochrome samples.

RESULTS AND DISCUSSION

Spectroscopic properties.

Absorption maxima and corresponding molar extinction coefficients of various states of cytochrome c_2 from *R. rubrum* and *Rps. palustris* plus the complex cytochrome 553 of *Chromatium* are listed in Table 1, and corresponding data for cytochromoid C of *R. rubrum*, *Rps. palustris* and *Chromatium* are listed in Table 2. The spectra of these cytochromes are presented in Figs. 1-6, respectively. These particular cytochromes were chosen because homogeneous samples were at hand for preparation of the spectra. Few other pure cytochrome preparations from photosynthetic bacteria have been reported; for none of these were extinction coefficients determined, nor were difference spectra presented which might aid in the study of the cytochrome in the native bound form. Incomplete spectra of *Rps. spheroides* cytochrome 553 and cytochrome *b* are reproduced in Figs. 7 and 8, respectively. In Table 3 are summarized some properties of incompletely characterized purified cytochromes obtained from both purple and green photosynthetic bacteria.

Molar extinction values listed in Tables 1 and 2 are calculated in two ways. For the *R. rubrum* and *Chromatium* proteins, values based on dry weight and molecular weight determinations are presented. In addition, values are calculated in terms of the heme concentration of each cytochrome sample. Heme concentrations were estimated from alkaline pyridine hemochromogen spectra, using Drabkin's value for the molar extinction coefficient of the 550 mμ absorption peak of the cytochrome *c* derivative, $a_M \times 10^{-3}$ liter/mol. cm. = 29 (9). To prepare the pyridine hemochromogen derivatives, the samples were diluted in 0.1 M NaOH-25% v/v pyridine and then 1-2 mg. dithionite per ml. was added. After a five-minute delay the absorption spectrum in the visible region was measured *vs*. a solvent blank. In every instance the α peak was located at 550 $\pm$ 0.5 mμ, indicating that the proteins contain heme groups like that of cytochrome *c*. The relatively close agreement between values determined in these two ways supports the notion that, if a rigorous determination of molecular weight is im-

TABLE 1.

Molar Extinction Coefficients at Absorption Maxima of c-*type Cytochromes of Photosynthetic Bacteria (pH 7)*

$\left(a_M \times 10^{-3}\right)$liter/mol.cm.

State of cytochrome	*R. rubrum* cytochrome c_2*				*Rps. palustris* cytochrome c_2		*Chromatium* cytochrome 553		
	$\lambda_{m\mu}$	a_M (2)	$\lambda_{m\mu}$	a_M/heme	$\lambda_{m\mu}$	a_M/heme	$\lambda_{m\mu}$	a_M (3)	a_M/3heme
ferri-	-	-	560 shldr.	6.6	560 shldr.	7.2	562 shldr.	-	18.9
	525	10.5	527	10.6	527	11.0	525	26	31.2
	-	-	-	-	-	-	480	-	31.5
	410	115	412.5	116	412	117	410	320	375
	357	30	-	-	-	-	-	-	-
	275	25	-	-	-	-	278	175	-
ferro-	550	28.1	551	26.9	552	26.7	553	61	75.6
	521	17.0	522	16.7	522	16.8	523	41.6	50
	415	143	416.5	132	417.5	136	416	364	435
	316	37	-	-	-	-	-	-	-
	272	34	-	-	-	-	-	-	-
Difference spectrum: ferro- minus- ferri-	-	-	565 trough	-4	566 trough	-4	568 trough	-	-9.4
	-	-	551	19.4	552	19	553	44.6	55
	-	-	536 trough	-2.7	536 trough	-2.5	538 trough	-	-3.9
	-	-	522	6.2	522	6.1	524	15.5	18.5
	-	-	451 trough	-7.6	450 trough	-8.8	452 trough	-	-39
	-	-	421	47	421	50	422	165	195
	-	-	404 trough	-20	403 trough	-22.6	405 trough	-	-124
CO-ferro- complex							555	50.5	62
							533 shldr.	38.8	37
							525	-	45.6
							414	500	580
Difference spectrum: CO-ferro- minus- ferro-							565	17.5	10.1
							552 trough	-	-14.2
							535	10.7	6.2
							522 trough	-	-6.1
							424 trough	-	-61
							414	165	165

* An unexplained systematic difference in the wavelength of absorption maxima for *R. rubrum* cytochrome c_2 has been noted between the results of Horio and Kamen, shown in the first column, and those determined for this report, which are shown in the third column.

TABLE 2.

Molar Extinction Coefficients at Absorption Maxima of Cytochromoid C *from Photosynthetic Bacteria (pH 7)*

$\left(a_M \times 10^{-3}\right)$liter/mol.cm.

State of cytochrome	*R. rubrum* cytochromoid C			*Rps. palustris* cytochromoid C		*Chromatium* cytochromoid C		
	$\lambda_{m\mu}$	a_M (2)	a_M/2heme	$\lambda_{m\mu}$	a_M/2heme	$\lambda_{m\mu}$	a_M (3)	a_M/2heme
ferri-	638	5.9	6	642	5.5	635	7.2	5.4
	497	21.5	22.3	500	20.8	495	24.5	20.4
	390	159	177	398	170	400	192	174
	282	44	-	283	42	280	63	-
ferro-	566 shldr.	-	22	570 shldr.	20	569 shldr.	-	17
	550	22	23	552	20.8	547	22.7	19.6
	432 shldr.	171	192	435 shldr.	184	437 shldr.	-	163
	423	175	196	426	192	426	210	188
Difference	640 trough	-	-3.7	642 trough	-3.8	635	-4.3	-3.6
spectrum:	563	-	15.6	567	14.2	565	5.4	12.6
ferro-	487 trough	-	-13.6	490 trough	-13.2	490 trough	-14.4	-12.8
minus-	433	-	165	436	162	437 shldr.	-	141
ferri-	426 shldr.	-	160	425 shldr.	150	429	158	146
	392 trough	-	-93	395 trough	-84	398 trough	-115	-92
CO-ferro-	564	-	24	570	22	565	23.8	23
complex	534	-	25.6	535	25.2	545	28	26.8
	416.5	-	480	418	524	418	583	564
Difference	590 trough	-	-2.8	592 trough	-4.8	590 trough	-0.3	-2.2
spectrum:	569	-	2.3	572	3.4	565	3.6	4.2
CO-ferro-	553 trough	-	-0.3	555 trough	-2	550 trough	0.3	2
minus-	534	-	6.2	534	8	534	7.2	7.7
ferro-	505 trough	-	.45	518 trough	0.2	508 trough	0.1	1.0
	434 trough	-	-130	436 trough	-151	437 trough	-	-120
	416.5	-	338	417	360	418	404	522

TABLE 3.

Absorption Maxima of Incompletely Characterized Purified Cytochromes from Photosynthetic Bacteria

Organism	*c*-Type cytochrome	CO complexing cytochrome	*b*-Type cytochrome	Ref.
Rhodomicrobium vannietii	cytochrome c_2 ferro α 550 mμ " β 521 " " γ 415 " partly purified	particle bound, not purified	particle bound, not purified	4
Rhodopseudomonas spheroides	cytochrome 553 ferro α 553 mμ " β 523 " " γ 418 " homogeneous MW ≃ 25,000 $E'_{m,7}$ = +0.12 volt	soluble, not yet described	cytochrome *b* ferro α 559 mμ " β 529 " " γ 426 "	5,6
Rhodopseudomonas palustris	cytochrome c_2 ferro α 552 mμ " β 522 " " γ 418 " crystallized, homogeneous MW ≃ 15,600 $E'_{m,7}$ = +0.33 volt	see Table 2	particle bound, not purified	7
Chlorobium thiosulphatophilum	cytochrome 554 ferro α 554 mμ " β 523 " " γ 417 " homogeneous MW ≃ 12,000 $E'_{m,7}$ = +0.14 volt cytochrome 553 ferro α 553 mμ " β 522 " " γ 416 " ~75% pure MW ≃ 45-75,000 $E'_{m,7}$ = +0.163 volt	not detected	not detected	8

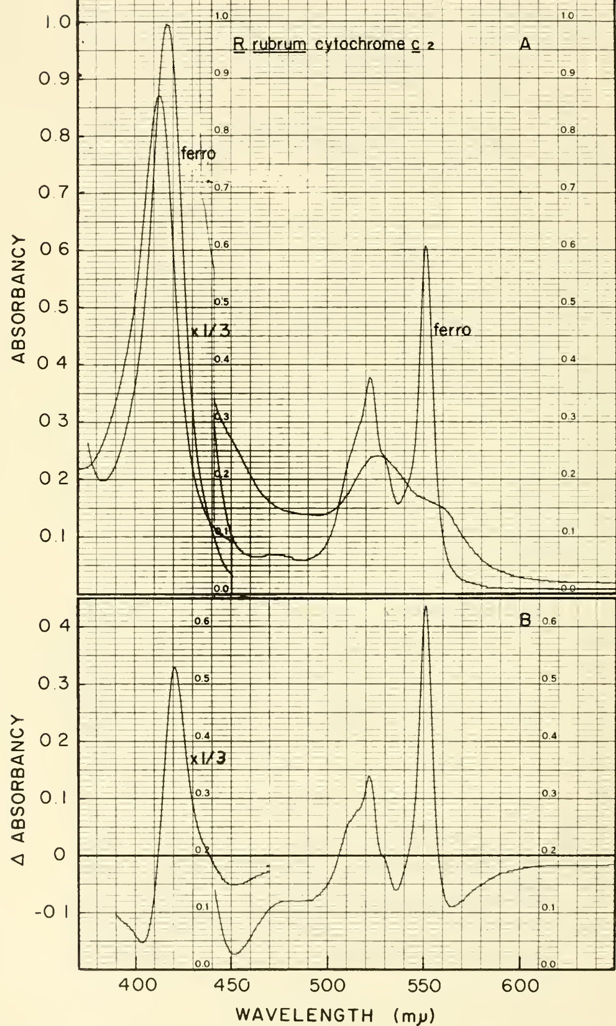


Fig. 1. *R. rubrum* cytochrome c_2 ferro- and ferri- absolute spectra (A) and ferro-minus-ferri- difference spectrum (B) at pH 7 (50 mM potassium phosphate buffer), d = 1.0 cm. The heme concentration for the visible region spectra is 22.6 μM and for the Soret region is 7.5 μM.

