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Appendix: Annual Progress Report, Contract Period Sept. 1, 1971 -
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CONTENTS

Page No.

ORIGINAL OBJECTIVES AND SUMMARY OF PROGRESS CORRESPONDING TO THESE OBJECTIVES

1. Photosynthetic reaction centers. 1
2. Use of viruses, antibodies and inhibitors of mem- 3
brane-bound enzyme activities, in studying photosynthetic
membranes.
3. Measurement of components of the "high energy state" 5
in photosynthetic tissues.

DETAILED REPORT OF PROGRESS

- I. Photosynthetic reaction centers. 8
 1. Preparation, composition and photochemical 8
parameters.
 2. Fluorescence. 11
 3. Secondary electron transfer. 12
 4. Absorption band shifts of pigments in reaction
centers. 14
 5. The primary electron acceptor? 15
 6. The primary electron donor. 16
- II. Viruses, antibodies, and analysis of the bacterial 17
photosynthetic membrane.
 1. Viruses 17
 2. An effect of LDAO on growing cells. 18
 3. Antibodies against photosynthetic membrane com- 18
ponents.

	<u>Page No.</u>
4. Fractionation and analysis of components of photosynthetic membranes.	20
III. Manifestations of the "high-energy state" in photo- synthetic tissues.	23
CURRENT OBJECTIVES RELATED TO PAST OBJECTIVES	26
I. Photosynthetic reaction centers.	26
II. Analysis of photosynthetic membranes.	31
REFERENCES	37
PRIORITIES	40
TRAINING	41
PUBLICATIONS	42
THE FUTURE OF THE FIELD AND ITS SIGNIFICANCE IN BIOLOGY AND MEDICINE	45
APPENDIX A: ANNUAL PROGRESS REPORT, Sept. 1, 1971 - Aug. 31, 1972	47
APPENDIX B: PREPRINTS AND REPRINTS OF ARTICLES PUBLISHED	48

ORIGINAL OBJECTIVES AND SUMMARY OF PROGRESS
CORRESPONDING TO THESE OBJECTIVES

The original (1969) objectives were listed as summaries on Science Information Exchange forms "Notice of Research Project". Titles of the individual summaries are listed here, together with outlines of relevant progress.

1. Photosynthetic reaction centers.

The main objective was to improve the preparation of reaction centers, eliminating unessential components and then reconstructing a system that may have physiological significance. We have had gratifying success in this area. Using the detergent lauryl dimethyl amine oxide (LDAO) applied to membrane fragments from carotenoidless mutant Rhodospseudomonas spheroides, we have isolated reaction center particles (rcp's) consisting of protein, bacteriochlorophyll (BChl), bacteriopheophytin (BPh), and possibly (analyses remain uncertain) ubiquinone (UQ). A particle of apparent molecular weight 70,000 contains 4 BChl and 2 BPh. The photochemical act entails the displacement of one electron from BChl to another part of the rcp, leaving the cation radical BChl^{+} , which we recognize as "oxidized P870" or " P^{+} ". The particle is composed of three protein components of apparent molecular weights 28,000, 22,000, and 20,000. The development of rcp's using LDAO was made simultaneously in our laboratory and that of G. Feher.

We have shown that cytochrome (cyt) and UQ can be added to these rcp's so as to reconstruct an electron transport system like that in whole cells. The donation of electrons from cyt to P^{+} , and from the reduced photoproduct (" A^{-} ") to UQ, happens far more rapidly than the interaction of primary photoproducts P^{+} and A^{-} . This explains how the energy of the primary products is

stabilized efficiently against wasteful recombination. Details about this model for photosynthetic electron transport are given later.

We have discovered two new categories of optical absorption changes related to the formation of reduced photoproducts in rcp's. In one category is the shifting of all the absorption bands of BChl and BPh, probably in response to an electric field associated with the electron in "A⁻". Another category suggests the formation of the anionic ubisemiquinone radical, UQ⁻·, either as a primary (A⁻) or closely associated secondary reduced product. Kinetic relationships between these changes and variations of BChl fluorescence in rcp's expose some new areas of study; see later. Electron spin resonance (ESR) studies by P. A. Loach and G. Feher have also suggested the formation of UQ⁻· as a reduced photoproduct, but discrepancies between the various observations await resolution.

Studies of the formation of P⁺ and its reaction with cyt, the latter in collaboration with W. W. Parson, have been combined with analytical spectroscopy of the pigments in rcp's and in organic extracts of rcp's with the following findings: Each rcp, containing 4 BChl, can effect the photochemical transfer of a single electron. The resulting P⁺ can be extracted, giving an entity resembling BChl⁺ in methanol. The extracted oxidized pigment can be re-reduced in vitro with ascorbate. This opens a new bridge between in vivo and in vitro studies of BChl photochemistry.

The photochemical formation of P⁺ proceeds with 70% quantum efficiency. This revised figure is consistent with a 3-fold to 4-fold rise in the fluorescence of BChl in rcp's when the photochemistry is prevented. The newly revised absolute optical absorption coefficients for BChl and for the reaction $P \longrightarrow P^+$ in rcp's will allow more reliable interpretation of experiments on photochemical activities of whole cells.

The variations in fluorescence of BChl in rcp's and the reactions of illuminated rcp's with cyt and UQ have revealed certain properties of the primary and secondary electron acceptors. Notably, the primary acceptor can hold just one electron in its reduced form and has a mid-point potential near -0.05 volt, independent of pH. We have delineated many details of interactions between light, rcp's, and endogenous and added electron transfer components, by the use of inhibitors and of variations of temperature and pressure. These details are recounted later.

Reaction center preparations from Rhodospirillum rubrum resemble those from Rps. spheroides in their absorption spectra and apparent photochemical activities.

2. Use of viruses, antibodies and inhibitors of membrane-bound enzyme activities, in studying photosynthetic membranes.

The main stated objectives were to explore the uses of specific viruses and antibodies that interact with photosynthetic bacteria and their components. This program has been expanded into an investigation of the structure of the bacterial photosynthetic membrane, using specific antisera and electrophoretic analysis.

The work with our newly isolated bacteriophage, which infect and propagate in Rps. spheroides, stands as follows: We are thus far the only laboratory (together with that of W. R. Sistrom, who developed the virology while on sabbatical leave in our laboratory) possessing phage that can be propagated in photosynthetic bacteria. Within a few minutes after infection, host cells clump together, signifying a change in the cell surface. The synthesis of new photosynthetic pigments, and presumably of many proteins, is arrested as the host is retooled for phage production.

We observed that the detergent LDAO in growing cultures of photosynthetic bacteria alters the synthesis of carotenoids, apparently by inactivating a membrane-bound hydrating enzyme.

We have fractionated broken Rps. spheroides cells by exposure to detergents, differential centrifugation, ammonium sulfate fractionation, column chromatography, and interaction with specific antibodies. We then analyzed the distribution of protein components among these fractions by electrophoresis in polyacrylamide with sodium dodecyl sulfate (SDS) and recorded spectra and photochemical activities of pigments associated with these proteins. Major results follow; many details are listed later.

The photosynthetic membrane of Rps. spheroides does not contain a specific pigment-protein ("photoreceptor subunit") with combined light harvesting and photochemical functions. Rather, the reaction centers and a major light harvesting fraction can be isolated separately as specific chromoproteins; the former with molecular weight 70,000 (three components) and the latter 10,000. Other distinctive chromoproteins are being recognized and isolated for study. A major 46,000 dalton protein is found in a nonpigmented fraction associated with the cell wall. The nonphotosynthetic mutant strain PM-8 lacks the three protein components that make up the 70,000 dalton rcp, and has no component that reacts with antibody specific toward reaction centers. Relations between these proteins will be probed further by examining the incorporation and redistribution of ^{14}C -labeled amino acids in whole cells during physiological variations such as the transition from aerobic (nonphotosynthetic) to photosynthetic growth.

