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ACCOMPLISHMENTS IN RELATION TO ORIGINAL OBJECTIVES

Objectives. The following plans were stated in the comprehensive report for 1969-1972. They deal mainly with photochemical reaction centers (RC's) isolated from the photosynthetic bacterium Rhodospseudomonas spheroides.

1. Measurements with RC's to coordinate the quantum efficiencies and kinetics of photochemistry as reflected by formation of oxidized bacteriochlorophyll (BChl), of triplet-state BChl, of excited singlet BChl (shown by the fluorescence), and of non-radiative quenching of the excited singlet state.

2. Exploration of physical and photochemical properties of RC's that have been altered by dehydration, by chemical inhibition, by mild denaturation, and by selective removal and replacement of components such as iron and ubiquinone (UQ), with a view to elucidating the nature of the primary photochemical electron acceptor.

3. Further study of the extraction of oxidized BChl from illuminated RC's.

4. Study of optical effects such as changes in the absorption spectrum of carotenoid pigments in photosynthetic membranes, that reflect ion movements and electrochemical gradients in the membranes. These phenomena are related to the coupling of electron transport to ATP formation.

5. Investigation of relations between pigments and specific protein components of the bacterial photosynthetic membrane. Use of specific antibodies in these studies, and to map functional sites on the protein components of the RC's.

6. Development of possible models of solar cells using components of the photosynthetic membrane, especially RC's.

7. A variety of lesser experiments related to single observations; not listed because they have not been pursued further.

Accomplishments. The past three years' progress will now be detailed in approximately the same sequence as the foregoing list of objectives. Progress since Sept. 1, 1974 is identified by vertical lines in the margins.

1. Characteristics of transient physical states and photochemical electron transport in reaction centers.

The quantum efficiency of photochemistry in RC's, measured by the rate of formation of oxidized BChl, was estimated earlier to be about 70% (1). This was approximately the value predicted from a threefold rise of BChl fluorescence seen when the photochemical pathway was blocked by lowering the redox potential so as to reduce the primary electron acceptor (2). This simple picture of photochemistry leading directly from the fluorescent singlet excited state was disturbed by the discovery of Dutton et al. (3) of an electron paramagnetic resonance (EPR) signal suggesting the formation of triplet-state BChl in illuminated RC's. This signal could only be seen below 20°K for technical reasons, and only in RC's set at low redox potential to block photochemistry ("closed" RC's). Our previous estimate of 70% photochemical efficiency would have led us to expect a triplet signal in photochemically active RC's, about 30% of that in closed RC's, or else no signal in any case. We therefore undertook to measure as carefully as possible both the photochemical efficiency in active RC's and the efficiency of triplet formation in closed RC's.

We found (4) that at room temperature the photochemical efficiency is $102 \pm 4\%$. This explains the absence of a triplet EPR signal in active RC's, but is clearly inconsistent with the 70% predicted by the rise in fluorescence. Apparently the rise in fluorescence signals the change of RC's from "active" to "closed", but its magnitude cannot be made part of a simple theory in which fluorescence and photochemistry are competing pathways for the dissipation of singlet excitation energy. The simple model, now shown to be incorrect for RC's, has been used axiomatically in photosynthesis research for decades.

We then found that, at least at low temperature, the quantum efficiency for triplet formation in closed RC's is as great as that for photochemistry in active RC's (5). The triplet seen by EPR could therefore not be dismissed as a minor side product. Recognizing that a triplet state seen by EPR must be manifested also by changes in the optical absorption spectrum of BChl, we sought evidence for such absorbance changes, in collaboration with W.W. Parson at the University of Washington. We first found appropriate absorbance changes in closed RC's at room temperature, with a spectrum resembling that for the formation of triplet BChl in vitro (6-8). These changes appear within 10 nsec after a laser flash and decay with 6 μ sec half-time*. The quantum efficiency for their formation is only about 10% at room temperature. We have denoted this triplet-like entity P^T .

With technical improvements Parson (in collaboration with us) discovered (6) another transient state in closed RC's, which we call P^F . Its spectrum is distinct from that of P^T ; it is formed with high efficiency (close to 100%) and decays in about 10 nsec at room temperature.

* The spectrum and kinetics are distinct from those reported earlier by Seibert and DeVault in photosynthetic membranes; the latter now seem to show the triplet state of carotenoids in the light-harvesting components of the membrane.

