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EFFECTS OF REVERSIBLE DENATURANTS ON THE PHOTOSYNTHETIC CELL MEMBRANE, FIGMENTS AND SUBSTRUCTURE, OF R. spheroides

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EFFECTS OF REVERSIBLE DENATURANTS ON THE
PHOTOSYNTHETIC CELL MEMBRANE, PIGMENTS
AND SUBSTRUCTURE, OF \textit{R. spheroides}

George Collins Ruben
(Ph. D. Thesis)

June 1972

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under Contract W-7405-ENG-48

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In memory of his scientific achievements among which is the discovery of carbon 14, I dedicate this thesis to my father, Sam Ruben, 1913-1943.
ABSTRACT

The light reactions of photosynthesis reside in the cytoplasmic and the intracytoplasmic membranes in R. spheroides. The efficient conversion of light into chemical energy depends on the high degree of organization within these membranes. This organization can be studied by biochemical isolations, by optical techniques and by freeze-etch and freeze-fracture electron microscopy. The photosynthetic apparatus in the membranes has been investigated with the reversible structural denaturants, octanoic acid and methyl octanoate. Characterization of the effects of these denaturants with optical techniques on biochemically isolated preparations made it possible to correlate and interpret the electron microscope results. In situ denaturation and its reversibility made it possible to relate the denaturant caused membrane changes to the original in vivo structure. The limitations of platinum-carbon shadowing for quantitative measurements of inner membrane particles are defined. Evidence for the molecular basis of the diversity of near infrared in vivo Bchl absorbance peaks is presented. An inner membrane particle is identified and other possible candidates are outlined.
ACKNOWLEDGEMENTS

A word of appreciation is due to the group of scientists who have passed through the laboratory of chemical Biodynamics and whose interdisciplinary views have uniquely shaped my graduate research career. I am indebted to my research adviser, Dr. Calvin, not only for his financial support but also for that extra dimension of scientific insight he has personally provided. To Rod Park and Dan Branton who have counseled me in the freeze-etch work, to Raj Sane who helped in the electrophoresis and ultracentrifugation experiments, and to Albert Pheifhofer who kindly assisted me in learning freeze-etch, I owe my thanks. For Hans Steffen whose electronic, technical and theoretical skills made the NMR and optical results in this thesis a big success, I owe an extra debt. To Ken Wiley who patched each new statistical calculation into the computer program and who I crewed with in numerous Flying Dutchman regattas, I owe special appreciation for upgrading my sailing skills. A thanks of special note goes to John Reiland who critically evaluated the thesis outline and who helped make the final draft of Opus Rex easier to write than expected. And, I am no exception to the countless graduate students who have in the past thanked Evie Litton for doing all those inked thesis figures.

Special thanks to my sister Connie, my future wife Faith and Sue Braunstein who typed various stages of the final draft.
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<td>Bchl</td>
<td>bacteriochlorophyll</td>
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<tr>
<td>BPh</td>
<td>bacteriopheophytin</td>
</tr>
<tr>
<td>B800</td>
<td>light harvesting bacteriochlorophyll with an 800nm absorbance peak</td>
</tr>
<tr>
<td>B850</td>
<td>light harvesting bacteriochlorophyll with an 850nm absorbance peak</td>
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<td>B860</td>
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</tr>
<tr>
<td>B890</td>
<td>light harvesting bacteriochlorophyll with an 890nm absorbance peak</td>
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<td>CD</td>
<td>circular dichroism</td>
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<tr>
<td>Cyt b</td>
<td>cytochrome b</td>
</tr>
<tr>
<td>Cyt c</td>
<td>cytochrome c</td>
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<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>FET</td>
<td>freeze-etch technique</td>
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<tr>
<td>FFT</td>
<td>freeze-fracture technique</td>
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<tr>
<td>LDAO</td>
<td>lauryl dimethyl amine oxide</td>
</tr>
<tr>
<td>MO</td>
<td>methyl octanoate</td>
</tr>
<tr>
<td>OA</td>
<td>octanoic acid</td>
</tr>
<tr>
<td>ORD</td>
<td>optical rotary dispersion</td>
</tr>
<tr>
<td>P800</td>
<td>photo-active bacteriochlorophyll absorbing maximally at 800nm</td>
</tr>
<tr>
<td>P870</td>
<td>photo-bleachable bacteriochlorophyll absorbing maximally at 865nm</td>
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<tr>
<td>RC</td>
<td>reaction center</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydroxymethyl) amino methane</td>
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1 INTRODUCTION

1.1 Purpose

Achieving a greater understanding of the primary light conversion process in photosynthesis was the primary aim of this thesis research. Chemically induced structural changes in the light conversion apparatus were studied by correlation of optical measurements with electron microscope measurements. Conditions for optimal structural alteration and its reversibility were sought. If the reversible nature of the chemically induced structural changes could be established, then the chemically induced structural changes could be related to the unchanged membrane associated primary light conversion apparatus of \textit{R. spheroides}.

1.11 Reversible Inhibition of Photosynthesis by Chemical Denaturants

It is known that fatty acids have fungistatic and fungicidal action at pH's below neutrality and are known to reversibly inhibit respiration and glycolysis in yeast cells (C. Hoffman, T. R. Schweitzer, and G. Dalby, 1939; A. L. Neal, J. O. Weinstock, and J. O. Lampen, 1965). Their effectiveness at low pH is probably due to the undissociated form of the fatty acid (Prince, 1959; Rahn, 1945). Inhibition of endogenous respiration was found with normal saturated chains of two to twelve carbon fatty acids with $2 \times 10^{-3} \text{M}$, pH 4.85 in \textit{Boletus variegatus} (T. A. Pederson, 1969). These same fatty acids inhibited photosynthesis in \textit{Chlorella pyrenoidosa} at concentrations of $3-6 \times 10^{-4} \text{M}$, pH 5 in phosphate buffer.

1.12 Denaturant Selection

T. A. Pederson, M. Kirk and J. A. Bassham (1966) treated \textit{Chlorella pyrenoidosa} with c-2 to c-12 normal chain saturated fatty acids at con-
centrations of $3-6 \times 10^{-4} M$, pH 5 in phosphate buffer. Inhibitory power increased with the length of the carbon chain, whereas its removability from the cells decreased. The best compromise for maximizing inhibitory power and reversibility was octanoic acid. Along with octanoic acid, its methyl ester was studied to assess the importance of the functional group. There was little difference in the inhibitory effects of methyl octanoate and octanoic acid (pH 5), although reversibility was greater with the acid because pH neutralization helped reverse its effects. Both the ester and the acid inhibited two enzymatic reactions in the CO$_2$ fixation cycle as well as photophosphorylation. Because of the dissimilarity in their functional groups, the inhibitory effects were attributed to the membrane solubility of the saturated hydrocarbon chains. Since direct mixing of the enzymes with these compounds does not inhibit their activity, T. A. Pederson, M. Kirk and J. A. Bassham concluded that inhibition probably occurs indirectly through a conformational change of the stroma membranes to which the carbon cycle enzymes may be attached. Conformational changes in membranes as well as inhibition of photophosphorylation suggested that octanoic acid and its ester might be used to reversibly alter the structure of photosynthetic membranes. In particular it was an excellent approach to studying the primary light conversion process by reversibly varying its structural parameter. The photosynthetic bacterium R. spheroides was selected for this study because it only has one kind of membrane (Gorchein et al, 1968), which probably contains only one light reaction, and this light reaction could be isolated. The light-induced changes in the reaction center Bchl and a cytochrome b and c were readily observable with no more than an ordinary Cary 14 spectro-
These then were the most important considerations, although the level of experience with this organism in our laboratory was also a deciding factor.

1.13 Unexplored Features

Like all studies that embark upon the study of a process of unknown configuration with chemicals which change that process in an undefined way, there are big problems which are solved through determination and luck. The studies began by looking for chemically induced changes in the *R. spheroides* membrane by analyzing for absorbance changes in the photosynthetic pigments. These pigments were slightly blue shifted, indicating some sort of membrane change. Another parameter of membrane alteration was of course the changes in the photoinduced absorbance changes of the electron transport intermediates. This parameter was only tapped to give a broader understanding of the chemical effects. A very interesting approach to the study of chemically induced membrane changes was via the FFT of the chemically treated membranes and observation of their membrane fracture faces by electron microscopy.

However before the effects of octanoic acid and its methyl ester could be studied, a great deal had to be known about their behavior with respect to solution pH and a way had to be found to optimize conditions for reversibility. A great deal of effort was expanded on optimizing the reversibility of effects of octanoic acid (OA) and methyl octanoate (MO). Very late in this study it was found that these chemicals could be separated from chromatophores by centrifugation at 250-300,000xg. Due to the lower density of OA ($\rho_{20^\circ C} = .91 \ \text{gm/cc}$) and MO ($\rho_{20^\circ C} = .88 \ \text{gm/cc}$), the chromatophores pelleted out on the bottom of the centrifuge
tube and OA and MO were found floating on the top. These chemicals probably interact with the membranes as micelles which can be separated by their difference in density. Although concentrations given in the text by their nature assume complete miscibility with water, they are nonetheless a convenient description but not a true one. The concentration of OA and MO around and in the cells is probably greater by a factor of 100 to 1000 because of the almost total association of these chemicals with the membranes (Section 3.32131).

Another factor of interest concerns the effect of pH alone and together with OA and MO. It is shown in Section 3.322 that there is a low pH effect on the chromatophores of R-26 R. sphaeroides and that the action of MO is greatly enhanced at low pH over its action at neutral pH. It appears that the effect of the fatty acids is a synergistic effect of low pH and the hydrocarbon nature of the fatty acids; that is, the sum of the two separate effects is much less than the effect of their concerted action.

1.14 Studies with Chemical Denaturants on R. sphaeroides Membrane

1.141 Effects on the Quantum Conversion Apparatus

Although both wild type and R-26 R. sphaeroides cells were treated with low pH, OA and MO, it was the R-26 chromatophore system which gave the most fruitful results. It was found that B860 could be completely denatured by OA at low pH. Since this effect was so large, it was studied by a variety of tools and it proved to be the best system to optimize reversibility of the denatured 770nm Bchl peak to its in vivo B860 form. Circular dichroism studies of various stages of B860 denaturation unexpectedly revealed that monomer Bchl can absorb at 860nm.
1.142 Reversibility

Although the reversibility of the effects of OA at low pH and MO on cells was tried over and over again in the initial stages of this study, it was never very successful. After various procedures, including dialysis, pH neutralization and fatty acid removal by Bovine serum albumin were tried, it was found that centrifugation at 250,000-300,000 times gravity could separate out more than 80% of the OA and MO (Section 3.32131). Although MO and OA can be withdrawn from the R-26 chromatophores, it was found that after a time period of about 2 hours the pigment absorbance changes in the membranes were at best partially reversible or irreversible. In other words, there is an irreversible time dependent change proceeding in the membranes which will not go away when a majority of the chemicals is removed.

1.143 Correlation with Electron Microscope Studies

At low concentrations of MO and OA there were not any recognizable changes in the membrane fracture plane or its surface particles. However at high MO concentrations (2x10^{-2}M), which denature about a third of the B860 peak, there is a decrease in particle frequency on the membrane fracture faces. At low pH, where there is about the same change in the absorbance at B860, there is a big change in the texture of the membrane fracture surface. However, no measurement of the change in the frequency of particles on the membrane fracture surface could be made. Even though membrane fracture face particles could still be seen, the rugged texture of the fracture plane obscured this type of measurement. OA at low pH seemed to combine the effects of low pH and MO as well as completely denaturing B860. Some chromatophore fracture
face particles remain under conditions which completely denature B860.

1.1431 Relationship Between P870 Reaction Center vis-a-vis Chromatophore and Cell Membrane Fracture Face Particles

The particles on the membrane fracture faces were of unknown function so their identification was attempted. This project was an unexpected dividend of the correlation experiments. Reaction center from R-26 R. spheroides was isolated by the method of D. Reed and R. K. Clayton (1968). The particle size of a purified fraction of RC particles was compared to the size of particles seen embedded in the chromatophore and cell membrane fracture faces. Within experimental error, these particles are the same size. In addition, RC is denaturation resistant to high concentrations of MO and OA at low pH. Under these conditions, there were still particles on the membrane fracture face, although their number was greatly diminished. In addition, chromatophores treated with triton X-100 in the RC extraction process showed only a few particles and a greatly changed membrane fracture face texture. Although these data are an indirect proof of the identity of some of the particles on the chromatophores and cell membrane fracture face, it is convincing because of its correlation with recent studies.

1.2 A Brief Review of Bacterial Photosynthesis

Since bacterial photosynthesis is a very extensive subject, the reader should find it helpful to visualize the specialized material of this paper within the broad scope of the field.