Reactions of anti-reaction center serum with membrane fragments ("chromatophores") support analyses showing that about 20 to 30 per cent of the pigmented membrane protein is reaction center protein. All of the antigenic

determinants of the rcp appear to be accessible to the antibody when the reaction centers are embedded in the photosynthetic membrane. Anti-reaction center impairs the interaction between purified rcp's and added cyt, but not added UQ. Rcp's precipitated with antibody show normal intrinsic photochemical activity.

3. Measurement of components of the "high energy state" in photosynthetic tissues.

Energy storage for photosynthetic phosphorylation is correlated with two electrochemical phenomena: the formation of a gradient of H^+ concentration across the photosynthetic membrane and the development of an electric (ion diffusion) potential across this membrane. These effects represent two forms of chemical potential energy that might be important for ATP formation. Changes of these energy states are accompanied by at least three optical manifestations: shifts of the absorption bands of carotenoid pigments, and related changes in the intensity of delayed fluorescence from Chl or BChl (these two are associated primarily with the membrane potential), and changes in the yield of prompt fluorescence (associated with the H^+ gradient). Many of the basic observations in this field were made originally in our laboratory, and we have continued to develop these correlations so as to use the optical effects as indices of the electrochemical state.

The observation of H^+ - dependent quenching of Chl fluorescence in spinach chloroplasts was confirmed and extended using sub-chloroplast preparations with various ionophorous antibiotics. We then observed the same set of effects in photosynthetic bacteria. This has allowed a clearer understanding of the ways that the fluorescence signifies both the intracellular H^+ concentration and the redox states of the components of the photochemical reac-

tion centers. It had not previously been suspected that the fluorescence in bacteria is affected by physiological factors other than the states of the reaction centers.

The relationship between carotenoid band shift and delayed fluorescence as indicators of electric fields in the photosynthetic membrane has been studied further; the two are related logarithmically, as if the band shift were a linear function of field strength, and the intensity of delayed fluorescence an exponential function. This is to be expected if the emission of delayed fluorescence requires an input of energy that is formulated in a Boltzmann factor, $\exp(-\Delta E/kT)$.

The view has become widely established that the carotenoid band shift is a simple electrochromic response to fields impressed on membrane-bound carotenoids. We have shown that a more specific interaction with the reaction centers is involved, by using a nonphotosynthetic mutant strain (PM-8) of *Rps. spheroides*. This mutant forms a normal complement of membrane-bound light harvesting pigments (BChl and carotenoids) but it lacks reaction centers (see earlier) and cannot grow photosynthetically. It can be grown as an aerobe, in darkness. We expected the mutant to show a carotenoid band shift in response to chemical perturbations (ion pulses) calculated to produce an electric potential across the membrane. It did not show such a band shift, whereas the parent strain (with reaction centers), grown in the same way as the mutant, did. Suspecting that the membranes of the mutant, lacking a major protein (reaction center), might have holes, we tested whether the membrane fragments of the mutant were capable of sequestering ions so as to generate (hypothetically) an electric field across the membrane. The membranes of the mutant appeared to be sound in this respect. Thus the lack of carotenoid band shifts in the mutant was associated with the absence of

reaction centers, but not with an absence of electrochemical gradients in the appropriate structures.

Light-induced movement of ions in photosynthetic systems can be signaled by the osmotic swelling and shrinking of membrane-bound structures, observed by the changes of light scattering. Using photosynthetic bacteria, we find that we can apparently discriminate between changes involving the entire cell and changes involving intracellular structures. When cells are suspended in 30% albumin solution, so that the index of refraction of the cells matches that of the suspending medium, the scattering by the cell surface is very much reduced. The residual scattering, in which the contributions of intracellular refractile surfaces are emphasized, shows light-induced changes that are qualitatively different from those exhibited by cells suspended in water (where the surface of the cell is the main scatterer). We have not explored this technique further, but it may prove to be valuable.

DETAILED REPORT OF PROGRESS

I. Photosynthetic reaction centers.1. Preparation, composition and photochemical parameters.

When the pigmented membranes of photosynthetic bacteria are exposed to the detergent LDAO, a characteristic chromoprotein identified with photochemical activity is disengaged from other pigments and proteins. This "reaction center particle" (rcp) is not sedimented when an aqueous-detergent suspension is centrifuged 3 h at 200,000xg; it is precipitated by ammonium sulfate at about 40-50% of saturation. These properties allow its purification and detailed study. This method of purification, an improvement over earlier procedures using Triton X-100 as detergent, was achieved simultaneously in our laboratory (1) and in Feher's (2), starting with carotenoidless mutant Rps. spheroides strain R-26 (3). We have made a similar preparation from carotenoidless mutant R. rubrum (4). The rcp's from R. rubrum have about the same ratios of pigments and proteins as those from Rps. spheroides, judging from absorption spectra. The difficulty in obtaining purified rcp's from normally pigmented strains of Rps. spheroides probably has a trivial basis (see later; Section II).

Purified rcp's from Rps. spheroides consist mainly of protein, BChl and BPh. We (and Feher's group) find that one particle contains three protein components of molecular weights 28,000, 22,000, and 20,000 dalton as judged from the mobility in acrylamide gels with SDS (5,6,7). In addition one particle contains four molecules of BChl and two of BPh (8,9). Okamura and Feher (7,10) showed that the 28 kilodalton protein can be removed, leaving the pigments bound, still photochemically active, to the conjunction of the two lighter proteins.

The photochemistry in rcp's results in the transfer of one electron from BChl to an acceptor site, A, not yet identified. It is possible that A is UQ (see later); the UQ content of rcp's has probably been underestimated in the past because of its degradation during the assay procedure. The photochemical removal of an electron from BChl in the rcp causes changes in optical absorption ("bleaching of P870" (11) and other changes) and an ESR signal appropriate for the formation of the cation radical, $\text{BChl}^{+\cdot}$ (12,13). The ESR band width (13) is $\sqrt{2}$ -fold narrower than that of monomeric $\text{BChl}^{+\cdot}$; this suggests that the + charge is delocalized over two BChl molecules in the rcp (14).

After removing extraneous BPh we found that the residual BPh was as effective as the BChl of rcp's in mediating the photochemistry (15).

The formation of $\text{BChl}^{+\cdot}$ in rcp's can be induced chemically (with ferricyanide) and follows a "one-electron" redox titration curve. The chemically induced and light-induced reactions are complementary; we have shown that if some $\text{BChl}^{+\cdot}$ is formed chemically, the light reaction of the remainder (up to a maximum of one $\text{BChl}^{+\cdot}$ per rcp) proceeds with "normal" efficiency (16). This rules out models (12) in which the light reaction forms $\text{BChl}^{+\cdot}$ and $\text{BChl}^{-\cdot}$, the two species giving similar ESR signals and absorption spectrum changes. This result also helps to establish a stoichiometry of one electron transfer per rcp. This stoichiometry is shown further by experiments in which the re-reduction of $\text{BChl}^{+\cdot}$ is coupled to the oxidation of added mammalian cyt c (17,18). We made this kind of experiment in collaboration with W. W. Parson at the University of Washington. The reaction kinetics of $\text{BChl}^{+\cdot}$ reduction (reversal of P870 bleaching, or " $\text{P}^{+} \longrightarrow \text{P}$ ") and cyt oxidation are identical and correspond to a maximum of one electron transfer per 4 BChl, or per rcp. These measurements, together with spectroscopic analysis of the pigments in

rcp's and in organic solvent extracts of rcp's (8,9), have afforded reliable ($\pm 5\%$) assignment of molar extinction coefficients to the absorption bands in rcp's, and differential extinction coefficients for the formation of BChl^+ . ($P \longrightarrow P^+$). The values at 865 nm are $\epsilon = 133 \text{ mM}^{-1} \text{ cm}^{-1}$ and $\Delta\epsilon(P \longrightarrow P^+) = 116 \text{ mM}^{-1} \text{ cm}^{-1}$. Using these coefficients, the previously measured quantum efficiency for $P \longrightarrow P^+$, based on the rate of bleaching of the 865 nm band, is recomputed to be 70% rather than the earlier value (12) of 90 - 100%.