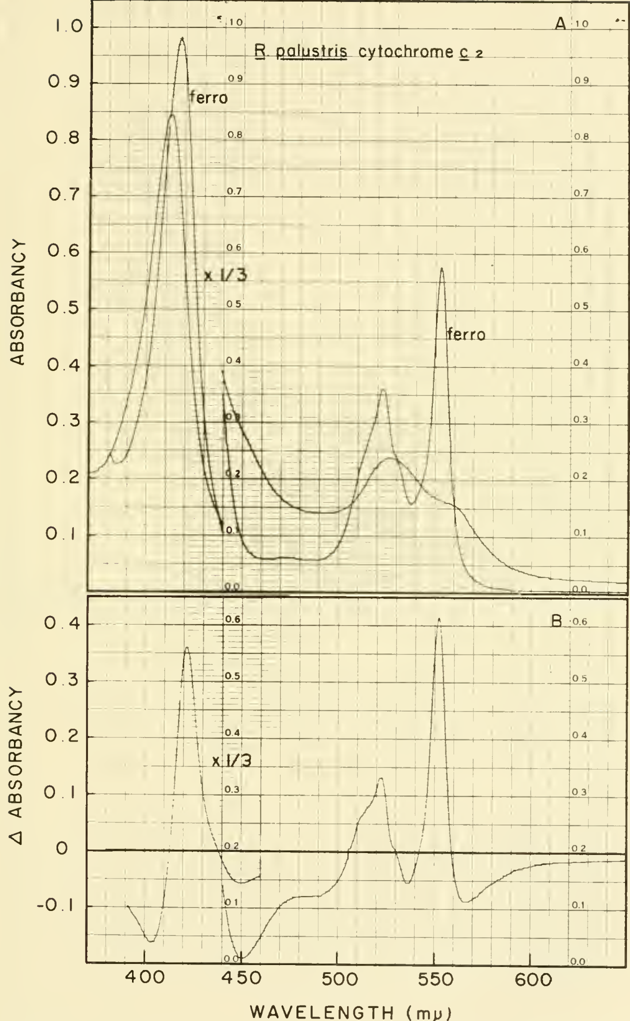
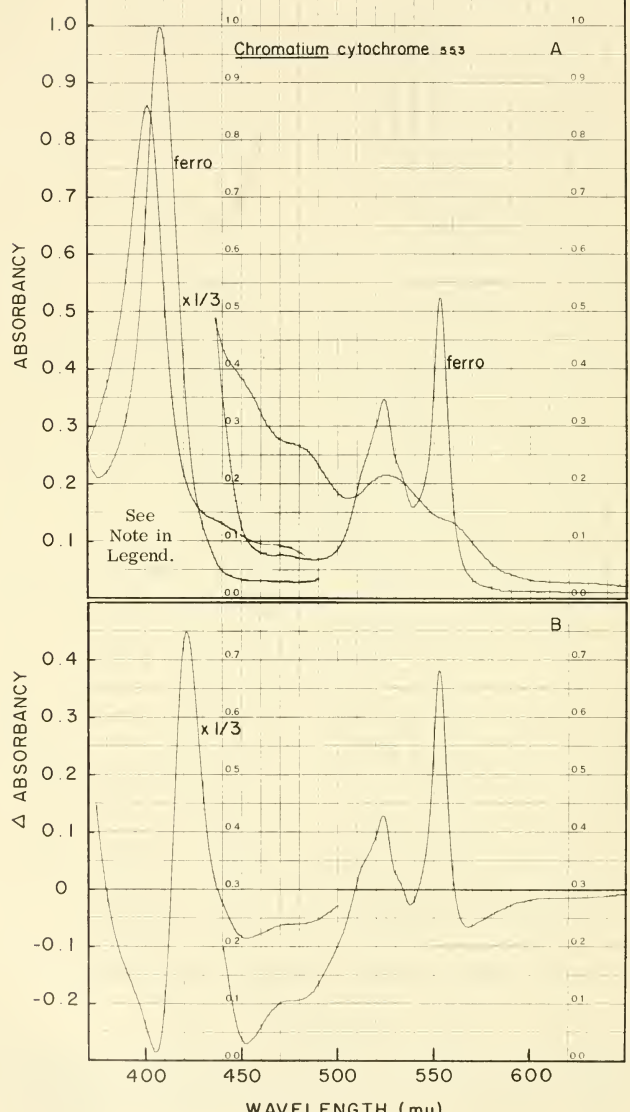


Fig. 2. *Rps. palustris* cytochrome c_2 ferro- and ferri- absolute spectra (A) and ferro-minus-ferri- difference spectrum (B) at pH 7 (50 mM potassium phosphate buffer), d = 1.0 cm. The heme concentration for the visible region spectra is 21.6 μM and for the Soret region is 7.3 μM.


Chromatium cytochrome 553
A
ferro
x 1/3
ferro
See Note in Legend.
ABSORBANCY
B
x 1/3
Δ ABSORBANCY
WAVELENGTH (mμ)
400
450
500
550
600

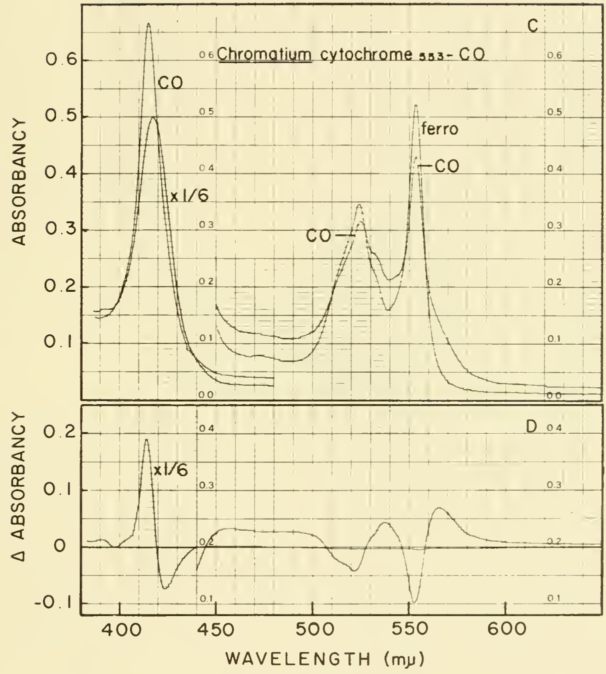


Fig. 3. *Chromatium* (strain D) cytochrome 553 ferro- and ferri- absolute spectra (A), ferro-minus-ferri- difference spectrum (B), CO-ferro- absolute spectra (C), and CO-ferro-minus-ferro- difference spectrum (D) at pH 7 (50 mM potassium phosphate buffer), d = 1.0 cm. For the CO spectra the gas phase is 1 atmos. CO. The heme concentration for the visible region spectra is 20.7 μM (x 1/3 = 6.7 μM cytochrome concentration) and for the Soret region is 6.7 μM for A and B, and 3.45 μM for C and D. *(Note added in proof.* The soret peaks in (A) are accidentally displaced 10 mμ to the left relative to the wavelength scale.)