Photosynthesis for bacteria, algae and plants is given by the net reaction:

\[ 2H_2A + CO_2 \xrightarrow{\text{light}} (CH_2O) + 2A + 2H_2O \] (Rxn 1-1)
As this equation applies to the photosynthesis in bacteria, it can be used to divide the photosynthetic bacteria into two classes according to the H₂A they can photometabolize. The Thiorhodacae can utilize sulfur-containing compounds such as H₂S. The Athiorhodacae, which R. spheroides is a member, can use organic compounds such as organic acids as electron donors. The photo-oxidation of organic and sulfur compounds in bacteria probably occurs for two reasons: they cannot utilize water as their electron donor and they probably only contain one light reaction. The algae and higher plants use a two or three light reaction process, and they can generate O₂. Consequently, the H₂A and 2A for algae and green plants are H₂O and O₂. A simplifying device which separates the general net reaction (Rxn 1-1) into light reactions (Rxn 1-2) and dark reactions (Rxn 1-3) conceptually divides photosynthesis into two parts. These two separate classes of reactions are:

a) The photochemical oxidation of H₂A and concomitant production of a strong reductant and "high energy" phosphate, and

\[ P + Y + H₂A + X \xrightarrow{\text{light enzymes}} P-Y + A + XH₂ \] (Rxn 1-2)

b) The enzymatic reduction of carbon dioxide using the reductant and phosphate carrier produced in (a).

\[ 3P-Y + 3XH₂ + CO₂ \xrightarrow{\text{dark enzymes}} (CH₂O)ₙ + X + Y + P \] (Rxn 1-3)

Since this paper is concerned with the structure and cellular location of certain intermediate steps in the light reactions the structural aspects of the light reactions, will be emphasized in this section. The dark reactions are relatively well understood. A generally accepted model for CO₂ fixation has been worked out for the algae, Chlorella pyrenoidosa, by M. Calvin and coworkers (M. Calvin et al., 1962; J. A.
Bassham and M. Calvin, 1957). This model is probably applicable to
*R. spheroides*, although the details of the CO₂ fixation cycle in this
organism have not been studied.

1.21 The Role of Chromatophores

The chromatophore is the location of the light reactions (Rxn 1-2).
Chromatophores are generally accepted as isolated pigmented material
from fractionated bacterial cells which retain the same pigment absorb¬
ance as the original cell. However, only carefully prepared chroma­
tophores supplied with added cofactors for the production of "high
energy" phosphate (ATP) and a "high energy" reductant (DPNH₂) have shown

1.22 Model for Light Associated Reaction Processes

For ease of discussion, the light reactions (Rxn 1-2) are divided
into three classes of reactions which differ mechanistically and which
on a relative time scale are listed by their order of occurrence. These
are:

1) Light absorption by antenna pigment (i.e., bacteriochlorophyll
and carotenoids) and resonance energy transfer.

2) Energy trapping in a P870 reaction center and conversion of
electronic energy to chemical potential.

3) Electron transfers from a terminal electron donor (i.e., H₂A,
organic acid) to a terminal electron acceptor (X) with asso­
ciated production of "high energy" phosphate.

These coupled and sequential reactions are located in the chromatophores.
The possibility that these classes of reactions are associated with
particulate structures which might be resolvable by present electron
microscopic techniques is explored below.

1.221 Light Absorbance-Resonance Energy Transfer

1.221.1 Characterization of Energy Transfer

Wild type and R-26 *R. sphaeroides*, which are similar to other purple photosynthetic bacteria, have absorption peaks in the ultraviolet at 375nm; in the blue at 450nm, 477nm and 512nm; in the orange at 580nm; and in the infrared at 800nm, 850nm, 860nm and 875nm (shoulder). Light absorbed at any of these wavelengths is transferred to the longest wavelength absorption peak. It is emitted as fluorescence at about 900nm, if it is not converted to chemical potential or degraded by other mechanisms.

In this process, there is no fluorescence at wavelengths shorter than 900nm. Non-radiative energy transfer takes place between 375nm, 590nm, 800nm, 850nm and 875nm and between the structurally dissimilar carotenoids (450nm, 477nm and 512nm) and Bchl molecules with very high efficiency. For reasons clarified later, B800, B850 and B890 in wild type *R. sphaeroides* are considered to be three different antenna Bchl species absorbing maximally at about these wavelengths. A pigment absorbing at 890nm can be shown to be the cause for the shoulder at about 875nm at the side of the 850nm.

Utilization of a broad spectrum of light for photosynthesis can be confirmed by phototactic response of the bacteria as a function of wavelength because phototaxis gives substantially the same result as a photosynthetic action spectra (J. B. Thomas, 1950; Manten, 1948, 1951). A 900nm fluorescence yield action spectrum is equivalent to the photosynthetic action spectrum. The fluorescence intensities at 900nm of an
organism irradiated at any absorption peak are a direct result of their 
energy transfer efficiency and of their ability to energize photosynthesis.

1.22111 Energy Transfer Efficiency

Energy transfer efficiency is measured as the absorbed light 
intensity divided into the fluorescence intensity at 900nm multiplied 
by 100. Quanta absorbed by carotenoids are transferred with an 
efficiency of 30-40% in *R. rubrum* and *Chromatium* (Duyens, 1952), 
but the efficiency is as high as 90% in wild type *R. spheroides* 
(Goldheer, 1959; Amesz, 1963). Transfer between the Bchl com­
plexes (i.e., from B800 to B850 and from B850 to B890) is approxi­
mately 100% efficient in *Chromatium, R. rubrum* and *R. spheroides*.
Because overlap of the B800 emission and B890 absorption bands (in the 
Förster theory of energy transfer) is poor, direct nonradiative energy 
transfer between these complexes is not considered significant. Bril 
(1964) treated *Chromatium* with deoxycholate demonstrating just this 
point. This type of mechanism must be operative generally in all photo-
synthetic bacteria if Förster's theory holds.

1.22111 Model for Pigment Arrangement

Theories proposed for the efficient nonradiative transfer of 
excitation energy to energy trapping sites invariably depend on the 
special arrangement of the photosynthetic membrane associated pigments 
Since we are primarily interested in interpreting the structural anatomy 
of the photosynthetic membranes (by electron microscopy and optical 
techniques), the focus of this section will be on the two most popular 
structural models of the antenna pigments.
Clues to the arrangement of the light harvesting pigments have generally come by way of biochemical and optical studies. The results generally have been interpreted against two theoretical pigment structural models which rely on pigment-pigment or protein-pigment interactions as their primary associations. The basic unit of macrostructure of the latter is the pigment-protein complex, and in the former, the two dimensional chlorophyll monolayer. Due to the very high chlorophyll concentration within membranes, (about 0.1 M), and due to the efficient energy transfer in photosynthesis, the Chl monolayer model was postulated. The monolayer theory can account for both properties (Bacon Ke, 1966). Nevertheless, there is no biochemical evidence for its existence in photosynthetic bacteria, whereas, pigment-protein complexes have been isolated from bacteria. Although, this model would influence the absorbance spectrum of Bchl it would be impossible to detect by the present methods of specimen staining and shadowed specimen replicas for electron microscopy. On the other hand, the pigment-protein complex model requires it to be of a size readily visible by electron microscopy. This latter model will be treated in detail because it might help account for the ubiquitous presence of globular particles within the photosynthetic membranes and the presence of a Bchl monomer at B860 in R-26 R. spheroides.

1.22112 Model for Antenna Pigments

1.221121 The Bchl-protein Complex

There are a number of Bchl and Chl pigment-protein complexes which have been isolated from photosynthetic organisms (J. P. Thornber and
J. M. Olson (1971). The Bchl-protein complex isolated from the green bacterium, *Chloropsseudomonas ethylicum* is probably the most thoroughly characterized (J. P. Thornber and J. M. Olson, 1968; J. M. Olson et al, 1969; L. Labaw and R. A. Olson, 1970; R. A. Olson et al, 1969). This Bchl-a protein complex is about 80 Å in diameter as measured by x-ray diffraction. It has a molecular weight of about 152,000 and it contains four subunits with five Bchl molecules each. This complex is isolated by washing it from the ruptured cells. It is a water soluble Bchl-protein complex.

Probably one of the most fruitful techniques for isolation of pigment-protein complexes comes from J. P. Thornber's (1970) work with a Thiorhodaceae organism. He fractioned *Chromatium* into three complexes with each fraction corresponding mainly to a major Bchl absorbance peak in the near infrared. It is surprising that the Bchl is not denatured by the levels of SDS used and that there are numerous carotenoids closely associated with each Bchl complex.

Of the Thiorhodaceae organisms, R. K. Clayton (1962) and Bril (1964) fractioned wild type *R. spheroides* into two Bchl complexes with deoxycholate. The 800nm and 850nm peaks and the 875nm shoulder were split into two fractions. In one the 800nm and the 850nm peaks were concentrated; in the other an 890nm peak was more concentrated than the smaller 800nm and 850nm absorbances. This evidence indicates that a B890 complex can be separate from a 800nm and an 850nm complex. From a part of the results of this study, the 800nm responds more readily to denaturation by octanoic acid than the 850 Bchl peak. This evidence strongly suggests that these three Bchl peaks are probably due to three
separate Bchl-protein complexes at 800nm (B800), 850nm (B850), and 890nm (B890). There is only one near infrared Bchl peak in R-26 R. spheroides at 860nm. Because there is strong evidence for its Bchl-protein nature, it will be indicated as a B860 Bchl-protein complex.

1.2211211 Molecular Associations Influencing the Absorbance Spectrum

There are changes in the near infrared Bchl absorbance in Chromatium cells grown under a variety of light intensities and nutritional conditions (Bril 1964; Fuller et al, 1963; Garcia et al, 1966; Wassink et al, 1939). Instead of these growth conditions changing the molecular associations in the cells, it appears that the relative amounts of three or more Bchl-protein complexes are varied. This serves to illustrate the fact that there is a genetic diversity of near infrared Bchl absorbance peaks among the purple and green photosynthetic bacteria and that they can be influenced by growth conditions. Wassink et al, (1939) suggested that the diversity of near I. R. Bchl peaks was due to Bchl-protein associations, although it was not clear whether Bchl-Bchl associations could account for the diversity of peaks. Bchl in vitro absorbs in the near I. R. at about 770nm, whereas Bchl in vivo can absorb at 800nm to 1012nm (J. M. Olson et al, 1966) which is a shift of 30-222nm to the I.R.

It is very difficult to choose whether Bchl or protein associations are the major influence causing the in vivo Bchl red shift. There will be evidence presented in Section 3.3214 which demonstrates the existence of a Bchl monomer at B860 in R-26 R. spheroides. A monomer form instead of the normal aggregated Bchl (Sauer, Dratz, Coyne, 1968) absorbing at B860 is in strong support of the protein-Bchl interaction as the major cause of the in vivo red shift.
Carotenoids

There are a number of carotenoids in wild type *R. spheroides*. Included are neurosporeine, spheroidene (Y), OH-spheroidene (OH-Y), spheroidenone, OH-spheroidenone, and 2-keto-spirilloxanthin (S. L. Jensen, 1963). They seem to serve a multitude of functions. Aside from their ability to absorb light and transfer its energy to a trapping site, they can protect Bchl from photo-oxidation.

Paul Mathis (1970) found that the triplet state of Chl readily transfers its energy to the carotenoids, whose triplet decay is accelerated by the presence of oxygen. This triplet quenching mechanism is probably the mechanism which protects Chl from oxidation. Plus, the photosynthetic membrane structure also becomes more resistant to detergent fractionation in the presence of carotenoids. The isolation of Reaction Center (R. C.) from carotenoidless R-26 *R. spheroides* by treatment with a triton X-100 is a good example because triton X-100 does not fractionate the wild type *R. spheroides* into R.C. The Bchl in the carotenoid-less mutant is readily photo-oxidized at high light intensities, whereas in the wild type it is resistant. These carotenoid functions give us reasons for their presence in photosynthetic organisms and also some idea of their spatial arrangement and proximity to Bchl because of energy transfer and triplet quenching. Limitations on spatial intermolecular distances are required for efficient radiationless energy transfer to take place and triplet state quenching to occur. Approximate separations might range as high as 60-100 Å (estimated from T. Trosper, 1966) for some organism such as *R. rubrum* which only transfers energy with 30-40% efficiency (Duysens, 1952). The maximum separations are
probably less for wild type *R. spheroides* which can transfer energy with close to 90% efficiency. It should be pointed out that these are large distances when compared to membranes of less than 100 Å in thickness. Since we have estimated the carotenoids maximum possible distances from Bchl and itself, it seems only fair to add a few more plausible estimates concerning its interactions within the membrane. These interactions are presented as a random list since none of them can be readily eliminated nor can they be listed in their order of importance.

1) Carotenoid-carrier interactions
2) Carotenoid-carotenoid interactions
3) Carotenoid-Bchl interactions
4) Carotenoid monomers in a hydrophobic environment

Although the ORD spectrum of the carotenoids exists for *R. spheroides* (E. A. Dratz et al, 1966), there does not exist work on model structures which could be used to interpret these results. More work is needed in this area.

1.221123 Location

The Bchl-protein complex of *C. ethylicum* can be about 80 Å in size. It is probably located mainly in a hydrophylic environment due to its water solubility. The Bchl complexes from *Chromatium* and wild type *R. spheroides* are apparently embedded deeply within a proteinaceous matrix because of their resistance to the membrane fractionating detergents SDS and deoxycholate. B860 in R-26 *R. spheroides* may also be embedded more deeply in a hydrophobic environment than just its protein shell. There is good reason to believe that the *C. ethylicum* 's Bchl-protein complex is unusual because it can reside in a hydrophilic environment. This
probably means that it resides on a membrane surface.

The other photosynthetic bacteria complexes discussed indicate by their hydrophobic nature that they more than likely reside within the membranes.