At temperatures below 20°K we found (6,8) that both P^f and P^T are formed with quantum efficiency near 100% in closed RC's, with P^f decaying in 20 nsec and P^T in 120 μ sec. Thus it appeared that P^f is a precursor of P^T . We identified P^T as triplet BChl, seen also as an EPR signal at low temperature. The EPR signal decays much faster than the optical signal P^T ; this can be attributed to equilibration among electron spin orientations. Because of its low quantum yield at room temperature we ruled out the triplet (P^T) as a photochemical intermediate, but we entertained the possibility that P^f mediates the photochemistry. P^f might be a biradical or charge transfer state showing rapid spin conversion. If so, P^f ought to be detected in active RC's, but with much smaller lifetime than the 10-20 nsec observed in closed RC's. We were gratified to learn that a state resembling P^f was detected subsequently in active RC's, in other laboratories possessing absorption spectrometers with picosecond time resolution (9,10). The lifetime of P^f in active RC's was reported to be 0.1 to 0.3 nsec, appropriate for a role as a photochemical intermediate. We believe that the discovery and partial characterization of P^f has been our most significant contribution in the past three years, because it opens the door to a detailed elucidation of the mechanism of the photochemistry of photosynthesis.

We are now engaged in measuring photochemical efficiency and fluorescence properties at temperatures down to 18°K for correlation with data on P^f and triplet formation.

2. Properties of altered reaction centers; possible "unnatural" reactions.

We have subjected RC's to dehydration by first preparing films of RC's on quartz slides and then exposing them to vacuum or to a drying

agent. Dehydration caused three obvious changes, all reversed by returning the films to a humid atmosphere. First, there is a partial inactivation. The absorption band at 865 nm is bleached only about 50%, rather than completely, in strong light. Bleaching of this band signifies the photochemical oxidation of BChl. Second, all the bands of BChl are shifted to shorter wavelengths. Third, there is some loss of absorbance at 865 nm which is compensated by greater absorbance at 800 nm. This is consistent with the view (11) that the absorption at 865 nm arises through interaction of BChl with water while the 800 nm band is due to BChl that is not hydrated. We are continuing these studies, preparing to examine the formation and decay of the excited singlet state of BChl and of P^f , by observing the fluorescence and through measurements of absorbance changes in the picosecond time domain (the latter in collaboration with P. Rentzepis and K. Kaufmann at Bell Laboratories).

Ubiquinone and iron have been implicated as alternatives or partners in the function of primary photochemical electron acceptor in RC's (12-14). We have shown that hydrocarbon solvents containing methanol remove UQ from RC's and abolish photochemical activity. Activity is restored when UQ is added back (15). While this experiment seemed to show an obligate role for UQ as primary acceptor, we were concerned that the restored reaction might not be the same as the natural one. This concern was strengthened because the restored reaction seemed not to be attended by an absorbance increase at 450 nm, seen in the natural reaction and indicative of the conversion of UQ to the anionic semiquinone, $UQ^{\cdot-}$.

Concern over "unnatural" reactions was deepened by the results of experiments involving pairs of laser flashes applied to RC's (16). In these experiments RC's were mixed with reduced cytochrome (cyt) to act as secondary electron donor producing the reaction sequence

Here the primary photochemical act in RC's is shown as the oxidation of BChl to BChl^+ and the reduction of the primary acceptor X to X^- . The conversion of $\text{BChl}\cdot\text{X}$ to $\text{BChl}\cdot\text{X}^-$ in this sequence is attended by a rise in BChl fluorescence, by the oxidation of cyt, and by an increase of absorbance at 450 nm due to the formation of X^- . We observed all of these effects in response to a single laser flash. Also, as expected, a second flash caused no further reaction until the system has returned from the state $\text{BChl}\cdot\text{X}^-$ to the active state $\text{BChl}\cdot\text{X}$, as indicated by relaxation of the fluorescence and the absorbance at 450 nm. However, a different pattern appeared when excess UQ had been added to the reaction mixture. Now the fluorescence did not rise in response to the first flash, although cyt was oxidized and the absorbance at 450 nm increased as expected. A second flash given immediately after the first caused no further change in absorbance at 450 nm, but did lead to the oxidation of more cyt (one molecule per RC). Also the reaction after the second flash was attended by the uptake of one proton per RC; there was no proton uptake in the response to the first flash. The most economical interpretation of these results is that in the presence of excess UQ, the RC's could respond to a second flash with an atypical reaction using external UQ as an adventitious electron acceptor:

We must ask, therefore, whether atypical reactions are misinterpreted as natural ones in a variety of situations involving RC's. One example is the case of solvent-extracted RC's that have been "restored" by adding UQ. Another is the "LM" particle derived from RC's by removing the heaviest of the three protein components (17). In another category we have RC's that have been inhibited chemically and then "restored" by adding UQ. We have found that the following agents inhibit the primary photochemistry: anilino naphthalene sulfonate, ortho-phenanthroline, lauryl dimethyl amine oxide (LDAO) at high concentrations, and quinoline-N-oxides. Ubiquinone completely reverses the inhibition caused by the first three of these agents. Perhaps solvent extraction (incidentally attended by removal of native UQ) should be put in the same general category of inhibitors. We are now exploring characteristics of the "restored" reaction in all of these cases, using the criteria of 450 nm absorbance change, rise in BChl fluorescence, proton binding, and kinetics at low temperature. We also plan to study collaboratively the absorbance changes in the picosecond time domain.