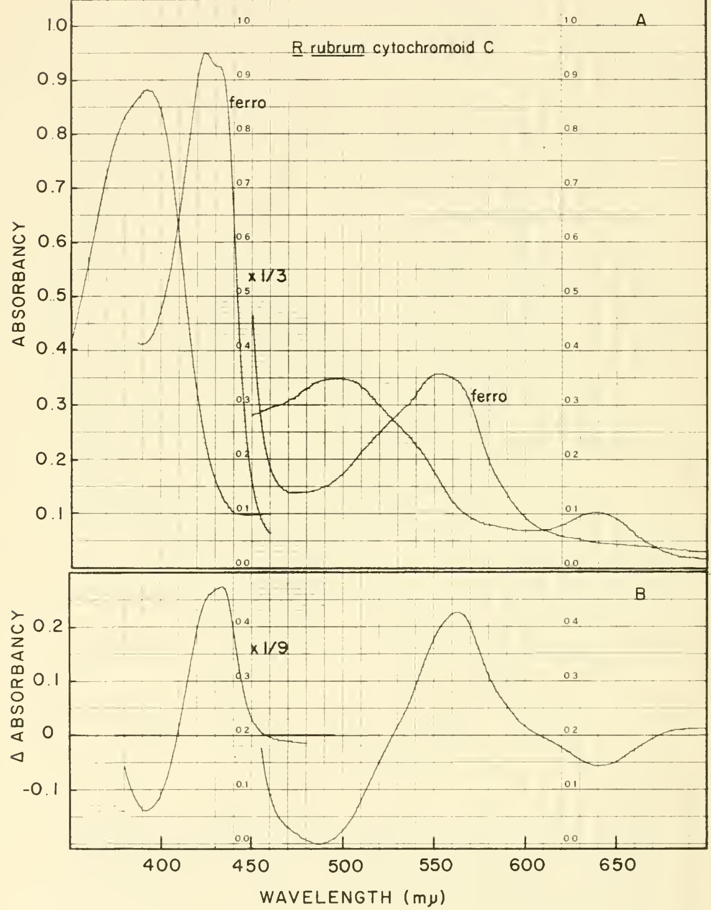
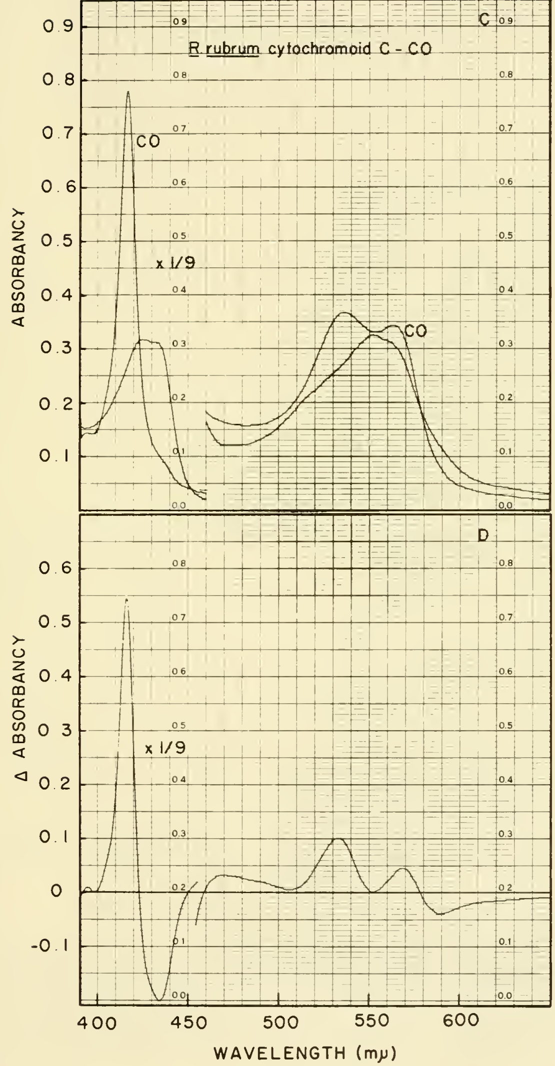


Fig. 4. *R. rubrum* cytochromoid C (RHP) ferro- and ferri- absolute spectra (A), ferro-minus-ferri- difference spectrum (B), CO-ferro- absolute spectra (C), and CO-ferro-minus-ferri- difference spectrum (D) at pH 7 (50 mM potassium phosphate buffer), d = 1.0 cm. For the CO spectra the gas phase is 1 atmos. CO. The heme concentration for the visible region spectra is 29.1 μM (14.55 μM cytochrome concentration) and for the Soret region is 9.7 μM for A and 3.23 μM for B, C and D.



R. rubrum cytochromoid C - CO
C
D
CO
CO
x 1/9
x 1/9
ABSORBANCY
Δ ABSORBANCY
WAVELENGTH (mμ)
400
450
500
550
600
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1

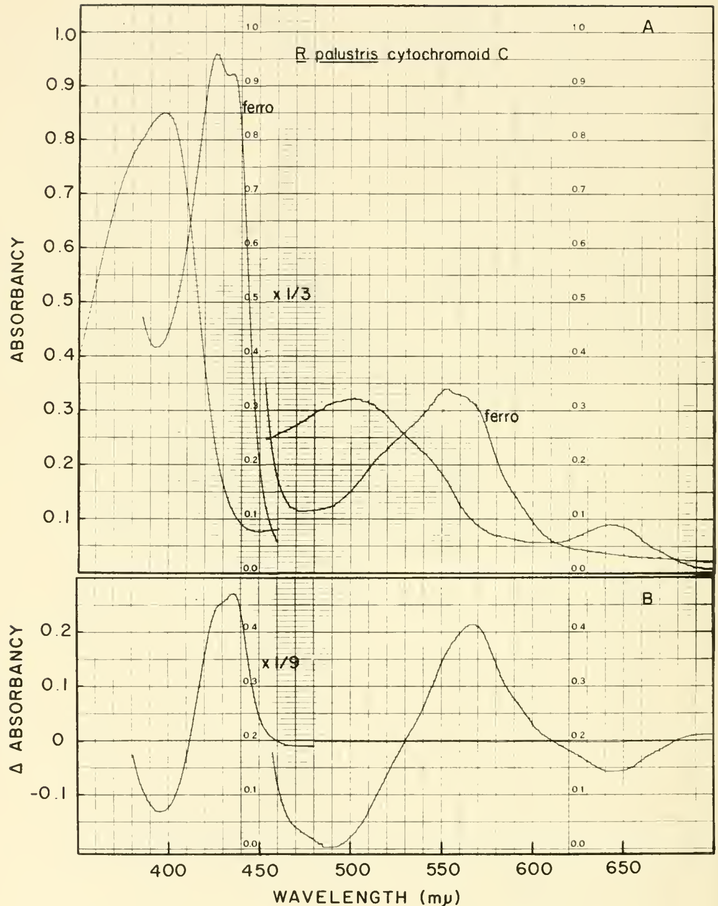
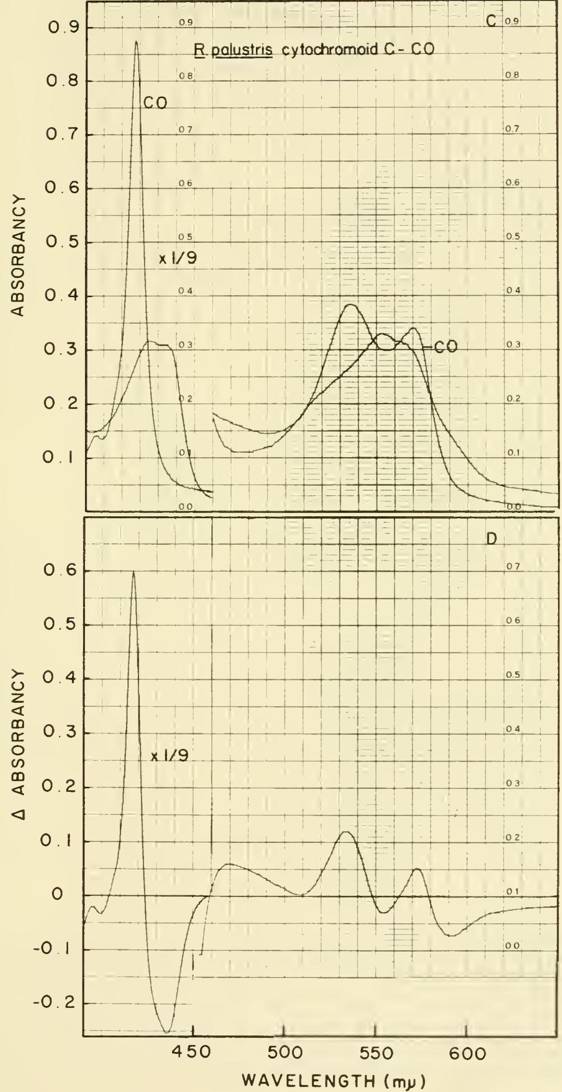


Fig. 5. *Rps. palustris* cytochromoid C (RHP) ferro- and ferri- absolute spectra (A), ferro-minus-ferri- difference spectrum (B), CO-ferro- absolute spectra (C) and CO-ferro-minus-ferro- difference spectrum (D) at pH 7 (50 mM potassium phosphate buffer), d = 1.0 cm. For the CO spectra the gas phase is 1 atmos. CO. The heme concentration for the visible region spectra is 30 μM (15 μM cytochrome concentration) and for the Soret region is 10 μM for A and 3.33 μM for B, C and D.