From measurements of the yield of fluorescence from BChl in rcp's we have inferred (19,26) that the lifetime of the singlet excited state is 7×10^{-12} sec, while the natural radiative lifetime (if fluorescence were the only mode of de-excitation) would be 2×10^{-8} sec. From these figures, together with a 70% photochemical efficiency, we can compute unimolecular rate constants for the three processes through which excitation energy is spent in rcp's:

$$\left. \begin{aligned} k_f (\text{fluorescence}) &= 5 \times 10^7 \text{ sec}^{-1} \\ k_d (\text{radiationless de-excitation}) &= 4 \times 10^{10} \text{ sec}^{-1} \\ k_p (\text{the first step leading to photochemical oxido-reduction}) &= 10^{11} \text{ sec}^{-1} \end{aligned} \right\} [1]$$

Recent evidence by Dutton et al. (20) suggests that the radiationless de-excitation involves conversion of singlet-excited BChl to the triplet state. The triplet is probably not an intermediate in the photochemistry, because when the photochemistry is blocked by chemical reduction of the acceptor, the fluorescence (singlet state population) rises together with the population in the triplet state. This is explained most economically if the triplet is associated with radiationless de-excitation, competing with the photochemistry, but there are other problems (see later).

2. Fluorescence.

We have measured the absolute yield of fluorescence from light harvesting BChl in cells and chromatophores of photosynthetic bacteria (21). The values of 2 to 10% are consistent with lifetimes of 0.4 to 2 nsec measured in other laboratories (22,23). However, this fluorescence may come largely from BChl that is not involved with the transfer of energy to reaction centers; the latter may have a much lower yield and shorter lifetime (24). We have sent cultures of carotenoidless mutant Rps. spheroides to Dr. Borisov in Moscow in order to pursue this question further.

The competitive relationship between fluorescence and photochemistry as fates of excitation energy in rcp's has been documented and exploited further. The yield of fluorescence from P870 (the 865 nm BChl band) increases threefold when rcp's are treated (chemically or photochemically) so as to reduce the acceptor: $P, A \longrightarrow P, A^-$ (19,25). This is compatible with the revised value of 70% for the quantum efficiency of photochemistry (ϕ_p), through the relationship (25)

$$\phi_p = 1 - \phi_f / \phi_f^{\max} \quad [2]$$

The high level of fluorescence can be attained through photochemical reduction by illuminating rcp's in the presence of reduced cyt:

This requires the transfer of just one electron per rcp, proving that the electron-accepting side of the system is saturated (except for slow processes) by one electron (25,27). This result is confirmed by the fact that just one molecule of cyt is oxidized per rcp in a fast reaction during steady illumination (25), provided that no secondary acceptors have been added. Sometimes there is evidence for a slowly reacting secondary acceptor in purified rcp's.

We have found three ways, two chemical and one physical, to lower the efficiency of the primary photochemistry (27,28). The addition of quinoline-N-oxides at about 10^{-6} M concentration, or of ortho-phenanthroline at 1 mM or more, causes a reduction in photochemical efficiency and a concomitant rise in P870 fluorescence. These agents appear to affect the photochemical mechanism directly, lowering the rate constant for photochemical utilization of the singlet excited state of BChl. In contrast, the application of high pressure (experiments made in collaboration with D. DeVault of the University of Pennsylvania), up to 6000 atm., lowers the photochemical efficiency indirectly, by promoting more rapid radiationless de-excitation (increasing the rate constant k_d ; Eqn. 1). We infer this because the efficiency of fluorescence, rather than rising, diminishes in proportion to the decline of photochemical efficiency under high pressure.

3. Secondary electron transfer.

When mammalian cyt c is added to rcp's, the two are bound together electrostatically (judging from effects of ions), and the cyt can transfer electrons to P^+ in times of the order of 10^{-5} sec (17,18,25). This rapid transfer effectively prevents a reaction between P^+ and A^- , which has a half-time of 20 msec or more depending on the temperature. The reaction of P^+ with cyt accounts for the ability of the living cell to consolidate the fruits of its photochemistry against possible wasteful interaction of the primary photoproducts.

If UQ is added to rcp's the two become associated, apparently through hydrophobic interactions that are weakened at high concentrations of LDAO (29). The presence of UQ retards the back-reaction between P^+ and A^- , probably because of the electron affinity of UQ, but there is no clear evidence

that the electron is transferred completely to UQ when P^+ is present. But if an electron from cyt has neutralized the charge of P^+ , the UQ can become reduced. Then if rcp's are illuminated with excess amounts of cyt and UQ, the photochemical system will drive electrons from cyt to UQ, with the expected stoichiometry of two cyt oxidized for every one UQ reduced to UQH_2 (25). This stoichiometry is confirmed by measurements to determine how many electrons must be driven through the system before the fluorescence rises in response to " $P, A \longrightarrow P, A^-$ ". These reactions provide a reasonable model for the primary photochemistry and associated secondary electron transport in living cells (26).

The fate of the electron on A^- can depend on the relative electron affinities of P^+ and other oxidants such as added UQ or ferricyanide (29). Thus ferricyanide can remove the electron entirely from the rcp, in spite of the attraction by P^+ , but UQ probably cannot (16,29).

The back-reaction between P^+ and A^- has a half-time of about 60 msec between room temperature and -40°C ; it decreases to 20 msec between -40° and -80°C , and remains at 20 msec down to 1°K (13,29,30). We do not know the mechanism of this change.

Secondary electron transport between A^- and UQ and perhaps between UQ and P^+ is affected by high pressure in a variety of ways (28). These effects deserve further study as they can reveal conformational (volume) changes attending these reactions.

In addition to reducing the efficiency of the primary photochemistry, ortho-phenanthroline blocks electron transport from A^- to secondary acceptors. The ability to isolate A^- from secondary acceptors in this way has proved useful, for example in determining the electron-holding capacity of A (27). These observations are consistent with some effects of ortho-phenanthroline

on cells and chromatophore preparations.

4. Absorption band shifts of pigments in rcp's.

We have found that all kinds of oxidations and reductions, photochemical or not, induce absorption band shifts of the pigments in rcp's (8,16). The bands of BPh are shifted toward greater wavelengths and the bands of BChl toward lesser wavelengths. Of course, the shift of the 865 nm band of BChl can be observed only when that band has not been bleached in the reaction $P \longrightarrow P^+$. Oxidation (chemical or photochemical) causes large shifts of the BChl bands and smaller shifts of the BPh bands, and vice versa for chemical or photochemical reduction. These shifts can be attributed to electric field effects. Then the field of the + charge in P^+ has a large effect on BChl and a small one on BPh, and conversely for the field of the extra electron in A^- . The dipole field of P^+, A^- has an effect almost the same as that of P^+ , whereas the monopole field of A^- has a greater effect on BPh. The blue-shift of the 865 nm BChl band can also be caused by high concentrations of LDAO or by dehydro-ascorbic acid. We hope that these effects will prove to be a valuable tool by reflecting changes in the distribution of electric charge in rcp's.

A small amount of carotenoid pigment associated with reaction center preparations from wild-type *Rps. spheroides* (see Sec. II) also shows band shifts to greater wavelengths during photochemical oxido-reduction.

The shift of the BPh band at 760 nm causes a conspicuous absorption increase at 770 nm in response to photochemical reduction, as when rcp's are illuminated in the presence of reduced cyt (Eqn. 3). The onset and decay of this absorption change shows the same kinetics as the change in fluorescence yield described earlier, which we have correlated with the transition between

P,A and P,A⁻ (8). But we shall note (next paragraphs) that another absorption change recovers more rapidly than either the high fluorescence or the band shift.

5. The primary electron acceptor?

The reduction of rcp's, either photochemically (Eqn. 3) or chemically, causes the aforementioned pigment band shifts and also a new set of absorption changes that includes the appearance of bands at about 310 and 450 nm (16,31). These changes suggest the formation of the anionic semiquinone radical of ubiquinone, UQ⁻. The reversal of these changes after illumination, measured at 450 nm, is actually more rapid than the relaxation of the band shifts (measured at 770 nm) or the decline of P870 fluorescence from the higher to the lower level (8). Thus at some time after the end of illumination, some reaction centers show high fluorescence and have not recovered from the band shift, but are ready once more to perform the reaction that resembles formation of UQ⁻. Possibly the semiquinone-like changes pertain to reduction of the primary acceptor, while the band shifts and the higher fluorescence are maintained through a secondary event in which A⁻ discharges its electron. If so, the higher fluorescence may sometimes reflect merely a reduced photochemical efficiency, and not a complete inability to perform photochemistry.