3. Oxidation of bacteriochlorophyll in extracts of illuminated centers.

We reported earlier (18) that the extraction of illuminated RC's with methanol yielded oxidized BChl (BChl^+) that could be re-reduced in

the methanolic solution. Now we have found (19) that the facts are different, revealing a curious reaction of BChl in vitro. If RC's are extracted with methanol, the BChl appears first in its usual reduced form. After a delay of a few min to an hour or more, one third of the BChl in the methanolic solution becomes oxidized in the dark. The delay is shorter if the RC's have been illuminated before extraction. Evidently the spontaneous oxidation is catalyzed by traces of BChl^+ that remained in the RC's after illumination. The kinetics can be described by

The reaction requires the presence of both oxygen and the detergent LDAO that was present in the RC preparation, but it does not occur in the strict absence of BChl^+ . The appearance of a delay followed by a sigmoid time-course of the reaction is explained by the autocatalytic role of BChl^+ in the reaction. Following this reaction there occurs a spontaneous conversion of BChl^+ to an altered reduced form (BChl_{al}). This reaction is bimolecular in BChl^+ :

The fact that only one third of the BChl becomes oxidized is a consequence of the dynamics of the two reactions, oxidation and re-reduction; there is no evidence that one third of the BChl extracted from RC's is different from the remainder.

4. Ion movements and electrochemical states in photosynthetic membranes.

We showed that the normal photochemistry of purified RC's is not attended or followed by H^+ binding or release, indicating that neither product, oxidized BChl or reduced primary acceptor (X^-) reacts with protons.

This is consistent with the fact that the redox mid-point potentials of $\text{BChl}/\text{BChl}^+$ and X/X^- are independent of pH in RC's (20). The mid-point potential of the primary acceptor, $E_m(\text{X}/\text{X}^-)$, is -30 mv from pH 6 to 9. Now, in collaboration with P.L. Dutton and J.S. Leigh at the University of Pennsylvania, we have found that the $E_m(\text{X}/\text{X}^-)$ measured in situ, but not in isolated RC's, the species X^- is in equilibrium with protons:

These experiments were conducted by incubating RC's or membrane fragments in redox buffers so as to establish an environment having a particular redox potential and a specified pH. The system reaches an equilibrium among X , X^- and XH ; the proportion of RC's with primary acceptor in the oxidized form X is revealed by the capacity for photochemistry. It must be emphasized that even though the adjustment to equilibrium is allowed to occur over many minutes, the test of photochemical capability is performed in less than a second. The photochemical products might be formed and discharged much more rapidly than any equilibration with H^+ can occur. Thus the measurements show the long-term capacity for X^- to equilibrate with H^+ , but this interaction with protons may fail to occur in the short time span of the photochemistry and closely coupled "dark" reactions. These equilibrium redox titrations can therefore be misleading as to the actual redox properties of X as it functions in nature. We do not know, however, why X^- can equilibrate slowly with protons when RC's are in their native state, but not when the RC's are isolated from the membrane.

This problem is now being pursued by Dutton, R. Prince, and K. Petty at the University of Pennsylvania. Present indications are that the

primary acceptor may function in vivo with a mid-point potential of -180 mv rather than -30 mv. This has an important bearing on the question of whether the photochemical system can effect the direct reduction of pyridine nucleotides, instead of relying of reductive dephosphorylation for NAD reduction.

5. Relationships between pigments and proteins of photosynthetic membranes: experiments with specific antibodies.

Two major proteins have been identified in membranes of Rhodospseudomonas spheroides, aside from the protein components of the RC's (22-24). A protein of molecular weight about 9000 is the bearer of the "antenna" BChl component absorbing at 800 and 850 nm; this is the major part of the light-harvesting apparatus in wild type Rps. spheroides. It is missing in carotenoidless mutants of Rps. spheroides, which poses a puzzle. The most obvious lesion in these single-locus mutants is the loss of an enzyme system for carotenoid biosynthesis, but the mutation has a broader consequence: loss of a major BChl-protein component of the membrane. This might be a useful system for studying cell development, posing the question "How is membrane development coupled to carotenoid synthesis?".