R. palustris cytochromoid C - CO
C
D
CO
x 1/9
-CO
x 1/9
ABSORBANCY
Δ ABSORBANCY
WAVELENGTH (mμ)
450
500
550
600

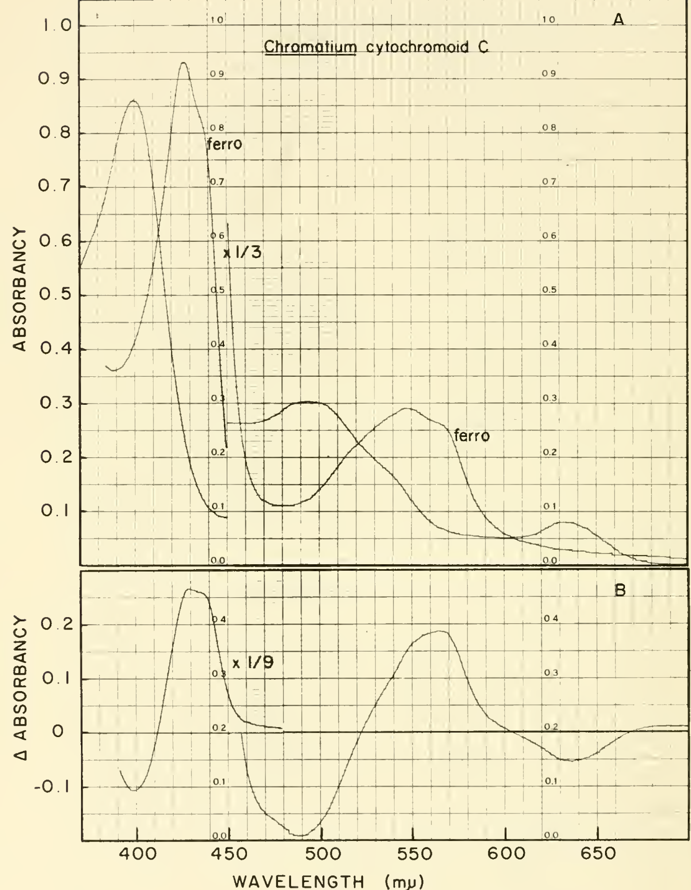
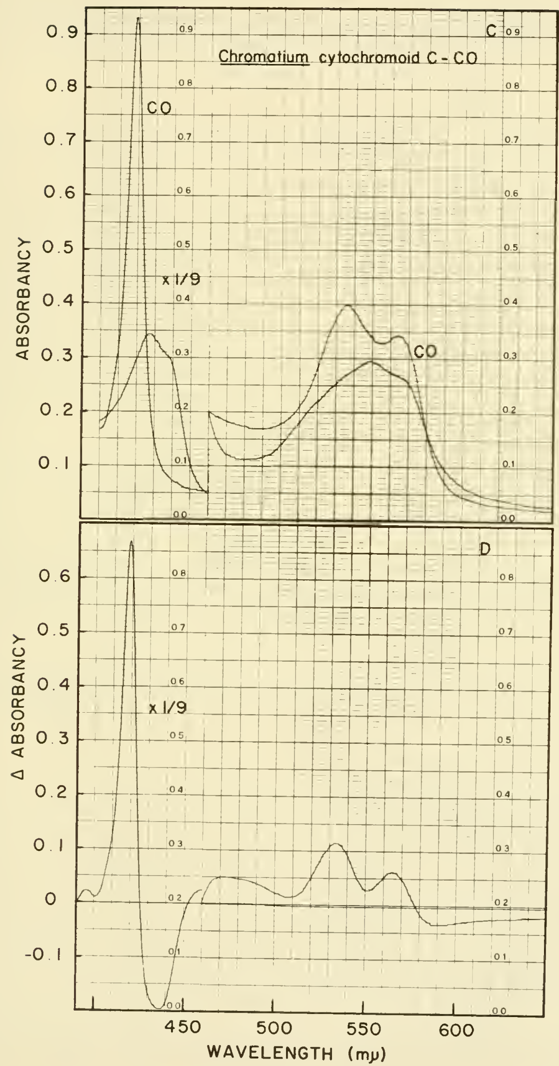


Fig. 6. *Chromatium* strain D cytochromoid C (RHP) ferro- and ferri- absolute spectra (A), ferro-minus-ferri- difference spectrum (B), CO-ferro- absolute spectra (C) and CO-ferro-minus-ferro- difference spectrum (D) at pH 7 (50 mM potassium phosphate buffer), d = 1.0 cm. For the CO spectra the gas phase is 1 atmos. CO. The heme concentration for the visible region spectra is 29.7 μM (14.85 μM cytochrome concentration) and for the Soret region is 9.9 μM for A and 3.3 μM for B, C and D.



Chromatium cytochromoid C - CO
C
D
CO
x 1/9
ABSORBANCY
Δ ABSORBANCY
WAVELENGTH (mμ)
450
500
550
600

practical, the sacrifice of a small amount of pure material for determining the extinction per heme gives a useable approximation to the true extinction coefficient of cytochromes which contain *c*-type heme groups.

Properties of c-*type cytochromes.*

Cytochrome c_2 is a heme protein of approximately 12,000 molecular weight (2) which contains one heme bound to the peptide chain by two thioether bonds as in cytochrome *c* (10). Cytochrome c_2 isolated from various sources has a relatively high oxidation-reduction potential, $E'_{m,7}$ = +0.29-0.33 volt (2,11). Inasmuch as cytochrome c_2 is the only *c*-type cytochrome found in *R. rubrum* and *Rps. palustris*, it is probably the *c*-type cytochrome which is rapidly oxidized when the bacteria are illuminated (12). Thus cytochrome c_2 may be considered to be a bacterial analog of green plant cytochrome *f* and may function as the primary reductant of light-activated bacteriochlorophyll.

Cytochromes with spectroscopic and chemical properties different from those of c_2 have been isolated from certain of the photosynthetic bacteria. *Chlorobium thiosulphatophilum* contains two, cytochrome 554 and cytochrome 553. In addition to cytochrome c_2, *Rps. spheroides* also contains a cytochrome 553 which may function in the aerobic metabolism of the organism (5). *Chromatium* may contain a c_2-type cytochrome, as is suggested elsewhere in this volume, p. 315, but such a cytochrome has not yet been isolated in soluble form. There is present in *Chromatium* a complex cytochrome 553 which contains three cytochrome *c*-type heme groups plus one FMN in a molecule of about 97,000 molecular weight (3,13). This heme protein forms a complex with carbon monoxide, perhaps involving but one of the three hemes, with resultant formation of an anomalous absorption spectrum (see Fig. 3). There is evidence that a cytochrome with these spectroscopic properties undergoes light-induced reactions in the *Chromatium* cell (14).

Properties of cytochromoids.

The cytochromoid C-type cytochromes are diheme proteins of about 26-28,000 molecular weight which form a complex with carbon monoxide (15,3,2) and are found in all the purple bacteria examined to date. The molecular weight of 37,000 (3) reported for *Chromatium* cytochromoid C may be too high on the basis of quantitative end-group analysis (personal communication from Dr. K. Dus). The heme groups possess many of the properties of *c*-type hemes as is indicated by heme splitting experiments (16) and by the properties of the diheme peptide prepared from *Chromatium* cytochromoid C (17). These heme proteins are autoxidizable with $E'_{m,7} \cong 0.0$ volt (2,3,15). Molecules with cytochromoid C spectroscopic properties do not engage directly

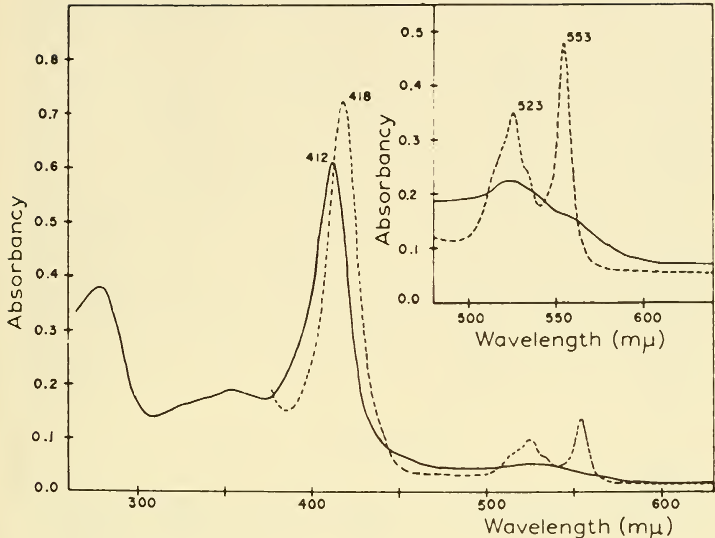


Fig. 7. *Rps. spheroides* cytochrome 553 ferro-, - - - -, and ferri-, ———, absolute spectra (concentration and pH of cytochrome not stated) (5). Reproduced by permission of *Biochimica et Biophysica Acta.*

in light-induced reactions (18) in the bacteria. The role of cytochromoid C is still unclear.