The idea that elevated fluorescence yield is associated not necessarily with reduction of the primary acceptor, but with a secondary event, has emerged also in Mauzerall's studies (32) of the time-course of changes in Chl fluorescence in algae after a brief flash. These findings may force us to revise our rather simple present interpretations of the fluorescence in relation to the redox states of reaction centers.

Loach has discovered (33) and Feher et al. have confirmed (10) the existence of an ESR signal, attending the formation of reduced photoproducts in sub-chromatophore particles and in rcp's, having the proper characteristics of $UQ^{\cdot-}$. To see this signal it was necessary to dissociate iron from the preparations; this could be done by adding 0.1% SDS. But we could observe the absorption changes that suggest $UQ^{\cdot-}$ without dissociating "bound" iron. It would seem that the primary photo-displaced electron can confront, and interact with, both UQ and Fe. The interaction with UQ causes absorption changes corresponding to $UQ^{\cdot-}$, but Fe interferes with the ESR signal that usually results from the interaction with UQ.

6. The primary electron donor.

We have considered evidence (part 1 of this Section) that the primary oxidized photoproduct is $BChl^+$, and that the single + charge in an rcp containing 4 BChl's is actually delocalized over two of the BChl's. We have now shown (8) that if rcp's are illuminated so as to oxidize some of their BChl, and mixed rapidly with an excess of methanol, the extract contains some oxidized BChl. Addition of ascorbate to the extract restores all of the BChl to its reduced form. We have thus achieved a link between the in vivo and in vitro chemistry of BChl:

Numerous chemical and physical studies are made possible by this discovery. We should make a detailed comparison of the properties of this $BChl^+$ (or " p^+ ") before and after extraction. At present we are perplexed because more than one fourth (about 35%) of the BChl of illuminated rcp's comes into the methanol extract in an oxidized form, while we believe that just one + charge (or electron vacancy) is formed in the rcp's for every 4 BChl.

A possible model for the oxidized substance in vivo is that of a BChl-water dimer carrying a single electron deficiency: $(BChl \cdot H_2O \cdot BChl)$. This model is suggested by the physical properties of such dimers, including the narrowing of the ESR band (14), and also by the observation that the photochemical activity of reaction centers is lost (and the fluorescence rises) when the material is dehydrated. This inactivation is reversible (34,35).

II. Viruses, antibodies, and analysis of the bacterial photosynthetic membrane.

1. Viruses.

We have isolated about 25 new strains of Rps. gelatinosa, capsulata, and spheroides, and have found several that yield viruses lysogenically and others that are sensitive to these viruses. As an ecological note, all of the lysogenic donor strains were from Cayuga Lake and its inlet, and all of the sensitive strains were from "upstream" sources. Our efforts have been concentrated on one virus that resembles T-even coliphage and that infects and can be propagated in the laboratory strain ATH 2.4.1 (van Niel) of Rps. spheroides.

A one-step growth experiment requires about 3 h from infection to lysis in Rps. spheroides. During the first hour we observed inhibition of various synthetic abilities by the host, including the ability to form the pigmented photosynthetic membrane and the capacity for induced synthesis of catalase (compare the effects of x-irradiation; 36). During the first 5 min we observed clumping of the host cells, signifying a change in the cell surface. This clumping was prevented by chloramphenicol and was absent in lysogenic variants of the host; therefore it involved virus-directed protein synthesis. As a viral-induced change of the host cell's properties it may have some

bearing on the cancer problem.

These experiments were made principally by Dr. W. R. Sistrom during his stay in our laboratory on sabbatical leave from the University of Oregon.

2. An effect of LDAO on growing cells.

At a concentration of 0.005%, the detergent LDAO prevented growth of Rps. spheroides and R. rubrum. At 0.001% it had no obvious effect. At 0.002% it caused a failure of terminal steps in carotenoid biosynthesis, so that neurosporene accumulated in Rps. spheroides, and lycopene in R. rubrum. These lesions probably represent the inactivation of membrane-bound hydrating enzymes. Such specific inhibition of a type of membrane-bound enzyme by a detergent suggests that a more general study would be rewarding, in which one explores the ways that activities of different sorts of membrane-bound enzymes depend on their relationships to lipids and to other proteins in the membrane. We have not yet pursued this further.

3. Antibodies against photosynthetic membrane components.

In collaboration with Drs. R. L. Berzborn and W. R. Sistrom we prepared rabbit antisera against various fractions from Rps. spheroides: purified rcp's, chromatophores, a pigmented membrane fraction lacking reaction centers, and a nonpigmented (aerobically grown) membrane fraction. These antisera were tested for precipitation with fractions from Rps. spheroides and from other photosynthetic bacteria. Major observations were as follows.

1. The nonphotosynthetic mutant strain PM-8 of Rps. spheroides contains no component that reacts with anti-rcp serum. The mutant therefore lacks the three reaction center proteins (assuming all three were antigenic when the serum was produced) and lacks any precursor protein with similar antigenic determinants.

ii. Other species of photosynthetic bacteria do not contain material that cross-reacts with anti-rcp from Rps. spheroides. This holds even for the closely related Rps. capsulata, and for R. rubrum, which yields reaction center preparations that bear a close resemblance to those from Rps. spheroides (see Sec. I). The "reaction center fractions" from Rps. capsulata and R. rubrum contain three proteins similar to those of rcp's from Rps. spheroides (as revealed by electrophoresis in polyacrylamide; see the next Section), but lacking any antigenic similarity. On the other hand, the reaction center proteins from twelve independent newly isolated strains of Rps. spheroides cross-react strongly with anti-rcp made from our older laboratory strain of Rps. spheroides. Thus the amino acid sequences in reaction center proteins appear to be constant among strains of one species, and utterly different from those of other species. Other membrane proteins bear some inter-species similarity; antisera against membrane fragments from Rps. spheroides cross-react weakly with fractions from Rps. capsulata.

iii. A major protein of molecular weight 46,000 associated with cell walls of Rps. spheroides (next Section), is variable among different strains of this species. Of 12 strains tested, extracts from 8 showed precipitation with antibody against this protein from laboratory strain ATH 2.4.1. In the four which did not cross-react, the "46,000 dalton" protein appeared abnormal, showing altered molecular weight or being apparently absent when examined electrophoretically.

iv. An amount of anti-rcp that could precipitate 40 μ g of rcp's could alternatively precipitate 200 μ g of chromatophores. This suggested that chromatophores are 20% rcp by weight, provided that all the antigenic determinants of rcp's in chromatophores are accessible to antibody. Electrophoretic analysis showed that reaction center protein comprises about 30% of the

chromatophore protein, or perhaps 20% of the total mass of chromatophores. This suggests that all the antigenic determinants of rcp's are accessible to the "outside"; i.e., that reaction centers are situated on the exterior surfaces of the membrane fragments.

v. Anti-chromatophore serum does not precipitate rcp's. This surprising result probably has a trivial quantitative explanation.

vi. Anti-rcp can be used to separate reaction centers from detergent-treated chromatophores of wild type Rps. spheroides; see the next Section.

vii. Interaction or precipitation with antibody does not impair the photochemical capability of rcp's, nor the ability of rcp's to react with added UQ. The antibody does interfere with electron transfer from added cyt c to P^+ in rcp's.

4. Fractionation and analysis of components of photosynthetic membranes.

Photosynthetic bacteria were subjected to various fractionations, and the fractions were tested for the presence of pigments and protein components, the latter by electrophoresis in polyacrylamide, following the method of Laemmli (37). Before electrophoresis the samples were treated with SDS and mercaptoethanol in order to dissociate complex proteins into individual peptides.