1.222 Energy Trapping—Conversion of Electronic to Chemical Potential

Light quanta absorbed by the photosynthetic pigments are transferred with high efficiency to the longest wavelength absorbance. (This longest wavelength pigment is normally the only one which emits fluorescent quanta.) In *R. spheroides*, for instance, the Bchl absorbing at about 860nm and 890nm is responsible for a fluorescent maximum at about 900nm. In the region of the long wavelength Bchl absorbance, there is a specialized Bchl which can undergo rapid reversible photobleaching activated by light quanta absorbed by any of the photosynthetic pigments (Duysens, 1952). This specialized Bchl is about 1-3% of the total Bchl in mutant and wild type *R. spheroides* (R. Clayton, 1962; D. W. Reed et al, 1970). The specialized Bchl with a maximum at 865nm (P870) and at 800nm (P800) undergoes a photoinduced bleaching of the P870 and a concommitant ortho-chromic shift of the P800. The absence of this specialized Bchl in a mutated *R. spheroides* which has its full complement of pigments is considered the cause for its inability to grow photosynthetically. These specialized Bchl are considered essential to photosynthesis (W. R. Sistron and R. K. Clayton, 1964). Because of P870's and P800's necessity to photosynthesis, their designation as reaction center took on greater significance and urgency. There is abundant evidence that the P870 and P800 of reaction center (RC) are a primary light reaction.
The P870 bleaching is light dependent, and it can have a rise time faster than a microsecond. Bleaching occurs readily at liquid nitrogen temperatures where solution chemistry ceases, and it is rapidly cycled in the presence of light and an electron donor (R. K. Clayton, 1965, 1966).

Since the naming of the RC, we have also come to think of it in terms of a structural entity. In a number of bacterial chromatophores the light harvesting Bchl could be preferentially destroyed by oxidation, whereas the RC Bchl persisted unaltered (I. D. Kuntz, P. A. Loach, M. Calvin, 1964). It was because of this physical ability to resist denaturation by oxidants and detergents that the RC was first isolated by Triton X-100 extraction from R-26 R. spheroides. More recently RC has been isolated by lauryl dimethyl amine oxide (LDAO). The next section will discuss the results of these RC isolations.

1.2221 Reaction Centers (RC)

1.22211 Characterization

1.222111 Molecular Weight

Before a meaningful chemical analysis of RC can be made, any photoactive RC preparation must be carefully purified. Although D. Reed and R. K. Clayton (1968) were the first to isolate RC from R-26 R. spheroides, they did not purify it carefully. G. Feher and coworkers (1971) used a series of ammonium sulfate fractionations on LDAO isolated RC to minimize its protein (280nm) to RC (800nm) absorbance. Gel electrophoresis of this preparation gave one major band of RC in 0.1% SDS. Upon dissociation of RC, three subunits of 21,000, 23,000, and 28,000 molecular weight were found by electrophoresis. The molecular weight of RC is less than 100,000, although G. Feher estimates that it could contain three subunits.
instead of four and have a 70,000 molecular weight. A smallest active RC of 44,000 has been found by Feher, Okamura, Raymond and Steiner (1971). Although this smallest RC contains photoactive P800 and P870, there is some confusion about what molecular weight particle should be considered as RC. The original usage of the term RC merely specifies that it contain photoactive P870 and P800. The RC is at least a two subunit particle of 21,000 and 23,000 molecular weight according to this definition. A preparation isolated by D. Reed (1969) reestimated by D. Reed et al, (1970) as 440,000 molecular weight, contains cytochromes and other electron transport compounds. D. Reed has isolated a particle larger than RC, which is nonetheless interesting because of its complement of closely associated electron transport molecules, which will be mentioned in the chemical section.

1.222112 Chemical

The chemical composition of the smallest RC is very important because it can tell us the minimum number of Bchl molecules necessary for the light reaction and give us some clue to the identity of the primary electron acceptor. At the end of this section RC preparations of much greater molecular weight are discussed because they contain the closely associated electron transport intermediates.

The number of pigment molecules contained in the smallest functioning RC is still not 100% sure. By successive treatment of RC with metal chelating agents, G. Feher and coworkers (1971) find four firmly bound magnesium atoms per P870. This evidence suggests that the RC contains at least four Bchl. H. Yau (1971) found that RC prepared by G. Feher's method showed energy transfer to P870 from Bph at 757nm with 80% efficiency.
The BPh could not transfer its energy with this high an efficiency if it were dispersed randomly in solution. Using a reasonable extinction coefficient for BPh there could be between one to two BPh per four Bchl in each BPh.

The question of whether RC contains lipids, polysaccharides or other molecules as well as its Bchl, BPh and protein is still as open question although maximum limits can be placed on the percent by weight of these other compounds. Since the RC are at least 90% amino acids and 7±3% extractable Bchl and some LDAO, it is unlikely that the other types of compounds present can be greater than 3%. Phospholipids are not present because there is less than one phosphorous atom per P870.

D. Reed (1969) isolated RC in a high molecular weight particle of 650,000 and later gave it a value of 440,000 molecular weight when he reevaluated his original results. These preparations contained a number of the electron transport intermediates. Although his relative number of transport molecules per P870 is doubtful, his findings are nonetheless interesting in spite of his somewhat high molecular weight species with preparation of doubtful homogeneity (see Section 3.52).

The number of each electron transport intermediate is probably correct within a factor of two or three.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>P870</td>
<td>1 mole</td>
</tr>
<tr>
<td>total iron</td>
<td>16 moles</td>
</tr>
<tr>
<td>cytochrome b562</td>
<td>1.8 moles</td>
</tr>
<tr>
<td>cytochrome c552</td>
<td>1.0 moles</td>
</tr>
<tr>
<td>copper</td>
<td>9.0 moles</td>
</tr>
<tr>
<td>ubiquinone</td>
<td>13.0 moles</td>
</tr>
</tbody>
</table>

Composition of Reaction Center Complex
Prepared with 1% Triton X-100
G. Feher's group finds that the copper and iron can be almost completely separated from room temperature photoactive RC. Their results may eliminate these metals as candidates for the primary electron acceptor if they can show that this RC is photoactive at very low temperature. Ubiquinone was thought to be the primary acceptor for a long time, however, it is sensitive to solution redox potential changes whereas the primary acceptor is not (D. Reed, K. Zankel, R. Clayton, 1969). Unfortunately the primary electron acceptor remains unidentified. If it is protein that is acting as the primary acceptor, it will be very difficult to prove.

1.22212 Molecular Model

E. Dratz, K. Sauer and L. Coyne (1968) advanced a model assuming three Bchl molecules in the RC. This model was based on C.D. data which could now be interpreted in light of an estimated four Bchl and 1 or 2 Bph per RC. Although the Bchl and acceptor three dimensional relationships in the RC are unknown, there is a certain level of information that has been learned from Chromatium which also applies to RC in R-26 R. spheroides.

Parsons (1968) found that the primary photo-act occurs with a very fast transfer of an electron from Bchl (G. Feher, 1971; Bolton, Reed and Clayton, 1969) to an acceptor in less than a microsecond. A positively charged Bchl is then reduced in about 2 microseconds by a Cyt c552 molecule which is coupled to the electron transport chain which generates ATP and delivers electrons from solution oxidized compounds. Ubiquinone is probably the next electron acceptor in the sequence after the primary acceptor. The RC protein contains the Bchl and ubiquinone pigments and
is responsible for
\[
P870 + A \xrightarrow{\text{hv}} P870^+ + A^-
\]
and for the geometrical organization making possible the high efficiency of RC's photo-induced absorbance changes.

1.222 Location
RC has to be suspended in solution by detergent otherwise it will aggregate. Due to its hydrophobic nature, it may reside within the photosynthetic membranes.

1.223 Electron Transport System

1.2231 Role of Molecules in the Electron Transport Chain
The electron transport chain is at least two sequences of molecules which can transport electrons in and away from the light reaction. Transport chain delivered electrons are pumped up an energy gradient in the light reaction to another sequence of carriers. These final carriers deposit their electrons and energy in a high energy reductant. This reductant as well as ATP generated along the electron transport chain supply the power that operates the CO₂ fixation cycle.

1.22311 Molecules Implicated in Electron Transport
So far the only molecules found to be on the electron transport chain are those molecules found in reaction center preparation. These are a Cyt c⁵⁵₂ which feeds electrons directly to an oxidized P870, and a Cyt b⁵₆₀ which is on a pathway that can feed electrons to Cyt c⁵⁵₂. Ubiquinone may be next in sequence after the primary electron acceptor.
Not enough evidence is available to completely define the electron transport chain of wild type and R-26 R. spheroides; however they should be very much alike. Figure 1.22311 gives an overall view of the schematic sequence of the light reaction processes.

1.22312 Is there a Characteristic Electron Transport Structure?

The cytochromes in R. spheroides are molecules of about 13,000-21,000 molecular weight. These molecules can have diameters as large 32Å-37Å if \( \bar{V} \) is assumed to be 0.76 cc/gm. Even if \( \bar{V} \) were as high as 1 cc/gm, these diameters would only increase by about 10%. If two or three cytochromes were aggregated, they could easily form a large particle. It is not clear whether this is the case; however, it is a possibility that should not be excluded. Ubiquinone is assumed to be part of the RC particles. It seems that there could be a cytochrome electron transport particle but it would have to be in close proximity to the RC particles. Perhaps Feher's third subunit which the RC can function without is nothing more than a Cyt c or the primary electron receptor. He has found that separation of the 28,000 m.wt. subunit also takes with it about 90% of the iron or about 0.8 Fe/P870.

1.22313 Is There an Oxidative Metabolism Electron Transport Particle

The evidence for internal membrane particles in all electron transport membranes studies comes from Freeze-etch Technique (FET) and Freeze-fracture Technique (FFT) for electron microscopy (D. Branton, 1969; J. M. Wrigglesworth, L. Parker, D. Branton, 1969). These particles average about 80-85Å in width in platinum shadowed preparations. In nerve myelin, which can not perform oxidative metabolism or any electron
Figure 1.22311

Schematic Representation of the Light Reaction Processes

Wild Type

hv
590nm
375nm

B800 B850 B890

excitation transfer

90% bacteriochlorophyll efficiency

Carotenoids

hv
400-512nm

R-26 mutant

hv
590nm
375nm

B860

Reaction center Bchl

P800 and P870

P870* e-

UQ

P870+

DPNH2

DPN

cyt c (ox)

cyt b (ox)

cyt c (red)

cyt b (red)

organic acid

ATP

ADP
transport function, there are no internal particles.

According to other studies, the enzyme coupled reactions in mitochondria oxidative metabolism require a sequence of a cytochrome a, cytochrome b and a cytochrome c (J. Fruton and S. Simmonds, 1958). The cytochromes can have, according to the author's rough calculations, diameters of about 32Å-37Å. If the cytochromes form a closely coupled structure, then they could form a large membrane particle. It is not too surprising that R. spheroides, like mitochondria, can live on oxygen and a carbon source and that it contains Cyt c₂, Cyt c₃, Cyt b, Cyt a and Cyt o (F. R. Whole and O. T. G. Jones, 1970; T. Sasaki, Y. Motokawa and G. Kikuchi, 1970; R. G. Bartsch, 1971). R. spheroides has a cytochrome a or o as a terminal oxidase depending on the oxygen tension. It was thought at one time that the light electron transport system and the oxidative electron transport system utilized a common enzymatic pathway (Van Niel, 1941). This however does not seem to be the case because the consumption of oxygen in the dark and in the light (30% less) are about the same in R. spheroides while the cells are also photosynthesizing at a near normal rate (R. K. Clayton, 1955).

From this experiment and the fact that all the cytochromes are present for oxidative metabolism, the light conversion process and the oxidative metabolism probably operate using physically separate enzymatic pathways. The cytochromes of both systems could associate forming inner membrane particles.

1.23 Anatomical Features of Photosynthetic Bacteria

1.231 Cell Membrane Anatomy

The membrane anatomy of cells has been investigated through
electron microscopy. The first methods for viewing wild type *R. sphaeroides* cell membranes required fixing cell membranes with a heavy metal stain then thin sectioning the cells embedded in plastic for viewing in the electron microscope. In order to differentiate the membranes of *R. sphaeroides* from other cell matter, they are poststained with uranyl acetate. These techniques normally stain the membranes in a "dark-light-dark" three-line pattern. These patterns are very readily distinguished in G. Drew's and P. Giesbrecht's (1963) work on wild type *R. sphaeroides*. These cells contain, just inside the cell wall, a cytoplasmic membrane and a variable amount of circular pieces of membrane in the cytoplasm whose frequency is dependent on the light intensity during growth. G. Drews et al (1963) demonstrate that the vesicular membrane structure sliced as circles by thin sectioning are attached to the cytoplasmic membrane at sites on the periphery of the cell. Gerald Peter's thesis (1970) shows pictures of cells that have been converted from aerobic dark growth to light growth. These cells in the beginning light growth phase show practically no vesicular membrane structure and vanishing amounts of Bchl. In response to light, synthesis of Bchl occurs with a concomitant appearance of vesicular (circular cross section) structures in the bacteria.

As part of this thesis work, the structures of wild type and R-26 *R. sphaeroides* were done by the Freeze-Etch Technique (FET). Micrographs of the wild type and the R-26 mutant demonstrate a slightly different membrane anatomy in the mature cells. The Freeze-Etch Technique (FET) the Freeze-Fracture Technique (FFT) are used for membrane localization because of two very distinctive characteristics. Membranes fracture
internally showing a surface containing particles of approximately 95 Å. Membranes fractured in cross section show ridges slightly larger than their estimated thickness of less than 100 Å. These two distinctive features are the membrane's signature for establishing its identity and will be used for establishing its anatomy in cells prepared by FET.