Small differences are noted among the spectra of the three examples of cytochromoid C presented above, but the spectra clearly belong to members of a single class of heme proteins. The doublet character of the absorption bands is most apparent in the Soret band of reduced *Rps. palustris* cytochromoid C. Presumably small differences in the immediate environment of the hemes in the several proteins account for variations in the contribution of each heme to an absorption peak, with consequent small shifts in extinction values and absorption maxima. The affinity of *R. rubrum* cytochromoid C for carbon monoxide is such that about 1.5 atmos. CO is needed for complete reaction (19). The relatively higher CO-ferro-complex Soret extinction values obtained with *Rps. palustris* and *Chromatium* cytochromoid C may indicate that these heme proteins have greater affinity for CO than does that of *R. rubrum.*

It has been suggested that cytochromoid C is identical with cytochrome *o* (20), a bacterial oxidase which remains a spectroscopic

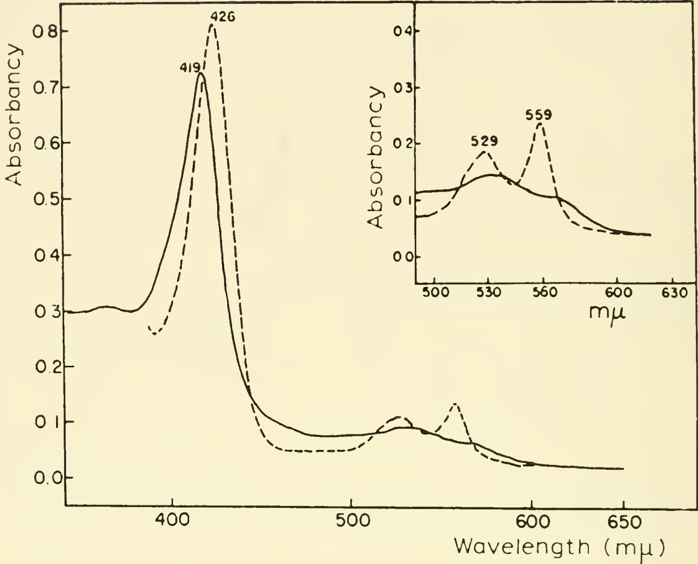


Fig. 8. *Rps. spheroides* cytochrome *b* ferro-, - - - -, and ferri-, ———, absolute spectra (concentration and pH of cytochrome not stated) (6). Reproduced by permission of *Biochimica et Biophysica Acta*.

entity, best detectable from the action spectrum of the relief of carbon monoxide inhibition of oxygen uptake by intact bacterial cells (21). Taylor and Horio concluded from the close similarity between such an action spectrum determined with *R. rubrum* and the absorption spectrum of *R. rubrum* CO-ferro-cytochromoid C complex that the two cytochrome types were probably identical (22). It would be of interest to test the obligate photoanaerobe *Chromatium* in this way to determine if *Chromatium* cytochromoid C could mimic cytochrome *o* properties. Cytochrome *o* activity is widespread among bacteria, including purple bacteria, and deserves intensive study.

Properties of b-*type cytochromes.*

Cytochromes of the *b* type have been detected in intact cells of photosynthetic bacteria and have been implicated in light-induced electron transfer processes (12,18). Both cells and chromatophore preparations of *Rps. palustris* and *Rps. spheroides* show a clearly

differentiated *b*-type ferro-minus-ferri- difference spectrum with peaks at approximately 560 and 430 mμ. With *R. rubrum,* a cytochrome *b* component is less obvious. Interfering pigments are first removed by washing cells or chromatophore preparations with acetone, and the residue is suspended in alkaline pyridine. After dithionite is added to reduce the heme derivatives, a distinct pyridine hemochromogen spectrum of a *b*-type cytochrome can be seen, with an α peak at 556 mμ (11). With *Chromatium* as well as with *Chlorobium thiosulphatophilum* (8) even this strategem is fruitless, and perhaps no *b*-type cytochrome is present in these species. Possibly cytochromoid C may be mistaken for cytochrome *b* in whole cells or particle preparations because ferro-minus-ferri- difference spectra of both types of cytochromes produce marked peaks or shoulders in the region of 560 mμ and 430 mμ. A small amount of soluble cytochrome *b* has been isolated from *Rps. spheroides* (6), but in general the cytochrome remains tightly bound to the cellular particles of several species that have been examined.

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ABSORPTION SPECTRA OF PHOTOSYNTHETIC BACTERIA AND THEIR CHLOROPHYLLS

RODERICK K. CLAYTON

The materials selected for presentation are from *Rhodospirillum rubrum, Rhodopseudomonas spheroides, Chromatium, Rhodomicrobium vannielii, Chlorobium thiosulfatophilum,* and *Chloropseudomonas ethylicum.* Figs. 1-3 pertain to chromatophores suspended in distilled water. The *Chromatium* chromatophores were donated by Dr. R. C. Fuller. Figs. 4 and 5, for whole cells, were submitted by Dr. J. M. Olson. The relative heights of the bands at 800, 850, and 870 to 890 mμ in *Rps. spheroides* and *Chromatium* vary considerably according to the culture conditions.

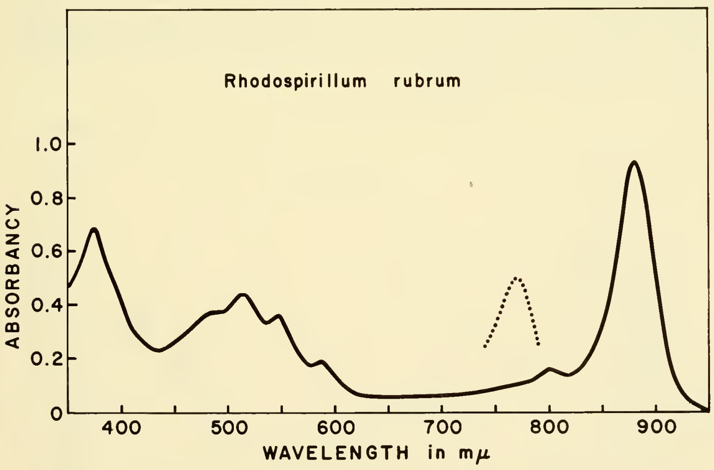

Fig. 1. Absorption spectrum of *Rhodospirillum rubrum,* strain S-1. Solid curve, chromatophores in water. Dotted curve, the same concentration of BChl in acetone/methanol, 7 vol/2 vol. A blue-green mutant of *R. rubrum,* isolated by Dr. J. W. Newton, has essentially the same near-infrared spectrum.

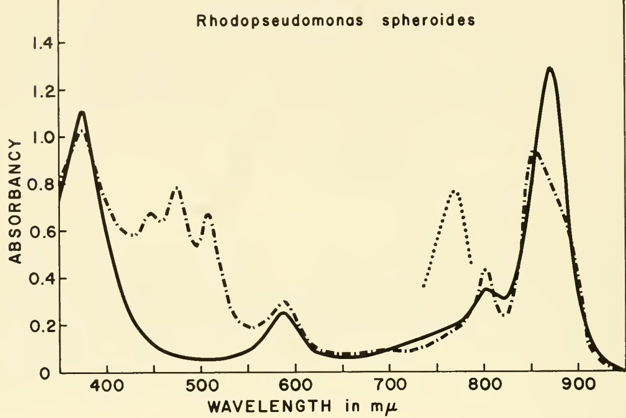

Fig. 2. Absorption spectra of *Rhodopseudomonas spheroides*, strain 2.4.1 and blue-green mutant strain 2.4.1/CC1/R-26. Solid curve, chromatophores of the blue-green mutant. Dashed curve, chromatophores of the wild type. Dotted curve, BChl in 7 acetone/2 methanol. The three preparations were adjusted to the same BChl concentration.

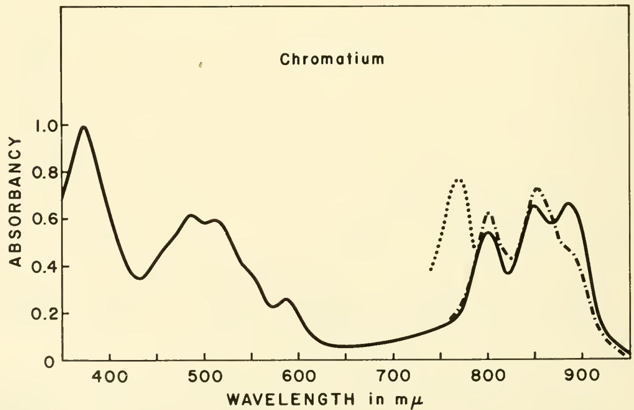

Fig. 3. Absorption spectra of *Chromatium*, strain D. Chromatophores from cells grown with CO_2 (solid curve) or malate (dashed curve) as carbon source. Dotted curve, BChl in 7 acetone/2 methanol. The three preparations were adjusted to the same BChl concentration.