In the course of this work we discovered that the Coomassie Blue, used to stain the proteins in the acrylamide gels, was fluorescent on some of the protein bands and not on others (6). We speculate, from some superficial correlations, that the more hydrophobic proteins render the stain nonfluorescent. If a reliable correlation can be made, the information content of the electrophoretograms will be augmented.

Crude cell extracts of Rps. spheroides, when analyzed in this way, displayed at least 50 distinguishable protein components including the three

reaction center proteins. A purified pigmented membrane fraction equivalent to Worden and Sistrom's "light chromatophores" (38) or Fraker and Kaplan's "purified chromatophores" (39) showed about 15 bands; predominant were the three of reaction centers and one at about 10,000 dalton (6). "Crude" chromatophores contained these and others; especially a major component at 46,000 dalton. When crude chromatophores were subjected to Loach's alkali-urea-Triton procedure (40), the resulting "photoreceptor subunit preparation" contained all the proteins of crude chromatophores; there was no indication that a selection of certain proteins had taken place.

Fractions from Rps. spheroides enriched for rcp's showed the expected three "reaction center" proteins. The nonphotosynthetic mutant strain PM-8 showed no trace of these proteins, confirming the serological finding (6). The triad of proteins characteristic of rcp's was conspicuous in extracts from R. rubrum and Rps. spheroides, capsulata, and palustris, but not Rps. gelatinosa or viridis.

In Rps. spheroides the 46,000 dalton protein was associated with non-pigmented fractions, especially those containing cell wall fragments. Recall its serological properties and inter-strain variability, mentioned earlier.

All fractions from Rps. spheroides that were rich in light harvesting pigments contained large amounts of a 10,000 dalton protein (6). This pigment-protein complex could be isolated in nearly pure form by a suitable exploitation of its solubility in relation to ammonium sulfate, Triton X-100, and LDAO. It is probably the same as that obtained by Fraker and Kaplan (39) after dissolving chromatophores in acidified chloroethanol. Prior to dissolution with SDS, our 10,000 dalton chromoprotein was present in larger aggregates. Controlled dissolution and study of the molecular weights of "characteristic" aggregates is in progress. We appear to be studying (as

have Fraker and Kaplan) the major chromoprotein that serves a light harvesting function in Rps. spheroides.

When anti-rcp serum was added to LDAO-treated extracts of Rps. spheroides, a precipitate was obtained that had the absorption spectrum typical of reaction centers, with little "contamination" by light harvesting BChl or carotenoids. Thus the light harvesting pigments seem to be bound preferentially to proteins (mainly the 10,000 dalton one) other than the reaction center proteins. The difficulty in obtaining "purified reaction centers" from carotenoid-containing strains of Rps. spheroides and R. rubrum appears to be a trivial one: with carotenoids present, the "light harvesting chromoprotein" accompanies the rcp's in the usual method of purification beginning with density gradient centrifugation. In carotenoidless mutants the light harvesting fraction is less buoyant and becomes separated from the rcp's during centrifugation.

We are beginning ¹⁴C-incorporation experiments, using autoradiography of the acrylamide electrophoretograms, to study the new synthesis and inter-conversions of proteins including those of rcp's and the 10,000 dalton protein. This can be done in conjunction with physiological variations (such as an increase or decrease of light intensity or a transition between non-photosynthetic (aerobic) and photosynthetic growth) that suppress or stimulate selectively the synthesis of the pigmented membranes. This has been done by Oelze and Drews (41), but with a technique of electrophoresis that does not allow us to recognize the proteins that we have characterized.

Hopefully the light harvesting and reaction center chromoproteins can be separated, purified, and then recombined so as to restore their functional interaction. Of course, there may be no single entity called a light harvesting chromoprotein. For example, prior to the addition of a detergent, the

light harvesting pigments might be bound hydrophobically to the reaction center protein as well as to the 10,000 dalton protein.

III. Manifestations of the "high-energy state" in photosynthetic tissues.

The "high-energy state" that mediates phosphorylation in photosynthetic tissues can be correlated with two interrelated sources of electrochemical energy: a gradient of $[H^+]$ across the thylakoid membranes and an electric potential across these membranes. In spinach chloroplasts the translocation of H^+ across the membrane is signaled by a quenching of Chl a fluorescence, at least if diaminodurene (DAD) is present as a carrier of electrons and protons (42). We have extended this correlation to sub-chloroplast fractions (43) and to the quenching of BChl fluorescence in photosynthetic bacteria (44). In chloroplasts the quenching associated with the redox states of system II reaction centers can be separated experimentally from that associated with H^+ movement. In the bacteria this separation cannot be made, but the quenching due to "open" reaction centers and to H^+ movements can be distinguished by their kinetics and their different responses to ionophoric uncouplers. The BChl quenching that we associate with H^+ movement is undone by agents such as NH_4^+ or nigericin, which abolish trans-membrane $[H^+]$ gradients. With these insights we can judge more effectively how the fluorescence of BChl in vivo indicates both the conditions of the reaction centers and the "high-energy state".

It has been suggested that DAD is important in the foregoing phenomenon not because of its ability to mediate electron transport, which can be done as well by the closely similar tetramethyl-phenylenediamine (TMPD), but because owing to its pK values it is an excellent H^+ absorber at physiological pH, and thus promotes H^+ uptake. However, pyridine, which has this buffering

property, did not appear to support the fluorescence quenching.

DAD was also found to cause a large increase in the intensity of delayed fluorescence (see 45) from BChl in Rps. spheroides chromatophores, and a corresponding increase in the light-induced band shifts of carotenoid pigments (46). This effect was intensified by uncouplers (like NH_4^+) that dissipate $[\text{H}^+]$ gradients; it was diminished by those agents (like valinomycin in the presence of K^+) that are believed to cause a collapse of trans-membrane electric potentials. We therefore believe that DAD applied to chromatophores augments the light-induced membrane potential as well as the $[\text{H}^+]$ gradient. In these experiments the carotenoid shift and the delayed fluorescence both varied over wide ranges as the pH and the "state" of the chromatophores varied. A linear-exponential relationship was found, as if the carotenoid shift were a linear function of membrane potential, and the delayed fluorescence an exponential function. This is expected if the emission of delayed fluorescence requires an input of energy governed by a Boltzmann factor, $e^{-E/kT}$ (see 47).

The nonphotosynthetic mutant strain FM-8 of Rhodospseudomonas spheroides, grown under limited aeration so as to contain an abundance of light harvesting BChl and carotenoids, was compared with the photosynthetically competent parent strain grown in the same way, for ability to show chemically induced carotenoid band shifts ^a(48). The chemical perturbations, all known to cause carotenoid shifts in photosynthetically grown Rps. spheroides and R. rubrum, included acid-base transitions, pulses of valinomycin, K^+ pulses after valinomycin, and pulses of pyrophosphate. In chromatophores from the parent (wild type) strain all of these treatments caused band shifts as expected from a correlation with hypothetical changes of electric potential in the membranes. None of these treatments caused a band shift in chromatophores from the

mutant. We then tested the ability of the mutant chromatophores to maintain ion gradients across the membrane, in two ways. The distribution of H^+ across the membrane, before and after a pH shift, was monitored by changes in the fluorescence of added atebrin (48). The efflux of K^+ induced by adding valinomycin was measured directly, with a K^+ -selective electrode. These experiments showed that chromatophores of the mutant were "normal" in that they matched the wild type chromatophores in their ability to control the permeation of H^+ and K^+ . We have concluded that the reaction centers, missing in the mutant, are involved somehow (not necessarily in their photochemical capacity) in mediating the carotenoid band shift. It had been generally accepted that the shift requires only that an electric field be impressed across carotenoids in the photosynthetic membrane.

Concerning light scattered by internal bodies and external surfaces of cells, the present status of these experiments has been described adequately in the "summary of progress" earlier in this report.