1.232 Chromatophores

1.2321 Definition

Ambiguity in the usage of the term "chromatophore" has crept into the literature since the electron microscope pictures first established vesicular pieces of membrane within the photosynthetic bacteria. H. K. Schachman, A. B. Pardee and R. Y. Stanier (1952) isolated a pigmented fraction from photosynthetic bacteria which they called chromatophores. Certain workers concluded too quickly that the vesicular pieces of membrane were the chromatophores. As it turns out, chromatophores can be isolated from bacteria of any internal membrane structure: lamellar, vesicular, or otherwise (J. Oelze and G. Drews, 1972). Also the size and molecular weight of a chromatophore fraction is strongly dependent on its method of isolation. Depending on the amount of sonication or disruption the cellular membrane sustains the chromatophores can range in size from 300 Å to 600 Å in diameter. There is evidence that they are derived from the cytoplasmic membrane as well as the vesicular and lamellar membrane. In order to clarify the term "chromatophore," the term "membrane fraction" will be considered equivalent and used interchangeably from time to time.

1.2322 Structure

It appears that the vesicular and lamellar type membranes within
the cytoplasm are continuous with the cytoplasmic membrane (G. Drews and P. Giesbrecht, 1963; P. Giesbrecht and G. Drews, 1962; 1966; R. A. Dilley, 1969). This evidence suggests that they are probably of the same composition; although this may not be true in the initial stages of light growth after dark aerobic growth (Peters, 1970; J. Oelze and G. Drews, 1972). Gorchein's biochemical characterization of cytoplasmic membrane mixed with vesicular membrane is the strongest evidence that these membranes are probably of the same composition (Gorchein et al, 1968). This is a very important point since sonication of *R. spheroides* makes it impossible to distinguish between fractionated vesicular membrane and cytoplasmic membrane. Another very important feature of the results from Gorchein and coworkers (1968) is that the composition of the photosynthetic apparatus does not change with light intensity in wild type *R. spheroides* (*vide infra* not in the R-26 mutant). This also strengthens our assumption that all the membrane from which the chromatophores is derived, vesicular and cytoplasmic membrane, is composed internally of the same structural composition under a wide variety of light intensities.
2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial Strains

The wild type *R. spheroides* was originally obtained from Stanier and the R-26 *R. spheroides* carotenoid-less mutant was kindly supplied by R. K. Clayton.

2.1.2 Culture Media

The wild type was grown on a modified Hutner's medium (Cohen-Bazire, Sistrom and Stanier, 1957). Most labs have been able to grow the R-26 mutant on the modified Hutner's medium. We could only get it to grow, however, in a very rich medium of yeast extract and casein hydrolysate of the same concentrations as in agar slants, which is 0.3 gms. of yeast extract, 0.2 gms. of casein hydrolysate in 100 ml of water.

2.1.3 Chemicals and Chemical Denaturants

Highest grade octanoic acid and methyl octanoic were purchased from Fisher & Company. The Bio-gel A 1.5M 100-200 mesh agarose column material was purchased from Bio-Rad Laboratories in Richmond, California. The trizma base and the triton X-100 were purchased from Rohm and Haas. The other chemicals such as the acids and sodium hydroxide used were of reagent grade.

2.1.4 Instruments

A number and variety of analytical instruments were used in these studies. For convenience, they will be listed.

1) Balzers freeze etch machine of the type described by D. Branton (1966) and H. Moor and K. Muhlethaler (1963).

2) Siemans Elmiskop 1.
3) Cary 14 M with a model 1462 Scattered-transmission attachment modified for actinic light illumination from the side.

4) The side illumination device was a Bausch and Lomb monochromator with a red blazed grating illuminated with a 1200 watt tungsten projection bulb.

5) Cary model 14R could not be used with highly scattering systems. Its IR II mode routes white light directly through the sample after which it is separated into wave lengths in a monochromator and analyzed. The IR I mode directly passes a monochromatic beam through the sample for O.D. measurements.

6) An aminco fluorimeter modified for greater sensitivity with a phase sensitive amplifier and chopper; the fluorescence was detected with Dumont 6911 or an RCA 7102 photomultiplier tube cooled with dry ice.

7) The circular dichroism experiments were performed on a machine built and described by E. Dratz (1966).

8) Beckman Model E ultracentrifuge.

9) Gel electrophoresis device built after the design of Davis (1964).

10) Varian 220 nuclear magnetic resonance machine.

11) Bio-sonik Sonicator was used for fractioning the cells at (20K).

12) Sorvall, Spinco and Beckman centrifuges were used in the preparative techniques.

2.2 Methods

2.2.1 Bacterial Culture Techniques

The wild type R. spheroides were grown in modified Hutner's medium between two banks of fluorescent lights at 31°C in tightly sealed 125 cc
cylindrical bottles. These cultures were inoculated from uncontaminated bottle cultures with a few milliliters of inoculum. These cells could grow up in a two days to a week depending on the size of the inoculum.

The R-26 R. spheroides carotenoidless mutant would not grow in a modified Hutner's medium in our hands; instead a yeast extract medium was used. These cells seemed to grow best on a tungsten light table in a room held at about 65°F. The 5 to 10 liter bottles usually matured within about 10 to 14 days if they grew at all. In order to protect the fresh inoculum taken from its usual aerobic dark slant, the cultures were kept in the dark for 12-24 hours so the concerted action of light and dissolved oxygen wouldn't damage their ability to grow. If these precautions weren't taken the cells rarely grew. Cultures had to be harvested when they were a bright blue-green in color, otherwise within a day they had turned a sickly olive green which indicated cellular deterioration.

For reaction center preparations cells were freeze-dried, so it wouldn't be necessary to wait the 2 weeks to 2 months it sometimes took to grow a good culture of the cells.

2.211 MO Growth Experiments with Wild Type R. spheroides

Nearly full 125 ml cylindrical bottles containing modified Hutner's were inoculated with 15 ml of a full grown culture of wild type R. spheroides. The bottles were inoculated with a 0.2M Methyl Octanoate in 95% ethanol to final concentrations of Methyl Octanoate of 5x10^-4M, 10^-3M, 2x10^-3M. Controls were inoculated with equivalent amounts of 95% ethanol to check ethanol's concentration effect on cell growth. The bottle cul-
tures were grown on a fluorescent light table at approximately 31°C. They were harvested in 2 days, since that is near the end of their logarithmic growth phase. Before the absorption of the cultures was measured, they were routinely diluted by a factor of 2 or 4 so the optical density at the highest bacteriochlorophyll peak at 850nm was about one. The absorbance spectra were taken on a Cary model 14 M with Model 1462 Scattered-transmission attachment using a Dumont 6911 end window phototube.

2.22 Chromatophore Isolation

Wild type \textit{R. spheroides} chromatophores. The wild type \textit{R. spheroides} used to make chromatophores were grown in 5 liter bottles of Hutner's solution inoculated with 125 ml of 5- to 10-day old cell solutions. Ten to twenty liters of cells were grown in the same manner as the 125 ml bottle cultures. After 5 days, the cells were spun down in the Sharples continuous flow centrifuge and then washed once in 0.01 M tris pH 7.5 and stored at 0°C in the cold room until the chromatophores were to be prepared.

One ml of this paste was resuspended with 15 ml of buffer to form a very fluid suspension of bacteria. This suspension was sonicated for 5 minute intervals for a total of 20 to 30 minutes on a Bio-sonik sonicator. The probe was cooled with a dry ice solution at 5 minute intervals to keep the ice-cooled sonicate solution from going above 20°C. Next, this solution was centrifuged at 40,000 x g for 30 minutes (in a Sorvall centrifuge). The pellet formed was discarded. The supernatant containing chromatophores is very dense, nearly opaque. The supernatant was centrifuged for 60 minutes at 140,000 x g (41.5 K in a Spinco head).
The supernatant from this centrifugation was discarded and the pellet was resuspended in buffer to give an absorbance of 50 at 850nm. Not all the chromatophores could be resuspended, so this solution was centrifuged at 40,000 x g for half an hour. The final solution was stored at 5°C.

**R-26 R. spheroides chromatophores**

The R-26 R. spheroides chromatophores were made similarly to the wild type, the only difference being that these cultures took from ten to fourteen days to grow before harvesting.

**2.23 P870 Reaction Center Isolation from R-26 R. spheroides**

Freeze dried cells were sonicated in 10 ml of a 10% cell solution in 0.01 M tris pH 7.5 at 0°C for one minute intervals for a period of 15 minutes. The sonicate was centrifuged at (40,000 x g) in the Sorvall centrifuge for 30 minutes. The supernatant was recentrifuged at 41.5 K (140,000 x g) in the Spinco 50 head for 1 hour. The chromatophore precipitate was resuspended at a concentration of approximately 50 O.D. at 860nm.

Dissolution of the chromatophores into reaction centers was accomplished by adding 0.3 volumes of a 10% solution of Triton X-100 to one volume of the OD$_{860nm}$ = 50 chromatophore solution. The Triton X-100 solution was slowly added at 0°C with stirring. Stirring was continued for 1 hour. Four milliliter portions of this suspension were layered onto a discontinuous sucrose gradient formed with 4 ml of 1 M sucrose and 4 ml of 0.6 M sucrose in 13.5 milliliter Spinco centrifuge tubes. The tubes were centrifuged for ninety minutes in a Spinco SW41 head at 260,000 x g (38,000 rpm). A band separated in each gradient layer. The
top band, S1, was green in color in the 0.01 M tris solution without sucrose; this is the reaction center containing band. The 2nd band, S2, was green in color and layered in the 0.6 M sucrose. The 3rd band, S3, also was slightly green with a white pellet material on the bottom. Both S2, and S3 contained green material that had chromatophore like absorption except for the appearance of 757nm and 683nm band. The S1 fraction was placed in the cold room in a dialysis bag and dialyzed for 3 days against four one liter changes in buffer. The color of the solution changed from green to brown over this period. The dialyzed S1 fraction was then concentrated to 4 milliliters by pressure dialysis. This concentrated solution was chromatographed on a 1.0 x 94 cm. Bio-gel A 1.5 m 100-200 mesh column. Instead of 2 bands, only one large band appeared which contained reaction center. By taking successive one milliliter fractions from the column, eighteen fractions were obtained. By measuring the 757nm band to 800nm peak, the fractions with too much 757nm Bph were set aside. Fractions 1-16 were combined and concentrated by pressure dialysis. Four to five milliliters of 0.D.\textsubscript{860nm} = 0.17 normally constituted the final yield.

2.24 Instrumental Methods

2.241 Electron Microscope Methods

2.2411 Freeze-Fracturing and Freeze-Etching

A droplet of the centrifuged material was mounted on a 3 mm striated copper disk or 3 mm cardboard disk and quickly frozen in Freon 22 at liquid nitrogen temperatures. Samples were stored under liquid nitrogen until they were used. Freeze-etching was carried out on a Balzers apparatus using one minute of etching at -100°C. Freeze-fract-
turing experiments were carried out at -120 to -150°C without etching. Although these experiments could be carried out at -150°C, this condition was in greater jeopardy of oil contamination from the vacuum of 2x10^{-6} mm of mercury (D. W. Deamer et al, 1970). The composition of the solutions is contained in the captions with the figures. The replicas were examined in a Siemans Elmiskop I at 80 KV. Direct plate magnifications range from 30,000 to 50,000. Magnifications of 40,000 made focusing easiest, and it also gave sufficient data per plate. An arrow in the upper right hand corner of each photomicrograph indicates the platinum-carbon shadow direction. If the arrow is not present then the shadow direction is from the bottom to the top of the page. The shadows are white.

The precision of the magnifications is about ± 5% and the absolute accuracy of these magnifications on the Siemans Elmiskop I is about the same.

2.242 Photometric Methods

2.2421 Chemical Difference Spectrophotometry

2.24211 Experiments with the Wild Type Organism

2.242111 Chromatophores, D(MO)-D

The effect of methyl octanoate concentration of the wild type chromatophores was measured in the following way. Three ml of chromatophore solution diluted to an O.D. \text{850} = 0.8 were placed in two cuvettes. 15 \mu l of methyl octanoate in 95% ethanol was added to the sample cuvette to give some concentration of MO and the reference cuvette was simultaneously diluted with 15 \mu l of 95% ethanol. The following solutions were made up in 95% ethanol.
The ethanol concentration was held constant throughout these experiments.

### Table

<table>
<thead>
<tr>
<th>MO Solution Concentration in 95% Ethanol, $M$</th>
<th>MO Concentration in 3 ml of Solution, $M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>$1 \times 10^{-2}$</td>
</tr>
<tr>
<td>.8</td>
<td>$4 \times 10^{-3}$</td>
</tr>
<tr>
<td>.4</td>
<td>$2 \times 10^{-3}$</td>
</tr>
<tr>
<td>.2</td>
<td>$1 \times 10^{-3}$</td>
</tr>
<tr>
<td>.16</td>
<td>$8 \times 10^{-4}$</td>
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<td>.12</td>
<td>$6 \times 10^{-4}$</td>
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<td>$3 \times 10^{-4}$</td>
</tr>
<tr>
<td>.04</td>
<td>$2 \times 10^{-4}$</td>
</tr>
<tr>
<td>.02</td>
<td>$1 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

**2.242112 Cells**

$D(10^{-3} M \text{ MO}) - D$

Methyl octanoate stock solutions were 0.2 $M$ in 95% ethanol solvent. Fifteen $\mu l$ quantities were injected into three ml of cell solution to give a final concentration of $10^{-3} M$ in all experiments. At the same time the sample solution was injected with 0.2 $M$ methyl octanoate, fifteen $\mu l$ of 95% ethanol was used to dilute the 3 ml of cell solution in the reference cuvette. Absorption difference spectra of this type will be called dark methyl octanoate difference spectra [$(D(+MO) - D)$]. These samples were bubbled with nitrogen before the MO was injected. Their tightly fitting caps were sealed carefully. What little air that may have reached the cells is quickly metabolized. None of the effects in absorbance difference spectrum are due to oxygen (G. Ruben LCBQ-7, 120, 1965).
D(pH 5) - D(pH 7) and D(pH 5 + pH 7) - D(pH 7)

Wild type *R. spheroides*, inoculated in modified Hutner's medium (Hutner's), was grown in 125 ml bottles between 2 banks of fluorescent lights for 2 days. The bottles were each inoculated with 10 ml of 5- to 10-day-old cultures. These young, healthy cultures were used in the dark pH 5 difference spectrum, D(pH 5) - D(pH 7), and in the dark pH 5 reversibility difference spectrum, D(pH 5 + pH 7) - D(pH 7), in which base was added to neutralize the sample. The reference was simultaneously diluted by an equivalent amount of Hutner's. Neutral pH is the normal growing environment for these cells. This type of experiment was conducted in fresh Hutner's with twice washed cells so that cell growth products in the culture were eliminated. A 25 ml vessel was constructed with pH electrodes and a nitrogen bubbler. The pH of the sample could be monitored directly throughout the experiment. The solution in this vessel could be moved along a side arm to the sample cuvette, which was seated in front of the sample beam in the Cary 14M scattered transmission attachment (Model 1462). A cuvette with 3 ml of cell solution was used for the reference in all cases. After bubbling the 3 ml of solution with nitrogen, its top was taped to ensure anaerobiosis.