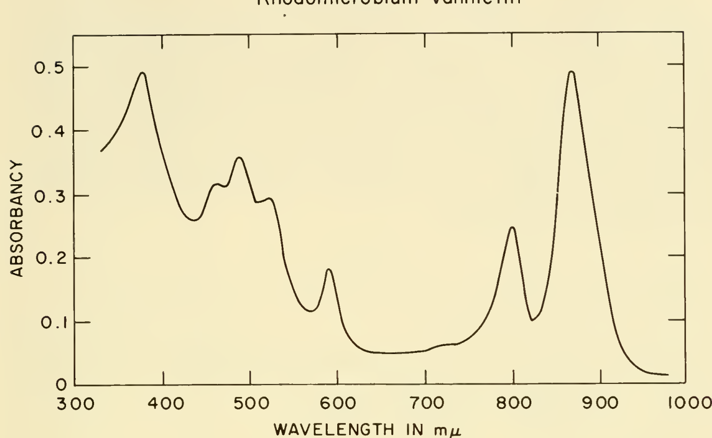

Fig. 4. Absorption spectrum of a suspension of *Rhodomicrobium vannielii*. Spectrum obtained by the opal glass method on a Cary 14R spectrophotometer. Prepared by Dr. J. M. Olson.

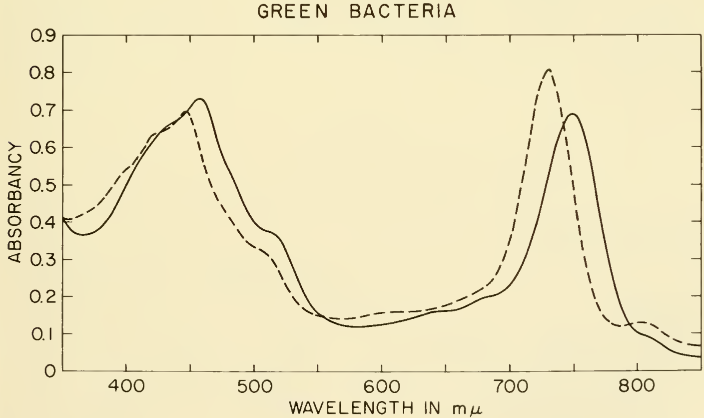

Fig. 5. Absorption spectra of whole-cell suspensions of green bacteria. Solid curve, *Chloropseudomonas ethylicum*, strain 2K. Dashed curve, *Chlorobium thiosulfatophilum*, strain L. Spectra obtained by the opal glass method on a Cary 14R spectrophotometer. The spectrum of *C. thiosulfatophilum*, strain PM is like that of *Cps. ethylicum*. Prepared by Dr. J. M. Olson.

The extinction coefficient of BChl in ether at 773 mμ (Table 2) is based on Weigl's value (1) of 96 $mM^{-1}cm^{-1}$. The values for BChl in acetone/methanol and *in vivo* were obtained by direct comparison with BChl in ether (R. K. Clayton, unpublished). The extinction values serving as ordinate in Figs. 6 and 7 are in units of liters per gram-cm. They differ from ϵ ($mM^{-1}cm^{-1}$) by a factor of 1000/Mol.Wt. The molecular weights of BChl and bacteriopheophytin are 911 and 889 respectively (2). Those of the chlorobium chlorophylls are not known.

Abundant references can be found in reviews by Smith and Benitez (2) and Smith and French (3).

TABLE 1.

Infrared Absorption Maxima of the Chlorophylls of Photosynthetic Bacteria

Reference	Pigment	Source	Absorption Maxima in mμ in vivo	in ether
4	chlorobium Chl 650	*Chlorobium thiosulfatophilum*, strain L.	725	650
4	chlorobium Chl 660	*C. thiosulfatophilum*, strain PM. *Chloropseudomonas ethylicum*, strain 2K.	747	660
5	chlorobium Chl 770 (Bchl?)	*C. thiosulfatophilum* and *Cps. ethylicum*.	810	770
many (see 2,3)	BChl	purple photosynthetic bacteria.	800 to 890	773

TABLE 2.

Extinction Coefficients of BChl in vitro and in vivo

For the Soret band in vivo, at 375 mμ, the millimolar extinction coefficient is 100 ± 10 (1 cm path).

	Wavelength, mμ	ϵ, $mM^{-1}cm^{-1}$
in solution		
ether	773	96
acetone/methanol, 7 vol/2 vol.	772	75
in vivo		
R. rubrum, strain S-1	880	140
Rps. spheroides, strain 2.4.1	850	approx. 95
Rps. spheroides, blue-green mutant strain 2.4.1/CC1/R-26	870	127
Chromatium, strain D	850	approx. 70

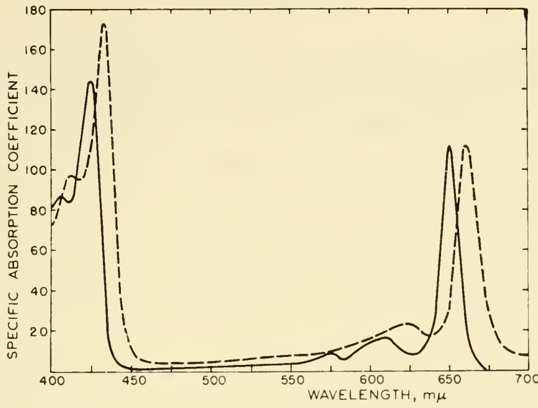

Fig. 6. Absorption spectra of chlorobium chlorophylls in ether. Solid curve, chlorobium Chl 650. Dashed curve, chlorobium Chl 660. The ordinate is in units of liters per gram-cm. Reproduced from Reference 4 by permission of Dr. J. H. C. Smith.

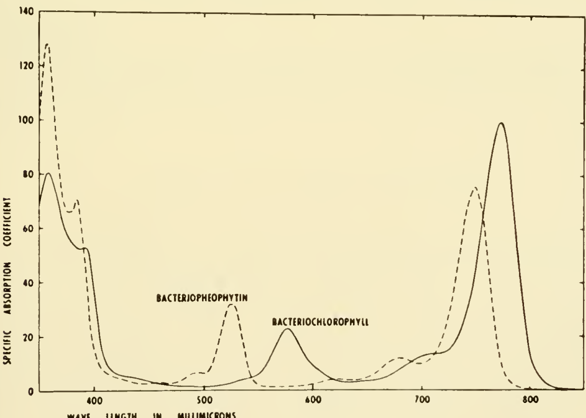

Fig. 7. Absorption spectra of BChl and bacteriopheophytin in ether. The ordinate is in units of liters per gram-cm. Reproduced from Reference 2 by permission of Dr. J. H. C. Smith.

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MEDIA FOR ANAEROBIC GROWTH OF PHOTOSYNTHETIC BACTERIA

SUBIR K. BOSE

A large number of media, both semi-synthetic and synthetic, have been described for growth of photosynthetic bacteria. Recipes are constantly being modified (usually in some minor way) and, accordingly, only a few media representative of those in common use are given below.

Anaerobic conditions are established by one of the three following methods: (a) Growth in completely filled glass-stoppered reagent bottles. (b) Growth in completely filled bottles (250 ml milk dilution, 1 liter Roux or 5.6 liter Diphtheria Toxin) which are sealed with a 1/2-3/4 inch layer of sterile paraffin. Molten paraffin is poured directly onto the medium and on cooling a plug forms in the neck of the bottle. This method is particularly useful with gas-producing cultures, since the plug loosens slightly and acts as an escape valve. The plug can be kept in place with a strip of adhesive tape. (c) Growth under an oxygen-free gas phase.

Illumination is conveniently provided by banks of 60-watt, 120-volt Lumiline lamps placed on both sides of the inoculated culture vessels. A fan is ordinarily used to aid in dissipation of heat generated by the lamps.

NONSULFUR PURPLE BACTERIA

These organisms are ordinarily grown with an organic carbon source. Some types are capable of growing with carbon dioxide as the primary carbon source if an "accessory donor" such as molecular hydrogen and appropriate growth factors are provided (e.g., see Ormerod and Gest, *Bacteriol. Rev., 26,* 51 (1962)). Depending on the particular organic carbon source employed, carbon dioxide may also have to be furnished (e.g., with ethanol or butyrate). Malate is an excellent carbon source for many nonsulfur purples, and with this compound, addition of carbon dioxide is unnecessary. In many instances, other compounds such as succinate, fumarate or lactate can be substituted for malate with good results. Complex supplements such as yeast extract frequently enhance growth rates, and there is little doubt

that further definition of *optimal* growth requirements would be a worthwhile endeavor.

Rhodospirillum rubrum.

Stock cultures are maintained as stabs in a medium containing 0.3% yeast extract (Difco), 0.3% Bacto-peptone and 1.5% agar in deionized water.

A. Synthetic liquid medium. After Ormerod, Ormerod and Gest, *Arch. Biochem. Biophys.*, *94*, 449 (1961).

Stock Solutions

1. Basal salt solution:

Deionized water	800.0	ml
$MgSO_4 \cdot 7\ H_2O$	2.0	g
$CaCl_2 \cdot 2\ H_2O$	0.75	g
$FeSO_4 \cdot 7\ H_2O$	118.0	mg
Ethylenediamine tetraacetic acid (dissolve separately and adjust pH to 6.8 with NaOH)	200.0	mg
Trace elements solution	10.0	ml

Deionized water to 1 liter.