CURRENT OBJECTIVES RELATED TO PAST OBJECTIVES

We shall continue to study the composition, structure, and photochemical mechanisms of reaction center particles from photosynthetic bacteria. We shall extend our analysis of the structure of the bacterial photosynthetic membrane and its relationship to the rcp's, using a combination of techniques including interaction with specific antibodies. We shall pursue the study of the electrochemical properties of the photosynthetic membranes, and of their relationship to virus infection, detergents, and other agencies. We shall explore the value of certain new techniques and observations: light scattering with differential suppression of the cell surface, fluorescence of protein stains in relation to the hydrophobicity of the protein, and perhaps the factors that would govern the crystallization of hydrophobic proteins. Details follow.

I. Photosynthetic reaction centers.

Our understanding of the composition, structure and photochemistry of photosynthetic reaction centers is nearing a major clarification. We propose to pursue this clarification by a variety of physical and chemical approaches.

Pursuant to our discovery (8) that the oxidized photoproduct in rcp's ("oxidized P870") can be extracted as a stable entity in methanol, we shall compare the properties of this entity with those of oxidized BChl made chemically or photochemically in vitro. The extensive studies of J. J. Katz and collaborators (see 14), characterizing the optical, ESR and photochemical properties of Chl and BChl monomers, dimers, hydrated dimers, etc., will provide an invaluable background to these efforts. Pursuing the hypothesis that the + charge of "oxidized P870" is delocalized over two of the four

BChl's in a reaction center, we shall return to our observation (35) that dehydration reversibly abolishes photochemistry in chromatophores and rcp's. We can ask, does the removal of H_2O break up a system of the kind

essential for the photochemistry in rcp's? If so, can the BChl still be oxidized chemically? What happens if the hypothetical $(BChl \cdot H_2O \cdot BChl)^{+}$ is dehydrated? Does an oxidized BChl remain, but with altered properties (e.g., a broader ESR band)? We shall examine several physical parameters: optical absorption, fluorescence, circular dichroism (CD, soliciting the help of Dr. Kenneth Sauer), and ESR, before and after the removal of H_2O from rcp's. We shall do this while the rcp's are reduced, oxidized, subjected to photochemical oxido-reduction, or relaxed in the dark (neither oxidized nor reduced). Water might be involved in dimer-interactions of "P800", of BPh, and of the bleachable "P870 + P815" system. Following the removal of H_2O we shall test the consequences of introducing other possible ligands such as alcohols or pyridine.

The bleachable band at 815 nm, evident at low temperature, deserves closer study. Does it, in cooperation with the 865 nm band, represent the spectrum of the two BChl's that share a + charge when oxidized? Does this pair of bands complete the pattern of dimer-like symmetry seen in the absorption spectrum of BPh (25) and the CD spectrum of P800 (50)? This pattern is in harmony with the finding of Feher *et al.* (7) that the pigments are associated with two similar protein components in rcp's.

If the "natural" oxidized product in rcp's is $(BChl \cdot H_2O \cdot BChl)^{+}$, can this be converted reversibly to a form in which both BChl's are oxidized? If so, under what conditions and with what consequences?

We shall continue to explore the effects of varying the temperature,

pressure and H^+ concentration on photochemical and secondary electron movements in rcp's, with and without added electron donors and acceptors. The pressure dependence may be correlated with volume changes, and the pH dependence may reflect the presence or absence of such endogenous entities as $UQ^{\cdot-}$.

The changing properties of rcp's under mild denaturation with SDS will be studied fully. Feher *et al.* (7,10) have shown that 0.1% SDS disengages the nonpigmented 28,000 dalton protein from rcp's, leaving the remainder capable of photochemistry. They have suggested that iron is disengaged as well. Dutton *et al.* (49) have seen an ESR signal in illuminated chromatophores, resembling that of a reduced iron-sulfur protein (g value 1.82). Dutton maintains (49; also verbal communication) that this is an expression of the primary electron acceptor, and that the photochemistry in rcp's devoid of Fe is an unnatural variation using some other (perhaps adventitious) acceptor. We wish to determine whether the Fe (and some sulfur?) is associated specifically with the 28,000 dalton protein, whether the $g=1.82$ ESR signal is associated with that protein, and whether any UQ is associated with that protein or with the rest of the rcp. Also we would like to know whether the primary photochemistry in SDS-treated rcp's is a "deranged" variant of the "natural" photochemistry. To these and other ends we propose a number of experiments: Starting with ^{57}Fe - and ^{35}S -labeled cells, derive rcp's and separate their component proteins for assay of radioactivity (lability of the Fe bonding may be a serious problem here). See whether the $g=1.82$ ESR signal is present in rcp's and is lost when SDS is added. See whether the absorption change that suggests formation of $UQ^{\cdot-}$ is retained after SDS is added. Using chemically induced difference spectra in the UV, assay the component proteins of rcp's for UQ *in situ*. Measure the quantum

efficiency for photochemistry (bleaching of the 865 nm band) and for fluorescence in SDS-treated rcp's, noting whether the temperature dependence (down to liquid helium temperature) has been altered by SDS. Determine pH dependence of the various effects: has SDS opened new access of H^+ to sites in the rcp's?

We have found that 0.1% SDS abolishes a dimer-like relationship between two molecules of BPh in an rcp. Are other associations between pigments affected? Specifically, does SDS alter the double-cotton effect near 800 nm (50) in the CD spectrum? Does it change the ESR band width of oxidized BChl in the rcp? Does it affect the bleachable 815 nm band? Perhaps the natural photoact involves donation of an electron by a BChl dimer, and SDS changes the donor to one or two monomeric BChl's in each rcp.

The entire question of fluorescence in relation to photochemistry should be reexamined in the light of our finding (8) of a fluorescence increase associated with secondary rather than primary processes in rcp's. Perhaps this is a mechanism by which reaction centers avoid trapping (and wasting) excitation energy from the light harvesting apparatus until the reaction centers have completed certain secondary reactions. The mechanism might involve conformational rather than redox changes (8). We should measure the efficiency of photochemical oxidation of phenazine methosulfate coupled to reduction of P^+ , during the time that the fluorescence is relaxing (post-illumination) from a high to a low level.

Dutton et al. (20; also verbal communication) have shown by ESR measurements that an entity like triplet BChl appears in rcp's when they are illuminated under reducing conditions that block the photochemistry. Photochemically active rcp's do not show this ESR signal even though 30% of the excitation energy is probably being dissipated in non-radiative and non-photochemical processes. Seemingly the interruption of photochemistry

allows triplet BChl to accumulate, as an intermediate or a derangement of the events that could lead to photochemistry. Meanwhile the blocking of photochemistry by reducing agents also increases the population of singlet excited BChl, as shown by the higher fluorescence. This leads to the non-parsimonious assumption that reduction slows down two processes: the one by which singlet energy is converted (to triplet?) and the one in which triplet energy is translated into a redox event. Possibly the fluorescence does not rise in response to the primary event, but only to a closely coupled secondary event. In any case we should examine the yields of photochemistry, of fluorescence (singlet excited BChl), of triplet BChl (perhaps Dutton and collaborators will do this), and of radiationless de-excitation, while affecting the photochemistry in many independent ways. The photochemistry can be inhibited by chemical reduction (presumably reducing the primary acceptor), by adding hydroxyquinoline-N-oxide or orthophenanthroline (27), by raising the pressure (28), or by dehydration (35). Lowering the temperature or adding SDS may also affect the photochemical efficiency. To estimate the triplet BChl at room temperature we may have to rely on an optical absorption change centered at 424 nm as described by DeVault (51). This assignation will be more convincing if the increased absorption at 424 nm proves to be accompanied by bleaching of the usual (singlet, ground state) bands of BChl.

We have sent Dr. A. Yu. Borisov at Moscow cultures of our Rps. spheroides strain R-26 and are prepared to assist him with the preparation of rcp's. He finds very fast excitation transfer in the light harvesting apparatus of whole cells of several types of photosynthetic bacteria including Rps. spheroides, with lifetimes approaching the 10^{-11} sec value typical of purified rcp's. This would give us a picture of an exciton that is delocalized

so well over the light harvesting antenna and the rcp that it can be trapped as rapidly from the antenna as it can in the isolated rcp. Heretofore we have believed that energy transfer in the light harvesting apparatus requires about 10^{-9} sec. Borisov believes that the fluorescence with lifetime 10^{-9} sec represents a component of BChl that does not communicate with the reaction centers.