The D(pH 5) - D(pH 7) and the D(pH 5 + 7) - D(pH 7) experiments were prepared in the following way. Titration from pH 7 to pH 5 in the 25 ml of cells in Hutner's was done with approximately 75 μl of 50% H₃PO₄. The reference cuvette was simultaneously diluted by approximately 9 μl of Hutner's. Approximately 125 μl of saturated NaOH solution was used to titrate the sample to pH 7, and about 15 μl of Hutner's was used to dilute the reference cuvette.
D(1.5x10^{-3} M OA, pH 5)-D(pH 5) and
D(1.5x10^{-3} M OA, pH 5 + 7)-D(pH 5 + 7)

Wild type *R. spheroides* cells, grown as above, were used in the dark. 1.5x10^{-3} M octanoic acid difference spectrum, D(1.5x10^{-3} M OA, pH 5)-D(pH 5), and the dark octanoic acid reversibility difference spectrum D(1.5x10^{-3} M OA, pH 5 + 7) -D(pH 5 + 7). When both sample and reference were made pH 5, 28 ml of cell solution were titrated to pH 5 of which 3 ml were transferred to the reference cuvette. 125 μl of 0.3 M octanoic acid in ethanol was added to the remaining 25 ml to make the total octanoic acid and octanoate present 1.5x10^{-3} M. The reference cuvette was bubbled with nitrogen as 15 μl of 95% ethanol was added. The reversibility experiment was performed by titrating both cuvettes to neutrality. The sample and reference solutions were diluted from beginning to end by no more than 1.5%. This dilution of the changes in the difference spectrum is negligible. Since it is the free acid that enters the cells and the amount of free acid is governed by its equilibrium constant (K_a = 4x10^{-6}) and the hydrogen ion concentration, addition of 1.5x10^{-3} M octanoic acid is approximately 1x10^{-3} M free acid in solution.

2.24212 Experiments with the R-26 Organism

2.242121 Cells

D(MO)-D concentration study and time study

Since these cells are grown on yeast extract medium, they had to be centrifuged and washed to eliminate the yellow yeast extract medium. Once they have been resuspended, they are made anaerobic. The vessel made for the D(pH 5)-D(pH 7) experiments for the wild type cells was used with two modifications. A small side arm was put on the reference
cuvette which was constantly bubbled. Both the sample and the reference side arms were bubbled with helium. Helium is better than nitrogen in that its extremely small oxygen contamination is less by a factor of 10. Its oxygen content is vanishingly small. The MO solutions made for the wild type chromatophore experiment (Section 2.232111) were inoculated in the proper quantities to give the appropriate concentrations. The reference cuvette with its 6 ml of solution was diluted by a proportionate amount of 95% ethanol. The ethanol concentration in all the experiments was held invariant. The effects of MO on R-26 cells are independent of the ethanol, oxygen and light scattering effects. The absorbance difference spectra were measured on a Cary 14M Scattered-transmission attachment. The time study is simply the time taken from the time of inoculation of MO.

$$D(\text{pH} 6) - D(\text{pH} 7.3) \text{ and } D(\text{pH} 6.5) - D(\text{pH} 7.3)$$

These experiments used the same apparatus used in the preceding experiment. Acidification of the sample and the reference solution was accomplished in the same manner as the acidification of the wild type cells with the only difference being that the modified Hutner's medium in these experiments contained no organic compounds which could act like octanoic acid.

$$D(\text{OA, pH} 6) - D(\text{pH} 6)$$ concentration and time studies

OA solutions in 95% ethanol were made up like the solutions in Section 2.232111. The pH was adjusted to 6 in a similar fashion as the preceding experiment. Modified Hutner's media without organic compounds was used. The same procedure for adding the OA to the sample and the 95% ethanol dilution to the reference was used just as in the wild type cells.
Both the sample and the reference were kept anaerobic with Helium bubbling. The time study was accomplished by taking the inoculation with OA as time zero and each spectrum following was recorded at some later time. The time resolution isn't better than ± 4 minutes. The sample and reference cuvettes were shaken and mixed with their side arm solutions before each spectrum. The spectra were taken on a Cary 14M with the attachments mentioned before. Helium was bubbled and the pH was recorded constantly throughout the experiment. For each spectrum fresh cells were used. The ethanol concentration was again held invariant through the series of experiments.

2.242122 Chromatophores

The series of MO solutions and OA solutions in 95% ethanol made up in a similar fashion to Section 2.232111 were used with the chromatophores. Since the chromatophores are suspended in 0.01 M tris pH 7.5, normally no further attempt was made to buffer the solution. Upon addition of the OA solutions the pH was decreased by the OA. The ethanol content of the samples throughout the experiments were held invariant. No attempt was made to keep the samples anaerobic since the absorbance changes in the pigments were so large in order to make changes in the electron transport intermediates unobservable. The OA concentrations changed the solution pH in the following manner.

<table>
<thead>
<tr>
<th>OA Concentration</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5x10^{-3}M</td>
<td>pH 6.55</td>
</tr>
<tr>
<td>5x10^{-3}M</td>
<td>pH 5.2</td>
</tr>
<tr>
<td>7.5x10^{-3}M</td>
<td>pH 4.85</td>
</tr>
<tr>
<td>10^{-2}M</td>
<td>pH 4.75</td>
</tr>
</tbody>
</table>

The MO solutions were mostly used in a neutral solution.
In the experiments where the MO concentration was fixed and the solution pH was varied, HCl was the titrating acid. Centrifugation of these samples was performed on a Beckman centrifuge with the Ti 60 head. This is the only head which can reach 300,000 x g. The absorbance spectra were taken on a Cary 14M with the Scattered-transmission attachment, model 1462. A Dumont 6911 was used as the photo-detector.

2.242123 Reaction Centers

D(2x10^{-2}M MO)-D

Since there were minute amounts of preparation, narrow cuvettes, 3 mm wide with a 1cm path length were used. Just enough sample was used in each to cover the analyzing beam. There was 10^{-4}M dithiothreitol in each cuvette to keep the RC in a reduced state. The sample was made 2x10^{-2}M MO and the reference was diluted with the appropriate amount of 95% ethanol. These experiments were done in the Cary 14M with a Dumont 6911 phototube.

2.2422 Light-Dark Difference Spectrophotometry

2.24221 Wild Type Organism

2.242211 Cells

Wild type cells, inoculated in modified Hutner's medium, were allowed to mature between two banks of fluorescent lights in 2-4 days in 125 ml bottles. These samples were inoculated with 15 ml samples of 5-10 day-old cultures. At the time of an experiment, solutions were diluted by half with fresh, modified Hutner's medium, deaerated with nitrogen and placed in cuvettes with their tops taped on to insure anaerobiosis. The steady-state light-minus-dark difference spectra were measured on the Cary 14M spectrophotometer equipped with a scattered
transmission attachment (model 1462). In taking the Light-Dark difference spectra, the sample cuvette was excited with actinic light at a wavelength several hundred nm removed from that of the analyzing beam. A weak analyzing beam passed through both the illuminated sample and a darkened reference. The photoinduced changes were recorded directly by this method. An RCA 6217 or a Dumont 6292 photomultiplier was used for detection in the visible region from 370-650 μm. A Dumont 6911 was used for the near infrared region, 650-1000 μm. A Bausch & Lomb grating monochromator with full slit settings illuminated the sample solution at right angles to the analyzing beams. The exciting source was a 1200 watt tungsten bulb. For visible spectra a Corning filter 9788 was placed in front of the photomultiplier and filter 2403 was placed in the exciting beam. For the near infrared region, Corning filter 2030 was placed in the analyzing beam and filter 4784 was placed in the exciting beam. The light ($+10^{-3}$M methyl octanoate)-dark($+10^{-3}$M methyl octanoate) spectrum will be abbreviated L(MO)-D(MO). This spectrum was taken by subtracting the D(MO)-D spectrum from L(MO)-D spectrum.

2.24222 R-26 Organism

2.242221 Chromatophores

L(OA)-D(OA)

In order to study the effects of OA on the P800 and P870 their photoinduced changes in the chromatophores were monitored. The electronics of the Cary 14M had to be modified. A phase sensitive amplifier with other appropriate electronics was hooked in directly to the Cary's photomultiplier circuit and the light-dark difference spectra were read externally on an X-Y recorder. The sensitivity of the Cary was increased
by more than a factor of two. At its very best a Cary can measure one part in $10^{-30}$ OD. The sample and reference were inoculated with the same concentration of OA. The sample was illuminated with actinic light at 590nm or 375nm from a Bauch and Lomb monochromator as in the preceding section. A Corning 2030 filter was placed in the analyzing beam and a Corning 4784 filter was placed in the actinic beam. Sample fluorescence did not interfere with these measurements because it was a constant background level and could be easily subtracted out.

2.242222 Reaction Center

L-D and L(2x10^{-2} M MO)-D

The R-26 mutant, reaction center (R.C.) absorption spectrum were taken on the Cary 14R, IR I mode. In the IR II mode, white light from a tungsten source is passed through the cuvette, and through the monochromator to the IR detector. The IR II mode completely bleaches the reaction center. $10^{-4}$M dithiothreitol is added to protect the R.C. preparation from irreversible bleaching. The sample was made 2x10^{-2} M MO with a 0.2 M MO solution in 95% EtOH.

2.2423 Fluorescence Spectrophotometry-Methods

2.24231 R-26 Organism

2.242311 Chromatophores

These samples were prepared in the same manner as the samples were for their absorbance spectrum (see Section 2.232122). No attempt was made to keep them anaerobic.

2.2424 Fluorescence Polarization Spectrophotometry

The Aminco fluorimeter was modified with a polarizer after the exciting monochromator and before the analyzing monochromator in the
same fashion that T. G. Ebrey and R. K. Clayton (1969) set up for their apparatus. The R-26 chromatophores samples were made up in the same manner as they were for the absorbance spectrum experiments.

2.2425 Circular Dichroism

Experiments on R-26 chromatophores and Reaction Center were run on the circular dichroism instrument described by E. Dratz (1966). These samples were prepared in the same fashion as in the chemical difference spectrophotometry Section 2.2421. A Dumont 6911 photomultiplier was used as the detector in these experiments.

2.243 Ultracentrifugation Methods

The RC samples were injected into the single sector cell in the highest g head. The temperature was held at 20°C, and a green filter was used in front of the schlieren optical system. The Head was run at a speed of 59,780 rpm. for one hour. The pigments in the RC absorbed the green light so they could confirm whether it was a pigmented fraction that was sedimenting.

2.244 Gel Electrophoresis Methods

An electrophoresis apparatus was fashioned after Davis' design (1964). The polyacrylamide gel was prepared by Clarke's methods (1964). These techniques were used in the electrophoresis experiments. The RC fraction 1 or fraction 2 were placed on the gel columns within the pH 8.6 buffer solution. The samples were run for about 2½ hours before they were removed and stained for proteins.

2.245 NMR Methods

Chromatophores were suspended in a D₂O solution so their proton spectrum could be observed. OA was suspended separately in D₂O for its
proton spectrum of OA's alpha carbon hydrogens. The chromatophores and the OA were mixed in a D_2O medium to a 10^{-2} M OA, pH 4.75 for NMR analysis. After centrifugation at 300,000 x g the supernatant was analyzed by NMR for OA and the chromatophores were resuspended in D_2O to see if there was still OA associated with them. The Varian 220 NMR was used at its highest sensitivity. In fact the highest concentration of OA was just great enough to make the proton NMR signal of the alpha hydrogens large enough to work with.

2.25 Data Interpretation

2.251 Electron Microscope Data

Each way one measures a particle of roughly 100Å in a shadowed preparation will give a different answer. In fact it is very difficult to measure the fracture face particles in a consistent way without observer bias distorting the measurements. This happened in the earliest results when the membrane fracture face particles were measured with a x6 eye piece on 100,000 magnification photomicrographs. When the data was coded it was less biased but the measurements were still very uncertain. Early in the experiments, it was decided that the only consistent particle measurement on the membrane fracture face was the particle widths measured orthogonally to the shadow direction. This is the measurement that was made in all the experiments. A large number of EM plates were taken of each preparation in order to obtain a large particle width population. These plates were projected on a screen at 45.6 magnification that of a plate magnification of 40,000x. The total magnification of the particles was 1,824,000x. The particles were measured with an ordinary clear plastic ruler of better precision than
the precision of particle measurement. The particles were measured perpendicular to the shadow direction as in Figure 5.13-1. The error of measurement of shadowed particles perpendicular to the shadow direction embedded in a nonplanar surface is about ± 20%. As a feeling for how difficult these measurements are, Figure 3.221-1A should be briefly studied. To assure unbiased distributions of such measurements, plates of a number of experiments were coded for the measurer while a second person recorded the particle widths. Distributions of particle widths are shown in Section 3.5 and in the appendix.