Another protein, of molecular weight 46,000, is found in nonpigmented parts of the membrane that are associated with the cell wall. Although it is a major membrane protein, we know almost nothing about it.

Tests with specific antibodies showed that the 9 kilodalton protein and the three proteins of the RC are specific within the species; any one of these proteins from one strain of Rps. spheroides cross-reacts serologically with its counterpart from another strain (14 independent strains were tested). There was little or no cross reaction with

seemingly similar proteins from closely related species such as Rps. capsulata or palustris. The 46 kilodalton protein proved to be more variable within the species. In some strains of Rps. spheroides this protein appeared to have a slightly altered molecular weight (44,000 to 49,000) or to be missing. There was no serological cross-reaction between the 46 kilodalton protein and the variant ones. The study of this conspicuous membrane protein remains for the future.

The non-photosynthetic mutant strain PM-8 of Rps. spheroides contains no material that cross-reacts with antibody against RC's. RC's can be precipitated from cell extracts of wild type Rps. spheroides by adding the specific antibody (24).

In preliminary experiments with anti-RC we found that the antibody interferes with electron donation from cytochrome to RC's but not with donation from RC's to added UQ. We have now undertaken a major experimental program: to prepare sufficient quantities of each of the three RC proteins electrophoretically, so as to generate antibodies specific against each of these proteins. The antibodies will be converted to f_{ab} fragments and these will be used as blocking agents, to explore sites of electron donation and acceptance on each component of the RC. This will help us to construct a picture of how the RC interacts with secondary electron transfer components in vivo. This program will be in collaboration with Dr. L. Smith of Dartmouth Medical School; she will provide purified cytochromes, f_{ab} fragments of antibodies against these cytochromes (some that block specifically the electron donating or accepting functions of the cytochromes), and expertise in the kinetic study of reactions involving cytochromes.

6. Experiments related to the development of solar cells.

In making a solar cell from photosynthetic materials we envision the use of photochemical reaction centers aided by a suitable admixture of "antenna" pigment. We have prepared mixtures of purified RC's and purified antenna BChl-protein (the 9 kilodalton component of the membrane) in various proportions. Energy transfer in some of these reconstituted systems proceeds with nearly 100% efficiency, as shown by the quantum efficiency of RC photochemistry and also by variations in the yield of fluorescence from the antenna BChl depending on the state (active or closed) of the RC's. These experiments show that we can use these components in any desired proportions in solar cells. They also give quantitative information about the rate of energy transfer from the antenna to the RC's, which helps to elucidate this process in the living cell. For example, it appears that the RC's are effective traps for excitation energy even when they are closed, which explains the low yield of antenna BChl fluorescence in vivo (25).

References. An asterisk denotes publications from our laboratory during the period covered by this report.

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FUTURE PLANS AND OBJECTIVES

1. We shall continue to study the relationships between singlet and triplet excited states, the state P^f (a possible photochemical intermediate), and photochemical oxido-reduction in photosynthetic reaction centers. We are developing absorption and fluorescence spectrometry with 5 nsec time resolution to this end, but will rely on collaboration with others for measurements in the picosecond time domain. We shall measure the photochemical efficiency as a function of temperature, and correlate this knowledge with information about the formation and decay of P^f . The fluorescence, an indicator of the singlet excited state population, will be measured as functions of time and temperature. Our principal motive here is to learn whether the fluorescence is prompt, reflecting decay of the first-formed excited singlet state, or delayed, perhaps resulting from conversion of P^f back to the dipole-allowed excited singlet state. Our objective is to expose the details of the mechanism of photochemistry in the photosynthetic bacteria.

2. We shall continue to study reactions of RC's that have been altered chemically or physically; our principal objective is to learn more about the way that UQ, with or without some participation by iron, functions as primary electron acceptor. Absorbance changes with picosecond

time resolution will be measured collaboratively with K. Kaufmann at Bell Laboratories and M. Windsor at Washington State University. The consequences of dehydration will be studied thoroughly to elucidate the role of water in association with the BChl that acts as photochemical electron donor. In these studies we will examine the BChl of RC's in its reduced and oxidized states by measuring optical absorption and absorbance changes, fluorescence, optical circular dichroism, and EPR. Oxidized BChl in dehydrated RC's will be generated both chemically and photochemically, if possible. Substitution of other solvating agents in place of water will be attempted. Details of the photochemistry will be examined in RC's that have been altered either by solvent extraction, chemical inhibition, or dissociation of protein components, with restoration by added UQ where possible. The properties to be studied will include fluorescence, proton binding, kinetics and quantum efficiency at low temperatures, and those absorbance changes that reflect reactions of UQ and physical responses of the chromophores.