II. Analysis of photosynthetic membranes.

Having effected purification of rcp's and a 10,000 dalton light harvesting pigment-protein* (10KDP), we shall ask how these proteins are disposed in the intact photosynthetic membrane, and how they are related to pigment-proteins obtained by other means in other laboratories. The presence of 10KDP as well as reaction center proteins will be sought in Loach's "AUT" ("photo-receptor subunit") preparation (40). Both rcp's and 10KDP will be subjected to Kaplan's technique of dissolving in acid chloroethanol, for correlation with the proteins that he has characterized (39). These proteins will also be run by the electrophoretic method of Oelze and Drews (41), for a better understanding of their experiments on ^{14}C -incorporation. Neither reaction center proteins nor 10KDP can be recognized at present in Oelze and Drews' electrophoretograms.

*We obtained this "10KDP" as follows: Purified chromatophores from Rps. spheroides were exposed to 1% LDAO and then to ammonium sulfate at 50% of saturation. The insoluble material was redispersed in 0.5% aqueous Triton X-100. This dispersion yielded, on centrifuging, a soluble phase enriched for reaction centers and a pellet devoid of reaction centers and containing most of the light harvesting pigments. The pellet was washed in 0.5% Triton X-100 and dissolved in 1% aqueous LDAO, and then centrifuged through a sucrose density gradient. The pigmented material, nearly pure 10KDP, was recovered at an interface between 1.25 and 0.9 M sucrose.

By subjecting chromatophores to relatively mild and more severe detergent attack, and attempting to remove rcp's and 10KDP selectively with specific antibodies, we hope to answer the question of whether the light harvesting pigments are bound to reaction center proteins as well as to the 10KDP. And by adding aqueous dispersions of BChl and carotenoids to purified rcp's we can see whether the pigments become bound to the rcp's in any functionally significant way as the detergent is removed. Possible binding between rcp's and 10KDP will also be tested in this way. Criteria of functional significance can include energy transfer from added or light harvesting pigments to reaction centers, measured by sensitized photochemistry; transfer in the reverse direction, measured by delayed fluorescence from the "bulk" BChl; absorption band shifts of the carotenoids in response to photochemistry in the reaction centers; and simply changes of absorption spectra that attend binding to protein.

Using electrophoresis of chromatophores and purified 10KDP with varying concentrations of SDS we shall see whether the 10KDP exists in specific, definable aggregates of higher molecular weight. It appears, in preliminary tests, that this is so. This would be a step toward reconstruction of the membrane.

Having shown that anti-rcp precipitates rcp's from detergent-treated chromatophores of wild type Rps. spheroides, we shall seek other ways of isolating these rcp's from the wild type. This would probably facilitate the purification of rcp's from a great variety of photosynthetic bacteria. It will be desirable to study the reaction center protein of Rps. viridis and gelatinosa since these species do not seem to yield the familiar triad of reaction center proteins seen in Rps. spheroides.

We have begun experiments in which growing cultures of Rps. spheroides are exposed to ^{14}C -labeled amino acids and the cell proteins are subsequently analyzed. This technique has already been applied by Oelze and Drews (41), but without recognizable identification of reaction center proteins or 10KDF (see earlier). By combining pulse-chase experiments with transitions between different light intensities or between aerobic and photosynthetic growth, we hope to trace the synthesis of specific proteins associated with photosynthetic activity. In this we will continue our already fruitful collaboration with Dr. Robert Haselkorn at the University of Chicago.

The relationship between c-type cytochromes and rcp's needs further study. It was shown (17,25) that mammalian cyt c is bound electrostatically to rcp's; this could be expected at pH 7-8 because the isoelectric point of the cyt is about pH 10 while that of rcp's is well below 7. But the isoelectric point of soluble cyt 551 (the c-type) from Rps. spheroides is also well below 7, so this cyt and rcp's should repel each other electrostatically at neutral pH. However, there might be a distinction between "free" and "bound" cyt 551 in Rps. spheroides. Most of this kind of cyt becomes freely soluble when the cells are broken, but an amount equalling about two molecules per reaction center is retained in reaction center preparations made with Triton X-100, through many washings (52). This "bound" cyt can be removed from rcp's with LDAO. We should examine these "free" and "bound" cyt components for differences in such properties as isoelectric point and mid-point redox potential.

The handling of photosynthetic membrane proteins has raised certain technical points that should be pursued. We noticed that Coomassie Blue applied to polyacrylamide gels, to stain the separated proteins, is strikingly fluorescent on some of the bands and not on others. All proteins known to be hydrophilic allowed fluorescence; ones known to be predominantly hydro-

phobic did not. If this generalization could be relied on, as it might after more examples have been compiled, one could tell at a glance which proteins in the preparation are hydrophobic.

The crystallization of a hydrophobic protein would allow analysis of its structure by x-ray diffraction. With current interest in membrane proteins, this would be a most valuable achievement. Perhaps the hydrophobic exterior of a particle such as rcp or 10KDP could be coated in a controlled way with a type of molecule having polar and nonpolar parts, so as to convert the protein into one having only a few hydrophobic patches on an otherwise hydrophilic exterior. If the hydrophobic patches were in specific locations, and not random, crystallization from an aqueous medium should become possible, in analogy with the crystallization of predominantly hydrophilic proteins. If we could achieve this we would of course try to analyze the structure of rcp's by x-ray diffraction.

The use of specific antibodies has already been mentioned in the context of biochemical analysis and preparation. We shall also try to use specific antibodies as agents to block specific sites on membrane fragments (chromatophores) and on rcp's. We shall enlarge our collection of antisera so as to have antibodies against rcp's, individual component proteins of rcp's, 10KDP, and the 46,000 dalton protein associated with cell walls in Rps. spheroides. Dr. Lucile Smith will make available, for a collaborative effort, antibodies and univalent antibody fragments against mammalian cyt c. Using the blocking action of the antibodies, we shall try to map sites of electron donation and acceptance and sites of ion translocation or binding on rcp's and chromatophores from Rps. spheroides.

We have shown (48a) that an electrochemical gradient generated in chromatophores of Rps. spheroides causes a shift of the absorption bands of caro-

tenoid pigments in the membranes, and that this response is missing in a mutant (strain PM-8) that lacks reaction centers. We can ask whether the reaction centers are needed in their photochemical capacity, or merely as protein components of the membrane. One experiment would be to oxidize or reduce the reaction centers of the wild type, so that they are unable to react photochemically (being either in the state P^+, A or P, A^-) and then see whether a suitable chemical perturbation, such as a K^+ pulse given after valinomycin, causes a band shift. We can also ask whether carotenoids infused into carotenoidless mutant Rps. spheroides show the appropriate electrochromic responses.

The quenching of BChl fluorescence that attends H^+ uptake in the presence of diaminodurene (44) may or may not occur in strain PM-8; this could be tested with H^+ pulses applied to the mutant and to the wild type.

The foregoing are representative of numerous experiments to probe the mechanisms of the optical correlates of electrochemical activities in photosynthetic tissues.

In conclusion we can list some other experimental avenues that await exploitation:

The clumping together of Rps. spheroides cells after bacteriophage infection depends on phage-directed protein synthesis. The agent responsible for this "transformation" should be sought.

LDAO inactivates enzymes that carry out hydration steps in terminal stages of carotenoid biosynthesis in photosynthetic bacteria. Does LDAO inhibit, more or less specifically, all membrane-associated hydrating enzymes? Does it affect the interconversion of malate and fumarate?

A major protein of molecular weight 46,000 dalton is found in nonpigmented fractions from Rps. spheroides that are rich in cell wall fragments.

This protein shows considerable variability among independent strains of Rps. spheroides. In carotenoidless mutant Rps. spheroides it fortuitously aids in the purification of rcp's by sticking to the light harvesting fraction and making that fraction denser. Do variations of this protein among different strains show any correlation with phenomena associated with cell walls and outer membranes, such as the susceptibility to infection by viruses or by Bdellovibrios, or the penetration of antibiotics? Are such variations attended by varying success in purifying rcp's?