2.2511 Statistical Methods

All of the statistical calculations were programmed for the CDC 6600 computer because of the time consuming nature of the calculations. There are calculations and comparisons tests in the program that were never put in the results section of this thesis. One such calculation is the W test. It can be used to show if two distributions of the same kind are the same or are different. The beauty of this test is that it can be used with any kind of a distribution. Before it was realized that the uncertainty in mean particle widths was due to variations in shadowing, this test was in operation testing the distributions. It was repeating over and over that the distributions were different. That's obvious in retrospect. The W-test can be found in any book on advanced statistics.

The ordinary parameters that were measured in each experiment were calculated as follows. The arithmetic mean of an individual particle population and of two particle populations taken together is as follows:
Their overall mean is

\[ \bar{x} = \frac{\sum_{i=1}^{n_1} x_i}{n_1} + \frac{\sum_{i=1}^{n_2} x_i}{n_2} \]

Under certain circumstances where the means were calculated for particle populations or individual cell membrane fracture surfaces, an overall mean was calculated by summing the individual cell means and dividing by the number of cells. This is the type of mean recorded in the sixth column of the table in Figure 5.21-1. In comparing the average mean of the particle width means in Figure 3.52-8, they were summed directly and divided by the total number of means. Their uncertainty was obtained by taking the largest difference of the average mean from one of its component means.

The standard deviation of each distribution was calculated below. A standard deviation for the two particle populations was calculated in the program for the W test which was never used.

\[ (s'_1) = \frac{n_1}{n_1} \left( \frac{1}{n_1} \sum_{i=1}^{n_1} (x_i - \bar{x}_1)^2 \right) \]

The true standard deviation is

\[ s_1 = \frac{n_1}{n_1 - 1} (s'_1) \]
The chi-square test of whether two distributions are the same or different is in every elementary statistics book. Each distribution was compared to a Gaussian by this method and was found to be very different. This test was also used in comparing the particle distributions on an individual cell membrane fracture surface to the sum of these individual distributions in particular replica. This was done for each of the experiments with the cells. Only the one with R-26 R. spheroides cells is recorded in the results section (Figure 5.21-7). A majority of the individual cell particle distributions were significantly different from the sum of their distributions. Indeed, the shape of these individually plotted distributions vary considerably among themselves as well as with their overall sum.

Since the distributions were definitely nongaussian it was felt that some measure of this behavior might give some information that wasn't extracted by the other methods.

Skewness and Kurtosis were calculated in the following manner where:

\[ g_1 = \text{skewness} \]
\[ g_2 = \text{Kurtosis} \]

\[ g_1 = \frac{1}{n s^3} \sum_{i=1}^{n} (x_i - \bar{x})^3 \]

a positive value indicated a right skewed curve
a negative value indicated a left skewed curve

\[ g_2 = \frac{1}{n s^4} \sum_{i=1}^{n} (x_i - \bar{x})^4 - 3 \]

a positive value indicated lepto-kurtosis
a negative value indicated platy-kurtosis
This method was taken from a text written by R. R. Sokal and F. J. Rohlt (1969). These then are the important calculations included in the computer program included in the next section.

2.2512 Computer Program

It took such a great deal of effort to get this program written and operating that I don't want to see someone else have to go through the same difficulty. The data has to be submitted in a certain manner behind the program before it will calculate the statistical parameters on the CDC 6600 at LRL.

This method of submission is briefly indicated for each distribution:

1) Title card is placed after the program.

2) First two digits are particle size and the second two digits are their frequency.

3) To signal the end of a distribution the third two digits have to end with a number.

4) A blank card is placed before the next distribution.
PROGRAM GEORGE
DIMENSION TIJ(12), TJI(12), TJ(2), TM(2), TS(2), SC(20)
DIMENSION CJJ(20), CJ(20), CM(20)
DIMENSION C(20), CC(20), F(20), Y(20), Q(23)
DIMENSION FCELL(2), XXX(2), X(2), S(2)

ISET=0
1 ISIT=ISF+1
WRITE(8*6) ISF
6 FORMAT(9H1) DATA SET, IT)
IT=0
2 IT=IT+1
TJJ(IT)=0.
TJI(IT)=0.
CJG=0.
DO 202 IT=1, 20
F(I)=0.
CTJ=0.
202 CONTINUE
READ(5*3) TITLE(I=1, 40)
3 FORMAT(40A2)
WRITE(8*4) TITLE(I=1, 40)
4 FORMAT(140*40A2, 8/37H EXP CELL MEAN SD

I=1, 5, 6, HCOUNTS+5X*2H1, 5X*2H2, 7/7
ICELL=0
10 ICELL=TCJL+1
CJL(ICELL)=0.
CJ(CELL)=0.
CJ2(CELL)=0.
DO 203 IT=1, 20
CC(CELL)=0.
203 CONTINUE
11 READ(5, 12) T1, JJ, KK
12 FORMAT(2I2, 15)
IF(JJ) 20, 20, 13
13 FI=FLOAT(JJ)
FJ=FLOAT(TJ)
CJ1(I, CELL)=CJ1(CELL)+FI#FJ
CJ2(CELL)=CJ2(CELL)+FJ#FJ
CJ(CELL)=CJ1(CELL)+FJ
CJ2(I, CELL)=CJ2(CELL)+FJ
CTJ1=CJ1(T)+FJ
CJSUM=CJSUM+FJ
IF(KK) 14*15, 14
14 T1J(I, IT)=T1J(I, IT)+CJ1(CELL)
TJ2(IT)=TJ2(IT)+CJ2(CELL)
TJ1(1T)=TJ1(1T)+CJ1(CELL)
CM(CELL)=CJ1(CELL)/CM(CELL)+CJ1(CELL)
CS=(CJ2(CELL)-CJ(CELL)/CM(CELL)**2)/(CM(CELL)-1.)
SC(CELL)=SQRT(CS)
WRITE(8,15)IT,ICELL,CM(ICELL),SC(ICELL),CJ(ICELL)
15 FORMAT(2110,F10.3)
GO TO 10

20 CONTINUE
ICELL = ICELL - 1
FCELL(IT) = FLOAT(ICELL)
TM(IT) = T(IJ(IT)) / T(J(IT))
TS(IT) = (T(J2(IT)) - T(IJ(IT)) * T4(IT) * 3) / (T(J(IT)) - 1)
TS(IT) = SQRT(TS(IT))
G1 = 0
G2 = 0
DO 330 I = 1, 20
G = FLOAT(I) - TM(IT)
G1 = G1 + C(IT) * G ** 3
G2 = G2 + C(IT) * G ** 4
330 CONTINUE
G = CJSUM * TS(IT) ** 3
G1 = G1 / G
G2 = G2 / G / TS(IT)
WRITE(8,21) IT, TM(IT), CJSUM, G1, G2
21 FORMAT(100, TOTAL EXP * 110, F10.3)
GO TO 500
X(IT) = 0
XX(IT) = 0
DO 35 I = 1, ICFLL
XX(IT) = XX(IT) + CM(I) ** 2
XX(IT) = XX(IT) + CM(I)
35 CONTINUE
X2(IT) = XX(IT) / FCELL(IT)
S2(IT) = 0
DO 36 I = 1, ICFLL
S2(IT) = S2(IT) + CM(I) - X2(IT) ** 2
36 CONTINUE
S2(IT) = SQRT(S2(IT) / (FCELL(IT) - 1) / FCELL(IT))
WRITE(8,23) X2(IT), S2(IT)
23 FORMAT(5HT X2= F10.3, S2=F10.3)
GO TO 500

DO 220 T = 1, 20
CALL FIXC(C)
IDF = 3
CHS = 0
FSUM = 0
DO 270 I = 1, 20
F(I) = 0
Y(I) = 0
IF(C(I)) 270, 270, 260
260 IF(IDF = 1)
CALL AREA(Q(I), A)
IF(IDF = 2) 261, 261, 262
261 F(I) = A * CJSUM
FSUM = FSUM + F(I)
GO TO 265
262 F(I) = A * CJSUM - FSUM
FSUM = FSUM + F(I)
265 Y(I) = (C(I) - F(I)) ** 2 / F(I)
CHS = C(I) + Y(I)
T = T
270 CONTINUE
SUBROUTINE TTEST1IC, CJ1, CJ2, CJ, CM, CS)
DIMENSION CJ(I), CJ2(I), CJ(I), CM(I), CS(I)
LIM=IC-1
DO 10 T=1,LIM
I=I+1
DO 50 J=IC+IC
T=CJ(J)+CJ(J)
X=(CJ(J)+CJ(J)+CJ(J)-2)/T
S=SQRRT5
W=CM(J)-X**2/(CJ(J)**2)
T=SQRRT(W**2/(T-1.0))

10 CONTINUE

CALL TTEST(T, CJ1, CJ2, CJ, CM, CS)
WRITE(8*31)

50 CONTINUE

WRITE(8*30)

DO 30 FORMAT(15X, 4HMEAN, 10X, 6HSTDEV, 12X, 1HMIN, 14X, 1HTOT, 12X, 6HCOUNTS, 10X, 13HK/P, 10X, 5HHT-X2)

30 FORMAT(8*2)

CALL AREA(I, I+1, A)
F(I)=F(I)*CJSUM
Y(I)=F(I)*Y(I)+F(I)**2/F(I)
CHS=CHS*Y(I)
WRITE(8,275)
275 FORMAT(6H SLOT, REAL FREQ, DIST, STD, DEV, THEORET FREQ)
150 IDF=+1
CHI=0
CALL FIXC(CJ(I), I)
391 CONTINUE
390 FORMAT(8*2)
100 STOP
END
WRITE(*,20) X,S,*,T*,I*,J*,T*,K*,DM
50 CONTINUE
RETURN
END
SUBROUTINE FIT(C)
DIMENSION C(1)
DO 250 I=10,20
J=21-I
IF(C(I)-5.5) 231,240,240
231 K$=I+1
IF(K$=20) 234,236
234 DO 245 K=K$,20
C(I)=C(I)+C(K)
C(K)=0.
235 CONTINUE
IF(C(I)-5.5) 236,240,240
236 C(I-1)=-C(I-1)+C(I)
C(I)=0.
240 IF(C(J)-5.5) 241,250,250
241 K$=J-1
IF(K$=1) 246,244,244
244 DO 245 K=1,K$.
C(J)=C(J)+C(K)
C(K)=0.
245 CONTINUE
IF(K(J)-5.5) 246,250,250
246 C(J+1)=C(J+1)+C(J)
C(J)=0.
250 CONTINUE
RETURN
END
SUBROUTINE ARPA(Q*A)
DIMENSION AR(61)
DATA AR/7.013*,0.019*,0.026*,0.035*,0.047*,0.062*,0.082*,0.107*,0.139*,
1.0179,0.0228,0.0287,0.0359,0.0466,0.0548,0.0668,0.0808,0.0968,0.1151,0.1357*,
IF(Q=3.0) 10,100,100
10 IF(Q=3.0) 200,200,20
20 D=Q+5.5
I=N*INT(D)
A=AR(I)+D-FLOAT(I)*AR(I+1)-AR(I)
RETURN
100 A=9.9999
RETURN
200 A=9.9991
RETURN
END
SUBROUTINE FIXC(C)
DIMENSION C(1)
DO 40 I=1,19
IF(C(I)-5.5) 5,20,20
5 C(I+1)=C(I+1)+C(I)
C(I)=0.
40 CONTINUE
20 CONTINUE
   DO 50 I=1,19
   J=21-I
   IF(C(J).LE.5.0) 25,60,60
   C(J-1)=C(J-1)+C(J)
   C(J)=0
50 CONTINUE
60 CONTINUE
   RETURN
   END
3 RESULTS

3.1 Spectrophotometric Studies on Whole Cells

3.1.1 Wild Type Organism

In determining the effects of chemical denaturants on cellular structure, it was initially thought that the study of wild type cells would provide certain advantages over R-26 mutant cells. Wild type cells are easily cultured and grow rapidly under laboratory conditions. They attain maximum growth in a few days whereas the mutant strain requires approximately two weeks under optimal conditions. For this reason, the wild type organism was studied in the initial investigations.

The effects of the chemical denaturants, MO and OA, on wild type cells generally exemplify their effects. Not only is MO an inhibitor of photosynthetic growth, but it also blue shifts the antenna pigment absorbance and interrupts the electron transport chain. The effects of OA on pigment absorbance can be made reversible by pH neutralization. The experiments with MO concentration effects on chromatophores and the R-26 mutant experiments were a direct result of these studies.

3.1.1.1 Studies with MO

3.1.1.1.1 Inhibition of Photosynthetic Growth

The growth of wild type *R. spheroides* in the presence of MO was measured both by the increase of light scattering at $\lambda = 1000\text{nm}$, an unpigmented wavelength, and by the increase of Bchl and carotenoid pigment absorbance. Growth occurred only at the lowest concentration of MO ($5 \times 10^{-4} M$), or at approximately 100 MO/Bchl. At the end of 48 hours, the cell population was only about one-fourth to one-sixth of that obtained in the absence of the inhibitor. No cell growth was obtained
Figure 3.111-1. Growth experiments with wild type *R. spheroides* cells.