3. We shall prepare f_{ab} antibody fragments specific toward the three protein components of RC's. These will be used to study which components carry sites at which the photochemical products interact with external electron carriers: sites for acceptance of electrons from cytochromes and for donation of electrons to the secondary pool of UQ. This will be part of an effort to visualize the detailed architecture of the bacterial photosynthetic membrane. It will be done in collaboration with Dr. L. Smith of Dartmouth Medical School.

4. The reconstitution of energy transfer in mixtures of purified antenna BChl-protein and RC's will be explored further, both to reveal

quantitative details of energy transfer in vivo and as an adjunct to the construction of a solar cell. We shall attempt to demonstrate light-induced charge separation in sandwiches composed of layers of metal electrodes, RC's, antenna, and secondary electron carriers such as iron compounds and quinones. This step toward solar energy conversion will be attempted also in liquid cells with metal electrodes coated with RC's and electron carriers.

5. As time permits, but with lower priority, we will make other experiments suggested by past studies. These may include study of the organization of the bacterial photosynthetic membrane with particular reference to the 9 and 46 kilodalton proteins, study of any coupling between carotenoid biosynthesis and the development of the complete membrane, study of specific inhibition of membrane components by the detergent LDAO (which can derange carotenoid synthesis without preventing growth, and study of the process (enzymatic?) by which antenna BChl in certain carotenoidless mutant strains of Rps. spheroides becomes converted to bacteriopheophytin.

GRADUATE STUDENTS TRAINED, DEGREES GRANTED, POSTDOCTORAL TENURES COMPLETED

Graduate students.

1. R.C. Downs, Sept. 1971 - June 1975. Awarded Master's Degree in Applied Physics.
2. T.C. Cook, March 1975 - June 1975. May return to our laboratory in Sept. 1975 as Ph.D. candidate after completion of rotation through other laboratories.

Postdoctorals.

1. L.A. Sherman, Sept. 1970 - Aug. 1972. Now Asst. Prof. of Biology at the Univ. of Missouri, Columbia, Mo.
2. C.A. Wraight, Feb. 1972 - Feb. 1974. Now Asst. Prof. of Biology at Univ. of Calif., Santa Barbara.
3. D.C. Brune, Jan 1973 - Nov. 1974. Now at Dept. of Biology, Brookhaven Nat'l. Lab.
4. R.J. Cogdell, Oct. 1973 - July 1974. Now in laboratory of W.W. Parson, Univ. of Washington.
5. T. Yamamoto, Oct. 1973 to present.
6. P. Heathcote, Oct. 1974 to present.

PUBLICATIONS, SEPTEMBER 1972 - PRESENT

These are denoted by an asterisk in the references listed at the end of the section "Accomplishments in Relation to Original Objectives". Additional ones are listed below.

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PRESENT STATE OF KNOWLEDGE, SIGNIFICANCE FOR BIOLOGY AND MEDICINE, AND
NEEDED FUTURE INVESTIGATIONS

We are on the threshold of understanding the mechanism of the conversion of light to chemical energy in bacterial photosynthesis, and probably in photosynthesis generally. This has come about through two major advances: the development of photosynthetic reaction centers as objects of study, and absorption spectrometry with nanosecond and then picosecond time resolution. The first of these advances was made chiefly in our laboratory over the past 13 years; in the second we have capitalized promptly on the advances of others through collaborative efforts. To understand this fundamental process of nature thoroughly ought to have far-reaching consequences for the improvement of agriculture and for the development of novel methods for solar energy conversion. We are optimistic that success in this area will come through a continuation of present lines of investigation. I believe that the most promising efforts are currently being made in our own laboratory and in the laboratories of P.L. Dutton (Univ. of Pennsylvania), W.W. Parson (Univ. of Washington), P. Rentzepis (Bell Laboratories), and M. Windsor (Washington State Univ.), with whom we have been or are collaborating.

Our store of knowledge about the photobiology of photosynthetic bacteria has had some impact on medicine in the past; specifically, it led to the use of carotenoids in the treatment of porphyria. This sort of unexpected impact might happen again where light is involved in disease or in therapy.

The study of arthritis and metabolic diseases intersects the study of cell membranes, especially where the coupling of electron transport to phosphorylation is concerned. While our work is not directed toward these considerations, we are concerned with the architecture and function of photosynthetic membranes in relation to the photosynthetic process. Thus we hope to continue making contributions to our knowledge of membrane biology.

OVERALL FEDERAL SUPPORT

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