Cells of Rps. spheroides suspended in dilute aqueous buffer solutions show light-induced changes of light scattering. In green plant tissues such changes have been studied extensively (see 53) because they result from osmotic swelling and shrinking of membrane-bounded spaces (thylakoids) in response to the translocation of ions. When we suspended Rps. spheroides cells in H₂O containing 30% bovine serum albumin, so as to match the index of refraction of the cell surface, the suspensions appeared non-turbid. The light-induced changes of scattering in such suspensions had entirely different kinetics, with some components of opposite sign, compared with the changes in dilute aqueous buffer suspension. This raises the possibility that we can discriminate between scattering associated with smaller internal structures and that associated with the cell surface. One should be careful, of course, to distinguish extraneous osmotic effects of the 30% albumin.

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PRIORITIES

The sum total of old and new objectives comprise an assortment of experiments far too numerous to be attacked simultaneously. We are equipped to pursue all of them, but priorities will have to be determined on the basis of interests and talents of the investigators, evidence of success, and so forth. We propose to be flexible about this, and to review our priorities from time to time in order to maximize the effectiveness of our program. At present I hold most urgent the detailed study of composition, structure, and photochemical mechanisms in photosynthetic membranes and reaction centers.

TRAINING

One graduate student, S. C. Straley, is completing her fifth year of study under the aegis of this program. She has effectively completed all requirements for the Ph. D. degree, and is putting her thesis into finished form. Two other students, M. R. Sheetz and R. C. Downs, have completed one year of graduate study (Mr. Sheetz has just returned from military service). Ms. Straley's field is plant physiology; Mr. Sheetz and Mr. Downs are students of applied physics. The general plan is to mix students of physics and biology in this laboratory in order that they may learn from each other as well as from the postdoctoral staff.

The following postdoctoral associates and fellows have been associated with this program during the past three years:

R. Wang, Postdoctoral Associate, Sept. 1968 - Aug. 1970.

H. F. Yau, Postdoctoral Associate, Sept. 1968 - Aug. 1971.

L. A. Sherman, mixed support as Postdoctoral Associate and as N. I. H. Postdoctoral Fellow, Sept. 1970 to present (will leave Aug. 1972).

C. A. Wraight, Postdoctoral Associate, Feb. 1972 to present (anticipate two years tenure).

W. R. Sistrom, Visiting Professor on sabbatical leave from the University of Oregon, Aug. 1970 - July 1971.

Since May of 1969 our research program has been involved in the training of three classes in Physiology at the Marine Biological Laboratory, Woods Hole, Mass. The tenure of this activity expired in Sept. 1971. This has been an especially stimulating factor for our program, and two of the students, H. Fleming and E. Z. Szuts, and one Course Associate, R. Haselkorn, have contributed to our published work.

PUBLICATIONS

No abstracts are included. Four articles published in this period but based on earlier work have been omitted.

1969 - 1971:Articles

1. R. K. Clayton and S. C. Straley, "An optical absorption change that could be due to reduction of the primary electron acceptor in photosynthetic reaction centers". Biochem. Biophys. Res. Commun. 39, 1114-1119 (1970).
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Review

R. K. Clayton, "Photochemical reaction centers and photosynthetic membranes". Adv. in Chem. Phys. 19, 353-378 (1971).

Books

R. K. Clayton, Light and living matter: a guide to the study of photobiology. Vol. I, The Physical Part, 1970. Vol. II, The Biological Part, 1971. McGraw-Hill, New York.

1971-1972:Articles

1. R. K. Clayton and H. F. Yau, "Photochemical electron transport in photosynthetic reaction centers: I. Kinetics of oxidation and reduction of P870 as affected by external factors". Biophys. J., in press (Rabinowitch Festschrift Volume, 1972).

2. R. K. Clayton, H. Fleming and E. Z. Szuts, "Photochemical electron transport.....: II. Interaction with external electron donors and acceptors, and a re-evaluation of some spectroscopic data". Biophys. J. 12, 46-63 (1972).
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14. L. A. Sherman and R. K. Clayton, "The lack of carotenoid band shifts in a nonphotosynthetic, reaction center-less mutant of Rhodopseudomonas spheroides". FERS Letters 22, 127-132 (1972).

Review

R. K. Clayton, "Physical mechanisms in photosynthesis: past elucidations and current problems". Proc. Natl. Acad. Sci. 69, 44-49 (1972).

Thesis

S. C. Straley, "Studies of photochemical reaction centers from photosynthetic bacteria". In preparation; completion expected by the end of the Contract Year.

THE FUTURE OF THE FIELD AND ITS SIGNIFICANCE IN BIOLOGY AND MEDICINE

Our original work with photosynthetic reaction centers, going back about ten years, represents a breakthrough in the sense that the isolation and study of reaction center preparations has become a major area of investigation in photosynthesis throughout the world. A better understanding of photosynthesis has led and will continue to lead to improvements in agriculture; examples were given in the Comprehensive Report for 1966-1969.

Our studies of the photosynthetic membranes in relation to energy conservation bear on those diseases associated with energy metabolism and with the ability of membranes to control fluxes and concentrations of ions and metabolites. Our discovery of a virus-induced transformation of the bacterial cell surface ("clumping") may also have medical significance.

To meet future worldwide power needs without polluting the atmosphere and the seas to the detriment of world health, scientists have turned to solar energy conversion as a major possibility. The silicon cell, able to convert sunlight to electrical energy with efficiency approaching 10%, is an excellent power source but is too expensive for practicality at present. Proposals have been made to use photosynthetic bacteria to generate H_2 , but the burning of H_2 to make electricity would represent an inevitable loss of efficiency and would contribute to thermal pollution. In a sense this is not far from the continued burning of fossil fuels. A photosynthetic reaction center can be viewed as a tiny solar battery, with efficiency comparable to that of a silicon cell. A useful device would consist of reaction centers aligned (in an artificial membrane?) so as to have the macroscopic electric polarity of a battery. In any event the better understanding of these living

solar cells may facilitate the construction of a feasible large-scale solar energy converter.

In a broader sense, our research is at a most stimulating focal point in the application of physics and chemistry to biological systems. Working at this intersection, we will continue to ramify in a variety of profitable biophysical directions, as we have in the past. This will probably have consequences for medicine; a recent example is found in the treatment of porphyria by administering carotenoids; a direct outgrowth of studies of the role of carotenoids in photosynthetic bacteria. Another recent example from physical photobiology is the inactivation of herpes (fever sore) virus by applying a sensitizing dye and visible light.

APPENDIX A: ANNUAL PROGRESS REPORT

FOR THE CONTRACT PERIOD FROM SEPTEMBER 1, 1971 TO AUGUST 31, 1972

This progress report is submitted as an appendix to the comprehensive three-year report in order to avoid needless repetition. The reader will be directed to appropriate sections of the three-year report.

Compliance with Terms of Contract

Research has been conducted in full compliance with the terms of Contract No. AT (11-1)-3162.

Current Summary of Work Plan.

For an outline of plans, a detailed account of proposed experiments, and a discussion of priorities refer to the sections in the three-year report entitled "CURRENT OBJECTIVES RELATED TO PAST STATED OBJECTIVES", and "PRIORITIES". The stated programs, in the approximate order of priorities indicated, will be pursued by a staff consisting of the principal investigator, one or two postdoctoral associates and two graduate students, plus continued collaboration with several investigators elsewhere.

Progress During the Period May 1, 1971 to May 31, 1972.

The activities of the past three years in relation to the originally stated objectives are outlined in the section entitled "ORIGINAL OBJECTIVES AND SUMMARY OF PROGRESS CORRESPONDING TO THESE OBJECTIVES". The specific progress during the past year is described under "DETAILED REPORT OF PROGRESS", and can be identified by vertical lines in the right margin.

APPENDIX B: PREPRINTS AND REPRINTS

Preprints or reprints of the publications are transmitted with this report.