Top. Growth of *R. spheroides* after 2 days without ethanol and in the presence of 0.24%, 0.49% and 0.98% 95% ethanol. Absorption spectrum of cells before growth is shown at the bottom.

Bottom. Absorption spectrum of *R. spheroides* before and after 2 days in the presence of $5 \times 10^{-4}$ M MO + 0.24% 95% EtOH, $10^{-3}$ M MO + 0.49% 95% EtOH, and $2 \times 10^{-3}$ M MO + 0.98% 95% EtOH.
Table 3.11

Changes in Optical Density Over 2 day Growth Schedule of Wild Type R. spheroides

A

<table>
<thead>
<tr>
<th>Wavelength, nm</th>
<th>870</th>
<th>850</th>
<th>800</th>
<th>590</th>
<th>510</th>
<th>477</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.67</td>
<td>2.45</td>
<td>1.74</td>
<td>.63</td>
<td>1.83</td>
<td>1.34</td>
</tr>
<tr>
<td>5x10^-4 M MO</td>
<td>.321</td>
<td>.36</td>
<td>.23</td>
<td>.13</td>
<td>.12</td>
<td>.18</td>
</tr>
<tr>
<td>1x10^-3 M MO</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2x10^-3 M MO</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Wavelength, mm</th>
<th>870</th>
<th>850</th>
<th>800</th>
<th>590</th>
<th>510</th>
<th>477</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>5x10^-4 M MO</td>
<td>10</td>
<td>15</td>
<td>13</td>
<td>21</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>1x10^-3 M MO</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2x10^-3 M MO</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

C

Change in Light Scattering at 1000nm as an Indication of Growth

<table>
<thead>
<tr>
<th>Scattering</th>
<th>Scattering Change</th>
<th>Percent Scattering Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>.10</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>.90</td>
<td>.80 (100%)</td>
</tr>
<tr>
<td>5x10^-4 M MO</td>
<td>.29</td>
<td>.19</td>
</tr>
<tr>
<td>1x10^-3 M MO</td>
<td>.12</td>
<td>.02</td>
</tr>
<tr>
<td>2x10^-3 M MO</td>
<td>.15</td>
<td>.05</td>
</tr>
</tbody>
</table>
when the MO concentration was $10^{-3} M$ (200 MO/Bchl). MO causes light scattering at concentrations of $10^{-3} M$ and $2 \times 10^{-3} M$. The increased light scattering is small (6%) at the highest MO concentration and it may be negligible at $5 \times 10^{-4} M$ MO. It was concluded that MO inhibits photosynthesis in *R. spheroides* at levels of about 200 MO/Bchl in wild type cells.

3.1112 Absorbance Difference Spectrum

MO affects the three Bchl absorbance maxima at 800nm, 850nm and 890nm just as it does in the chromatophores. Each of these three broad absorbance bands of antennae Bchl are slightly shifted to shorter wavelengths (Figure 3.111-2). There is also a loss of absorbance in the orange (590nm) and in the ultra violet (375nm) Bchl bands (Figure 3.111-3). The carotenoid bands at 512nm, 477nm, and 450nm are blue shifted as well. These same effects are recorded in the chromatophore study at a variety of MO concentrations. There is one absorbance change that does not occur in the chromatophore study where the MO absorbance difference spectrum of cells shows an oxidized minus reduced absorbance change for a cytochrome b. Its identification is made by a negative peak at 558nm in the MO difference spectrum. A concomitant negative peak normally at 428nm has been shifted to 422nm by the blue shift in the 450nm carotenoid peak. Since cytochrome b remains in the oxidized state in the presence of $10^{-3} M$ MO, the light induced absorbance changes under these conditions, do not show Cyt b oxidation by light.

3.1113 Light-Dark Difference Spectrum

A variety of light-induced absorption changes are reduced in magnitude by the presence of MO. The greatest change results from the
Figure 3.111-2.

A) Absorption spectrum of wild type *R. spheroides*

B) Dark $10^{-3} M$ Methyl octanoate absorbance difference spectrum ($10^{-3} M$, pH 7), $D(10^{-3} M \text{MO}) - D$ (anaerobic)
Figure 3.111-3

A) Absorption spectrum of wild type *R. spheroides*

B) Dark 10^{-3}M Methyl octanoate absorbance difference spectrum, pH 7. (abbrev. D(MO)-D) (anaerobic)

C) Two spectra: light minus dark spectrum (L-D) and light (10^{-3}M MO) minus dark (10^{-3}M MO), (abbrev. L-D(MO), (anaerobic) exciting $\lambda = 850$nm at 100 volts
blockage of the reduction of cytochrome b by MO (Figures 3.111-3, 3.111-4). In the presence of MO, cytochrome b accumulates in its oxidized state. When the light-induced absorbance changes are recorded in the presence of $10^{-3}M$ MO, there is no reduced Cyt b to be oxidized by light. A normal oxidized reduced absorbance spectrum of Cyt b shows negative absorbance difference bands at about 560nm and 428nm (J. A. Orlando et al, 1961). In the absence of the 428nm negative band due to Cyt b, a new light-induced positive band at 430nm appears. This band probably belongs to the oxidized Bchl of the RC (D. W. Reed, 1969). It appears at about 433nm in the RC light-dark spectrum. There is strong evidence in support of this hypothesis. *R. rubrum*, which has similar photoinduced RC changes as *R. spheroides*, has a light induced electron paramagnetic resonance (EPR) signal which has the same decay kinetics as its 433nm light induced absorbance change (R. H. Ruby, et al, 1964). Now this same type of EPR signal is found in R-26 *R. spheroides* RC. Not only does it have the same decay kinetics as the P870 Bchl photo-induced absorbance change (J. R. Bolton et al, 1969), but it also has the same rise kinetics at liquid nitrogen temperatures (G. Feher, 1971). This evidence is in strong support of the 433nm OD change belonging to the oxidized Bchl radical. The MO effects on the light-induced absorbance changes in the near IR and in the carotenoids will be discussed in the following section.

3.1114 Possible Photophosphorylation Indicators

The carotenoids in wild type *R. spheroides* cells show a photoinduced red shift in their absorbance maxima (Figure 3.111-3C). These changes in the carotenoids can be uncoupled while other photoinduced electron transport changes remain unaltered (W. Arnold and R. Clayton, 1960). It has been suggested that the photoinduced carotenoid shift and a photoinduced near infrared Bchl change in wild type
**Figure 3.111-4**

A) Absorption spectrum of wild type *R. spheroides*

B) Two spectra: light minus dark spectrum (L-D) and light

MO minus dark MO (abbrev. L-D(+MO)); exciting light 475nm

at 100 volts, $10^{-3} M$ MO, pH 7
whole cells are probably due to photophosphorylation-induced membranal changes (D. E. Fleischman and R. K. Clayton, 1968). We know for sure that MO is a photophosphorylation inhibitor at $10^{-3}$ M MO, pH 5 in *Chlorella pyrenoidosa* (Pederson et al, 1966). It has been suggested that a 520 nm photoinduced absorbance change is due to the carotenoids and/or Chl b and that it may be a photophosphorylation indicator. If this were true, then it should not be surprising that the 520 nm photoinduced absorbance change is completely inhibited by $10^{-3}$ M MO (unpublished results). If photoinduced changes in the carotenoids and in infrared Bchl bands of wild type cells are directly or indirectly indicators of photophosphorylation, then photoinduced changes should be correlated with MO titration of photophosphorylation to total inhibition. This hypothesis is consistent with results obtained at the one MO concentration tested. $10^{-3}$ M MO diminishes the magnitude of carotenoid and infrared Bchl photoinduced changes (Figures 3.111-3C, 3.111-4B). It is known that MO does not inhibit photoinduced reaction center (RC) absorbance changes (Section 3.511). This suggests that an experiment that correlates photophosphorylation with photoinduced carotenoid and infrared Bchl changes in cells by MO titration might well establish correspondence between these two phenomena.

### Conclusions

At $5 \times 10^{-4}$ M MO (100 MO/Bchl) wild type cells are just able to grow, but at $10^{-3}$ M MO (200 MO/Bchl) wild type cell growth is inhibited completely. At this same concentration, MO blue shifts the absorbance maxima of carotenoids and antennae Bchl and depresses the orange and the blue absorption maxima of Bchl. Also at this concentration of MO, a
majority of a cytochrome b is oxidized. MO can block the Cyt b reduction pathway. Since the presence of light can no longer induce cytochrome b oxidation in MO treated cells, a light induced positive 430nm absorbance change is isolated. By literature evidence the 430nm OD change is suggested to be due to the oxidized Bchl of P870. A good case can be made for the photoinduced carotenoid red shifts and the photoinduced near IR Bchl absorbance changes as being photophosphorylation indicators. Experiments are suggested.

3.112 Studies with OA

3.1121 Absorbance Difference Spectrum

The chemically induced absorbance changes caused by pH 5 and $1.5 \times 10^{-3} M$ OA at pH 5 are shown in Figure 3.112-1. These absorbance changes continue increasing with time; the absorbance changes shown were recorded in less than 20 minutes because base neutralization of these changes becomes less reversible at longer periods.

As a general interpretation of the MO induced near infrared changes in B800, B850 and B890, they are blue shifted and with a simultaneous loss of absorbance of the parent maximum. This kind of behavior is obvious for the 800nm absorbance peak which probably contributes the most to the newly formed band for denatured Bchl monomer at about 770nm. Oddly enough pH 5 effects the absorption spectrum in much the same manner that OA at pH 5 does, only to a much smaller degree. The explanation for this behavior becomes obvious when one checks the list of organic compounds in Hutner's medium. Unfortunately a 0.02M malate concentration is only part of the problem. All of the organic compounds have to be eliminated as they are in some of the experiments in later sections.
Figure 3.112-1. Wild type R. spheroides cells.

A) Absorbance spectrum

B) Dark OA difference spectrum at pH 5, 1.5x10^{-3} M OA (abbrev. D(1.5x10^{-3} M OA, pH 5)-D(pH 5)). Dark OA reversibility difference spectrum at pH 7 (abbrev. D(1.5x10^{-3} M OA, pH 5 → pH 7)-D(pH 5 → pH 7)) the pH was changed from 5 to 7.

C) Dark pH 5 difference spectrum (abbrev. D(pH 5)-D(pH 7)). Dark pH 5 reversibility difference spectrum (abbrev. D(pH 5 → pH 7)-D(pH 7)).

Note: Anaerobic under nitrogen.
Wild Type, *R. spheroides*, WHOLE CELLS

![Graph](image-url)
However, this change in the contents of Hutner's medium is enough to make the OA, pH 5 induced absorbance changes irreversible.

3.1122 Absorbance Changes Reversed by Base Neutralization

Although the absorbance changes cannot be directly attributed to pH 5, the chemically induced absorbance changes could be almost completely reversed by base neutralization of the acidic cell medium. This type of reversal of the OA, pH 5 and pH 5 induced changes only works in modified Hutner's medium within 20 minutes of acidification. It does not work in a phosphate buffer solution or Hutner's without organic compounds or at time periods much greater than twenty minutes. In Figure 3.112-1C the base neutralization of the acid induced absorbance changes is indicated by D(pH 5 + 7)-D(pH 7). In Figure 3.112-1B, the base neutralization of the OA, pH 5 induced absorbance changes is indicated by D(1.5x10^{-3}M OA, pH 5 + 7)-D(pH 5 + 7). To check if the cells subjected to OA, pH 5 remained viable after base neutralization, they were used as inoculum for bottle cultures. After a lag phase of a week, these cells grew normally.

Performed in parallel with these experiments was the FET preparation of pH 5, 1.5x10^{-3}M OA at pH 5 and its base neutralization treatment. The results of these experiments and 10^{-3}M MO treated wild type cells are reported in Section 3.22.

3.1123 An Interpretation of Selective B800 Denaturation

The largest pH 5 or 1.5x10^{-3}M OA, pH 5 induced absorbance change occurs at 800nm in Figure 3.112-1. R. K. Clayton (1962) and C. Bril (1964) concentrated the wild type's 890nm absorbance band in one fraction and the 800nm and an 850nm bands in a second fraction in their experiments.
This evidence is suggestive of at least two pigment complexes. However, in these experiments with low pH and OA, the 800nm Bchl absorbance peak can be denatured and renatured independently of the 850nm and 890nm absorbance maxima. This evidence, considered with R. K. Clayton's and Bril's work, supports the hypothesis that B800, B850 and B890 are separate Bchl complexes.

3.1.24 Conclusions

Since pH 5 and 1.5x10⁻³ M OA at pH 5 induce absorbance changes that are similar, it is concluded that one of the organic nutrients in modified Hutner's media could be responsible for the absorbance changes. In modified Hutner's medium base neutralization of the pH 5 and OA, pH 5 absorbance changes could normally be reversed in a time period less than twenty minutes. These reversal experiments were not successful at longer time periods or in phosphate buffers or Hutner's medium depleted of organic compounds.

The Bchl maxima at 800nm was selectively denatured by pH 5 and 1.5x10⁻³ M OA, pH 5 in modified Hutner's medium. It is concluded that its absorbance is independent of the 850nm and 890nm absorbance—just like B850 and B890, it can be considered as the independent Bchl complex, B800.