2. Trace elements solution:

$MnSO_4 \cdot 4\ H_2O$	2.1	g
H_3BO_3	2.8	g
$Cu(NO_3)_2 \cdot 3\ H_2O$	40.0	mg
$ZnSO_4 \cdot 7\ H_2O$	240.0	mg
$Na_2MoO_4 \cdot 2\ H_2O$	750.0	mg

Deionized water to 1 liter.

3. Phosphate solution:

KH_2PO_4	40	g
K_2HPO_4	60	g

Deionized water to 1 liter.

4. 20% DL-malic acid, pH adjusted to 6.8 with NaOH (stored in deepfreeze).

5. 20% L-glutamic acid, pH adjusted to 6.8 with NaOH (stored in deepfreeze).

6. Biotin stock solution containing 10 mg per 100 ml of 50% ethanol; dilute 1:20 before use (stored in deepfreeze).

Final Medium

Deionized water	800 ml
Basal salt solution	100 ml
Biotin (diluted)	3 ml
DL-malate	2-6 g
L-glutamate or ammonium sulfate	0.5-2 g
Phosphate solution (added last)	15 ml

Deionized water to 1 liter.

The pH of the medium is 6.8 before autoclaving (15 minutes at 15 lbs./sq. in.). Immediately after autoclaving, the medium is cloudy but becomes clear upon cooling.

Incubation temperature: 26-28°C.

Inoculum Medium
(for daily transfers)

Same as above, but with 2 g DL-malic acid and 0.5 g ammonium sulfate per liter. The inoculum is grown in completely filled screw-cap tubes. 24-hour-old cells are recommended as inocula.

B. The Cohen-Bazire, Sistrom and Stanier (1957) medium for *Rps. spheroides* has also been used for growing *R. rubrum.*

Rhodopseudomonas spheroides.

Stock cultures are maintained as stabs in a medium containing 0.3% yeast extract (Difco), 0.2% casamino acids (Difco) and 1.5% agar in tap water (see reference given for medium A below).

A. Semi-synthetic liquid medium. After Cohen-Bazire, Sistrom and Stanier, *J. Cellular Comp. Physiol., 49,* 25 (1957).

Stock Solutions

1. Potassium phosphate, pH 6.8	1.0	M
2. Ammonium DL-malate, pH 6.8	1.0	M
3. Concentrated base:		
Nitrilotriacetic acid	10.0	g
$MgSO_4$	14.45	g
$CaCl_2 \cdot 2\ H_2O$	3.335	g
$(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$	9.25	mg
$FeSO_4 \cdot 7\ H_2O$	99.0	mg
Nicotinic acid	50.0	mg
Thiamine · HCl	25.0	mg
Biotin	0.5	mg
Metals "44" (see below)	50.0	ml

Distilled water to 1000 ml.

The nitrilotriacetic acid is dissolved and neutralized with KOH (about 7.3 g), after which the rest of the ingredients are added. The pH is adjusted to 6.6-6.8 before making to volume.
Metals "44" contains, per 100 ml:

Ethylenediamine tetraacetic acid	250.0 mg
$ZnSO_4 \cdot 7\ H_2O$	1095.0 mg (250 mg Zn)
$FeSO_4 \cdot 7\ H_2O$	500.0 mg (100 mg Fe)
$MnSO_4 \cdot H_2O$	154.0 mg (50 mg Mn)
$CuSO_4 \cdot 5\ H_2O$	39.2 mg (10 mg Cu)
$Co(NO_3)_2 \cdot 6\ H_2O$	24.8 mg (5 mg Co)
$Na_2B_4O_7 \cdot 10\ H_2O$	17.7 mg (2 mg B)

A few drops of sulfuric acid are added to retard precipitation.

In order to prepare 1 liter of the complete medium, 20 ml each of solutions 1, 2 and 3 are mixed and diluted with distilled water, after which 1.0 g of vitamin-free casamino acids (Difco) is added. A copious precipitate forms when this medium is autoclaved, but it redissolves on cooling. The casamino acids may be replaced by a mixture of L-glutamic acid (0.1% final concentration) and sodium acetate $\cdot$ 3 H_2O (0.1% final concentration).

Incubation temperature: 30°C.

B. Medium S. After Lascelles, *Biochem. J.*, *62*, 78 (1956).

Sodium-L-glutamate monohydrate	3.8 g
DL-malic acid	2.7 g
KH_2PO_4	500.0 mg
K_2HPO_4	500.0 mg
$(NH_4)_2HPO_4$	800.0 mg
$MgSO_4 \cdot 7\ H_2O$	200.0 mg
$CaCl_2$	40.0 mg
Nicotinic acid	1.0 mg
Thiamine $\cdot$ HCl	1.0 mg
Biotin	10.0 μg

Distilled water to 1 liter.

The pH is adjusted to 6.8 with 1 *N* NaOH before autoclaving for 10 minutes at 10 lbs./sq. in.

C. Medium MS. After Lascelles, *Biochem. J.*, *72*, 508 (1959).
Medium S, plus: $MnSO_4$, 5 μM; sodium-L-glutamate, 0.01 M.

D. Ormerod, Ormerod and Gest (1961) medium for *R. rubrum*, supplemented with 300 mg (Difco) yeast extract per liter.

E. Keane *et al.* medium (1963) for *Rps. palustris*.

Rhodopseudomonas palustris.

Synthetic medium. After Keane, Zahalsky, Hutner and Lubart, p. 163 in *Studies on Microalgae and Photosynthetic Bacteria* (Japanese Soc. of Plant Physiologists, ed.), Univ. Tokyo Press, 1963.

$MgSO_4 \cdot 7 H_2O$	600 mg
$CaCl_2$	150 mg
$(NH_4)_2SO_4$	40 mg
Na_2-glycerophosphate $\cdot$ 5 H_2O (mixture of α and β isomers)	2 g
Sodium acetate $\cdot$ 3 H_2O	200 mg
Tripotassium citrate $\cdot$ H_2O	500 mg
L-glutamic acid	3 g
DL-malic acid, neutralized with KOH separately	4 g
L-histidine (free base)	2 g
L-tyrosine	100 mg
Monobutyrin	400 mg
p-aminobenzoic acid	200 μg
Homocysteine thiolactone $\cdot$ HCl	200 mg
"Metals":	
Fe	18.0 mg
Mn	14.0 mg
Zn	9.0 mg
Mo	1.8 mg
Cu	0.9 mg
Co	0.18 mg
B	0.18 mg
V	0.18 mg
I	0.18 mg
Se	0.036 mg

Glass-distilled water to 1000 ml.
pH: 6.2 to 6.5
Incubation temperature: 30-34°C.

Rhodopseudomonas gelatinosa.

After Siegel and Kamen, *J. Bacteriol.*, *59*, 693 (1950).

Stock Cultures: deep stabs in a medium containing 0.3% yeast extract (Difco), 0.3% peptone and 1% agar in tap water.

Basal Medium

$(NH_4)_2SO_4$	1.0 g
$MgCl_2$	0.2 g
$CaCl_2 \cdot 2 H_2O$	0.04 g
KH_2PO_4	2.0 g
H_3PO_4, 6 N	3.6 ml
Thiamine $\cdot$ HCl	4.0 mg

Nicotinic acid	4.0 mg
Biotin	5.0 μg
Bicarbonate solution	40.0 ml
(5 g $NaHCO_3$ + 5 g $KHCO_3$ per 100 ml). Added aseptically after separate sterilization.	

Tap water to 1000 ml.

The basal medium is supplemented with desired substrate (isopropanol (0.1-0.2%), acetate, acetoacetate, pyruvate, glycerate). The final medium should have a pH of 7 to 7.5.

Incubation temperature: 30°C.

Rhodomicrobium vannielii.

After Duchow and Douglas, *J. Bacteriol.*, *58*, 409 (1949); modified by Morita and Conti, *Arch Biochem. Biophys.*, *100*, 302 (1963).

$NaHCO_3$	5 g
$NaCl$	2 g
$(NH_4)_2SO_4$	1 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7\,H_2O$	0.1 g
$Na_2S \cdot 9\,H_2O$	0.1 g
Organic substrate (Ethanol, propanol, butanol, acetate, propionate, butyrate, valerate, caproate or lactate)	2 g
Yeast extract	1 g
Water	1000 ml

pH adjusted to 7.0 with H_3PO_4.

Incubation temperature: 28-30°C.

PURPLE SULFUR BACTERIA

The purple sulfur bacteria most commonly used in biochemical investigations belong to the genus *Chromatium*. These organisms can be readily grown either with carbon dioxide as the sole carbon source or with organic compounds. To obtain satisfactory growth in a reasonable time period, the media currently in use are sown with relatively large inocula (10-20%).

Stock cultures: stabs in medium B specified below; sufficient agar is added to solution 1 before autoclaving to give a final concentration in the complete medium of 1.5% (w/v).

Chromatium.

A. Inorganic medium used by R. Bartsch (unpublished). See also: Newton and Kamen, *Biochim. Biophys. Acta*, *21*, 71 (1956), and Newton, p.

73 in *Methods in Enzymology* (S. P. Colowick and N. O. Kaplan, eds.), Vol. V, Academic Press, New York, 1962.

NaCl	10.0 g
K_2HPO_4	0.5 g
KH_2PO_4	0.5 g
NH_4Cl	1.0 g
$MgCl_2 \cdot 6\ H_2O$	0.5 g
$CaCl_2$	0.05 g
$NaHCO_3$	2.0 g
$FeCl_3 \cdot 6\ H_2O$	0.005 g
$Na_2S_2O_3 \cdot 5\ H_2O$	2.0 g
$Na_2S \cdot 9\ H_2O$	1.0 g

Tap water to 1 liter; final pH adjusted to 7.8-8.0. If distilled or demineralized water is used, 1 ml of the trace elements solution described by Larsen (*J. Bacteriol.*, *64*, 187 (1962)) is added per liter of medium.

When molecular hydrogen is used as the accessory electron donor, thiosulfate and sulfide are omitted from the medium.

Incubation temperature: 30-35°C.

B. Inorganic medium used by R. C. Fuller, based on the medium of Hendley (*J. Bacteriol.*, *70*, 625 (1955)).

Solution 1

NaCl	10.0 g
NH_4Cl	2.0 g
KH_2PO_4	1.0 g
$MgCl_2$	1.0 g
$CaCl_2$	0.2 g
Trace elements solution (see below)	1.0 ml
Conc. HCl	9.0 ml

Distilled water to 500 ml.

Solution 2

Na_2CO_3	12.76 g
$Na_2S_2O_3$	6.0 g
Ethylenediamine tetraacetic acid	0.2 g
$Na_2S \cdot 9\ H_2O$	0.1 g

Distilled water to 500 ml.

Autoclave solutions 1 and 2 separately and cool. Add 1.0 ml of conc. HCl to 1 and pour solution 2 into 1 very slowly. Final pH should be between 7.8 and 8.0.

Trace Elements Solution

$ZnSO_4 \cdot 7\ H_2O$	440.0 mg
$CoSO_4 \cdot 7\ H_2O$	240.0 mg
$CuCl_2 \cdot 2\ H_2O$	13.5 mg
$MnSO_4 \cdot H_2O$	16.5 mg
$Na_2B_4O_7 \cdot 10\ H_2O$	880.0 mg
Versenol iron solution (see below)	110.0 ml

Distilled water to 1 liter.

Versenol Iron Solution

59 g of ethylenediamine tetraacetic acid (Versenol) are dissolved in 500 ml distilled water. 24.9 g of $FeSO_4 \cdot 7\ H_2O$ are added, the solution diluted to 1 liter with distilled water, and then aerated overnight. The final pH should be approximately 9.7.

C. "Heterotrophic" synthetic medium used by R. C. Fuller.

Stock Salt Solution

$CaCl_2$	0.25 g
NH_4Cl	1.2 g
$MgSO_4 \cdot 7\ H_2O$	0.66 g
NaCl	20.0 g
Hutner's trace elements solution (see below)	1.0 ml

Distilled water to 1000 ml.

Hutner's Trace Elements Solution
(*J. Bacteriol.*, *52*, 213 (1946))

Ethylenediamine tetraacetic acid	50.0 g
$ZnSO_4 \cdot 7\ H_2O$	22.0 g
H_3BO_3	11.4 g
$MnCl_2 \cdot 4\ H_2O$	5.1 g
$FeSO_4 \cdot 7\ H_2O$	5.0 g
$CoCl_2 \cdot 6\ H_2O$	1.6 g
$CuSO_4 \cdot 5\ H_2O$	1.6 g
$(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$	1.1 g

Boil in 750 ml distilled water, cool slightly and bring to pH 6.5-6.8 with KOH (do not use NaOH). The clear solution is diluted to 1000 ml with distilled water and should have a green color which changes to purple on standing. Stable for at least one year.

Final Medium

Solution I	120 ml of stock salt solution diluted to 800 ml with distilled water.

Solution II	50 ml of 1 M Tris, pH 7.5
Solution III	1 g K_2HPO_4 in 50 ml water.
Solution IV	0.2 g $Na_2S \cdot 9\ H_2O$ in 50 ml water.
Solution V	5.5 g of organic carbon source (neutralized; e.g., malate) in 50 ml water.

Autoclave the five solutions separately. Cool and mix in the order indicated. Final pH 7.5, adjusted with conc. HCl.

GREEN BACTERIA

Chlorobium thiosulfatophilum and *C. limicola.*

After Larsen, "On the microbiology and biochemistry of the photosynthetic green sulfur bacteria." *Kgl. Norske Videnskab. Selskabs Skrifter, 1953,* 1-205.

Stock cultures: stabs in a medium containing 0.1% NH_4Cl, 0.1% KH_2PO_4, 0.05% $MgCl_2$, 0 to 3% NaCl and 2% agar in tap water. The medium is sterilized and allowed to cool to about 45°C. Separately sterilized solutions of $NaHCO_3$, Na_2S, and $FeCl_3$ are then added aseptically to give the following final concentrations: 0.2% $NaHCO_3$, 0.1% $Na_2S \cdot 9\ H_2O$, and 50 μg % Fe. The pH is adjusted to 7.3 by adding a few drops of a sterile dilute solution of H_3PO_4.

Synthetic Liquid Medium

NH_4Cl	1.0 g
KH_2PO_4	1.0 g
$MgCl_2$	0.5 g
NaCl	10.0 g
$NaHCO_3$	2.0 g
$Na_2S_2O_3$ or $Na_2S \cdot 9\ H_2O$	1.0 g
(*C. thiosulfatophilum* can use either Na_2S or $Na_2S_2O_3$ as electron donors, whereas *C. limicola* utilizes Na_2S but not thiosulfate.)	
$Na_2S \cdot 9\ H_2O$ (if $Na_2S_2O_3$ is used)	0.1 g
$CaCl_2$	0.1 g
Fe (as $FeCl_3 \cdot 6\ H_2O$)	500.0 μg
B (as H_3BO_3)	100.0 μg
Zn (as $ZnSO_4 \cdot 7\ H_2O$)	100.0 μg
Co (as $Co(NO_3)_2 \cdot 6\ H_2O$)	50.0 μg
Cu (as $CuSO_4 \cdot 5\ H_2O$)	5.0 μg
Mn (as $MnCl_2 \cdot 4\ H_2O$)	5.0 μg

Pyrex glass-distilled water to 1000 ml.
The pH is adjusted to 7.0-7.5 with H_3PO_4.
Incubation temperature: about 30°C.

Chloropseudomonas ethylicum strain 2K.

Recipe for synthetic liquid medium used by J. M. Olson, based on the medium of Shaposhnikov, Kondrat'eva, Krasil'nikova and Ramenskaya (*Doklady - Biol. Sci. Sect.* (English Transl.), *129*, 1047 (1959)), which, in turn, is a modification of Larsen's inorganic medium (*J. Bacteriol.*, *64*, 187 (1952)) for green sulfur bacteria.

Stock cultures: stabs (1.5% agar) in the medium specified below, except that the final concentration of $Na_2S \cdot 9\,H_2O$ is 0.1% (w/v).

Stock Solutions

1. Versenol iron solution: see medium B for *Chromatium*.
2. Trace elements solution:

$FeCl_3$	1.6 g
$Na_2B_4O_7 \cdot 10\,H_2O$	880.0 mg
$ZnSO_4 \cdot 7\,H_2O$	440.0 mg
$CoSO_4 \cdot 7\,H_2O$	240.0 mg
$CuCl_2 \cdot 2\,H_2O$	13.5 mg
$MnSO_4 \cdot H_2O$	16.5 mg
Versenol iron solution	110.0 ml
Distilled water to 1 liter.	

3. Double strength basal salts:

KH_2PO_4	2.0 g
NH_4Cl	2.0 g
$MgCl_2 \cdot 6\,H_2O$	10.0 g
NaCl	40.0 g
$CaCl_2$	0.08 g
Trace elements solution (add last)	2.0 ml

Final Medium

I.	Double strength basal salts	500 ml
	Distilled water	450 ml
II.	10% (w/v) $Na_2S \cdot 9\,H_2O$	2 ml
III.	10% (w/v) $NaHCO_3$	40 ml
IV.	0.05% (w/v) $FeSO_4 \cdot 7\,H_2O$ in 0.3 N HCl	5 ml
V.	70% ethanol (sterilized by filtration)	3 ml

Autoclave solutions I to IV separately. Cool and mix. Add solution V, and adjust pH to 7.3 with conc. HCl.

Incubation temperature: 30°C.

Light intensity should be less than 100 foot-candles.

BIBLIOGRAPHY ON METABOLISM OF PHOTOSYNTHETIC BACTERIA

It is hoped that the following reference list will serve as a useful supplement to individual papers in this volume, particularly in connection with the carbon and inorganic nitrogen metabolism of photosynthetic bacteria. A group of papers dealing with miscellaneous topics of interest is also included. The bibliography is not exhaustive and is limited to papers published since 1949; references to earlier investigations can be found in the reviews cited.

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