3.12 R-26 Mutant Organisms

The uncomplicated absorbance spectrum of R-26 R. spheroides, unlike wild type, cells made it an ideal system for the study of MO and OA chemical difference spectra. R-26 cells do not contain carotenoids and only contain one major Bchl peak at 860nm. A small band at 800nm is probably part of the reaction center P800 Bchl.
It was the goal of this work to define the action of MO, acid pH, and OA on the primary quantum conversion apparatus. All of these experiments, unlike those with wild type cells, were done in buffered modified Hutner's minus its normal organic compounds. The pH 6 induced absorbance changes are possibly an acid pH effect, although the cells contain organic compounds which could be participating in this effect. An effect of this kind could happen to any actively growing cells in a culture media made slightly acid. Any organic acid within or without the cells can become an active surfactant at acid pH.

MO blocks the reduction of cytochrome b in the R-26 mutant cells, just as it does in the wild type cells. Consequently oxidized Cyt b accumulates in the cells. pH 6 and OA at pH 6 block the reduction of a cytochrome b and a cytochrome c. The cytochrome c is a dark step electron donor to the light reaction of P870 and the cytochrome b is on the electron transport chain in a less understood position.

3.121 Studies with MO

3.1211 Absorbance Difference Spectrum

The effects of MO on the absorbance spectrum are complicated by their time and concentration dependence. About 90% of the changes in the absorbance spectrum occur within about twenty-five minutes. Effects of cell settling and changes in oxygen tension and light scattering have been eliminated or minimized by experimental design. Thus, changes seen in the absorption spectrum can be attributed to MO.

3.12111 Time-Dependent Changes

There appear to be two antagonistic effects operative on the major absorption band of Bchl at 860nm (B860). The first, a loss of absorbance
at 865nm, can be resolved during a period of 1 to 12 minutes. After 25 minutes at concentrations $4 \times 10^{-4} M$ MO, absorbance in the B860 region increases (Figure 3.121-1). This increase is associated with increases in the Bchl peaks at 590nm and 375nm. As the concentration of MO is increased to concentrations $6 \times 10^{-4} M$, the antagonistic increase in absorbance at around 860nm diminishes and the decrease in absorbance at 865nm becomes dominant. At the higher concentrations of MO a cytochrome b becomes oxidized, and a decrease in absorbance is seen at 560nm and at about 428nm. This oxidation is probably caused by the analyzing light and blockage of the Cyt b reduction. An MO concentration of $3 \times 10^{-3} M$ causes the greatest 428nm and 560nm absorbance decrease due to oxidized cytochrome b, but the decrease at about 860nm is curiously smaller than that at the lower concentrations of $6 \times 10^{-4} M$ or $10^{-3} M$ MO. No explanation is given.

3.12112 Concentration-Dependent Changes

A complex concentration-dependent interaction of MO with B860 exists. At $10^{-4} M$ MO there is a small red shift in B860. As the concentration is increased to $4 \times 10^{-4} M$ MO there is an increase in absorbance in the B860 region and in the Bchl maxima at 590nm and 370nm (Figures 3.121-3, 3.121-4). At MO concentrations $6 \times 10^{-4} M$, there is an absorbance decrease at about 860nm. An absorbance decrease is also seen in the regions of 590nm and 375nm. The oxidation of a cytochrome b becomes greater with increasing MO concentration. The increasing magnitude of the negative 560nm and 428nm absorbance change with concentration indicates this behavior.

In summary, MO affects the pigments in the membranes of R-26
Figure 3.121-1.  R-26 *R. spheroides*, whole cells.

A) Dark methyl octanoate difference spectra (abbrev. D(M MO)-D), D(10^{-4} M MO)-D scanned at 1 min, 12 min, and 25 min.

B) D(2x10^{-4} M MO)-D scanned at 1 min, 12 min, and 25 min.

C) D(4x10^{-4} M MO)-D scanned at 1 min and 25 min.

Note: All samples under Helium.
Figure 3.121-2. R-26 R. spheroides, whole cells

A) Dark methyl octanoate difference spectra
(abbrev. D(MO)-D), D(6x10^-4 M MO)-D scanned
at 1 min, 12 min, and 25 min.

B) D(10^-3 M MO)-D scanned at 1 min. and 25 min.

C) D(3x10^-3 M MO)-D scanned at 1 min, 12 min, and
25 min.

Note: All samples under Helium.
Figure 3.121-3. R-26 *R. sphaeroides*, whole cells.

A) Absorption spectrum (near infrared)

B) Dark Methyl octanoate difference spectra
(abbrev. D(M.O.)-D) $10^{-4}M$ to $4 \times 10^{-4}M$ in methyl octanoate

C) Dark methyl octanoate difference spectra
(abbrev. D(M.O.)-D) $6 \times 10^{-4}M$ to $3 \times 10^{-3}M$ in methyl octanoate

Note: Samples anaerobic under Helium.
R-26 *R. spheroides* WHOLE CELLS

**METHYL OCTANOATE CONCENTRATION STUDY**

**D (M.O.) - D**

**ABSORBANCE**

**λ (nm)**

0.2 0.4 0.6 0.8

400 500 600

**Figure 3.121-4.** R-26 *R. spheroides* whole cells.

A) Absorption spectrum (visible to U.V.)

B) Dark methyl octanoate difference spectra (abbrev. D(M MO)-D) $10^{-4}M$ to $4x10^{-4}M$ in methyl octanoate

C) Dark methyl octanoate difference spectra (abbrev. D(M MO)-D) $6x10^{-4}M$ to $3x10^{-3}M$ in methyl octanoate.

**Note:** Samples anaerobic under Helium.
R. spheroides cells in a complex manner. At the higher concentrations of MO a cytochrome b becomes oxidized.

3.122 Studies at pH 6 and pH 6.5

3.1221 Absorbance Difference Spectrum

The effects of acid pH on the absorption spectrum of R-26 cells were determined in preparation for experiments with octanoic acid at low pH. All of the organic acids were removed from modified Hutner's medium so that changes in the absorption spectrum could be attributed to the effects of low pH. If the acids were not removed from the medium, changes seen in the spectrum are similar to those caused by OA at low pH (Figure 3.112-1). Acid pH values near pH 7 were originally chosen in order to minimize irreversible structural damage. Since reversal by acid neutralization is dependent on the physiological state of the organisms, time of exposure, and medium composition, the proper conditions for the reversal of the absorbance changes are restricted. The complete modified Hutner's medium appears to be necessary for the base neutralization of the absorbance changes. In the modified Hutner's depleted of all organic compounds, the absorbance changes sometimes increased with base neutralization.

Mildly acidic conditions were chosen to follow the time changes in the absorbance spectrum. pH 6 is more drastic than pH 6.5, as can be seen in Figures 3.121-5 and 3.121-6. Changes in the absorption spectrum vary in magnitude depending on the physiological state of the bacteria. The R-26 mutant cells in particular are sensitive to exposure at low pH values.
3.12211 Time-Dependent Changes

D(pH 6.5)−D(pH 7.3)

Except for a slight increase in absorbance at 860nm after 2 min. and 26 min., the absorbance changes at pH 6.5 are similar and of smaller amplitude than those caused by pH 6. The rationale given for absorbance changes at pH 6 is also applicable to those at pH 6.5.

D(pH 6)−D(pH 7.3)

The effects of low pH on chromatophores in Figure 3.32-13 are very similar to the effects of pH 6 on an anaerobic cell suspension. A decrease in B860 is paralleled by an increase in 785nm and appears to be a time dependent denaturation of B860 into a peak near the Bchl absorbance at 770nm. A small fluorescence peak at 800nm is the result of this new peak, as can be seen in Figure 3.32-14, for R-26 chromatophores treated with low pH. A loss of absorbance occurs in the in vivo Bchl bands at 590nm and 375nm. Since the cells are anaerobic (He gas), cytochromes absorbing at 550nm and 560nm have become oxidized by light or endogenous oxidants. These are probably a cytochrome c and a cytochrome b, (Orlando et al, 1961, 1963) respectively, which would have negative bands at 550nm and 560nm, respectively. These peaks are not evident in aerobic cultures of chromatophores treated with low pH. In anaerobic cultures the cytochromes are in a reduced state before they are treated with low pH. The presence of oxygen can oxidize Cyt b (G. Ruben, LCBQ-7, pg. 120, 1965).

3.123 Studies with OA at pH 6

3.1231 Absorbance Difference Spectrum

The time dependent and the concentration dependent effects of OA at
Figure 3.13-5. R-26 R. spheroides whole cells.

A) Absorption spectrum (near infrared)

B) Dark pH difference spectrum (abbrev. D(pH) - D(pH 7.3))

D(pH 6.5) - D(pH 7.3) scanned at 2 min., 26 min., 38 min.
50 min. after sample made pH 6.5.

C) D(pH 6) - D(pH 7.3) scanned at 2 min., 13 min., 24 min.,
43 min., and 56 min. after sample made pH 6.

Note: All samples are under Helium.
Figure 3.121-6. R-26 *R. spheroides* whole cells.

A) Absorption spectrum (visible to U.V.)

B) Dark pH difference spectrum [abbrev. 
\[D(pH) - D(pH\ 7.3)\] \(D(pH\ 6.5) - DpH\ 7.3\)] scanned at 2 min, 26 min, 38 min and 50 min after sample made pH 6.

C) \(D(pH\ 6) - D(pH\ 7.3)\) scanned at 2 min, 13 min, 24 min, 43 min and 56 min after sample made pH 6.

Note: All samples are under Helium.
at pH 6 on the absorbance spectrum of R-26 cells are exceedingly complex. Assignment of changes in the absorbance difference spectrum is generally made with pigments absorbing at about the same wavelengths.

3.1231 Time-Dependent Changes of D(OA, pH 6)-D(pH 6)

The time changes occurring at times longer than a minute and shorter than 25 minutes are confined to the antennae B850 Bchl bands. These changes occur at about 860nm, 590nm and 375nm. From the previous section we found that pH 6 can denature the 860nm Bchl peak, shifting it towards its denatured form, absorbing at about 770nm. Oddly enough, the presence of OA at low and high concentrations renatures 770nm monomer Bchl, shifting it back to about 860nm. This effect occurs within a minute. Two effects in B860 occur at time periods greater than a minute. At lower concentrations the extinction coefficient of the 860nm transition and the 375nm band can increase. At concentrations greater than $3 \times 10^{-3} M$ OA, pH 6, the extinction coefficient of these two bands decreases in spite of a shift of more of the 770nm Bchl back to 860nm. An oxidation of a cytochrome b and a cytochrome c appears in a time period of less than a minute. These changes occur more rapidly than changes in B860.

3.1232 Concentration Dependent Changes of D(OA, pH 6)-D(pH 6)

The concentration dependent D(OA, pH 6)-D(pH 6) absorbance difference spectrum changes, compared to the absorbance changes caused by $10^{-2} M$ OA at pH 4.70 in R-26 chromatophores, are mild conditions. These latter conditions can completely denature B860 forming Bchl absorbing at 770nm. OA at pH 6 can't decide whether to renature the 770nm Bchl formed by pH 6 preceding the addition of OA or to alter the B860 absorbance peak.
The OA, pH 6 absorbance changes at 860nm are caught in a region of limbo, part of the 770nm Bchl is being renatured to B860 and the 860nm peak is undergoing changes. B860 can increase its extinction coefficient at OA concentrations above $3 \times 10^{-3} M$ OA, pH 6. One can get a feel for these changes by using the experimental relationship that the 860nm Bchl shifted to 770nm has approximately half its 860nm extinction coefficient. Using this relationship (taken from chromatophore results), $10^{-4} M$ OA, pH 6 causes an absorption increase at about 860nm by red shifting 770nm Bchl. At concentrations of $5 \times 10^{-4} M$ and $10^{-3} M$ OA, the absorbance increase at 860nm is greater than twice the 770nm absorbance change. At these concentrations, the 860nm Bchl is undergoing a slight increase in its extinction coefficient. The same increase occurs at 375nm. A decrease in the extinction coefficient occurs with OA at concentrations of $3 \times 10^{-3} M$ and $10^{-2} M$ in addition to renaturation of 770nm Bchl to 860nm Bchl. At $10^{-2} M$ OA, pH 6 more of the 770nm Bchl is renatured to 860nm but the decrease in the extinction coefficient of 860nm is also greatly increased. There is also a loss of absorbance at 375nm which occurs in parallel to a decrease at 860nm. The 590nm Bchl peak is blue shifted by pH 6 and by OA at pH 6. In contrast MO is much less effective. A cytochrome b and a cytochrome c collect in their oxidized state in the presence of OA, pH 6. In fact the oxidized minus reduced bands at about 405nm, 428nm, and 560nm for cytochrome b and the oxidized minus reduced bands at about 405nm, 428nm, and 550nm for cytochrome c increase with increasing OA concentration. OA, just like pH 6, blocks the reduction of these cytochromes but allows their oxidation.
Figure 3.121-7. R-26 *R. spheroides*, whole cells

A) Dark octanoic acid difference spectrum (abbrev. D(OA, pH 6)-D(pH 6)) D(10^{-4} M OA, pH 6)-D(pH 6) scanned at 1 min. and 25 min.

B) D(5x10^{-5} M OA, pH 6)-D(pH 6) scanned at 1 min. and 25 min.

C) D(10^{-3} M OA, pH 6)-D(pH 6) scanned at 1 min. and 25 min.

D) D(3x10^{-3} M OA, pH 6)-D(pH 6) scanned at 1 min. and 35 min.

E) D(10^{-2} M OA, pH 6)-D(pH 6) scanned at 1 min. and 25 min.

Note: All samples under Helium.
R-26 R. spheroides WHOLE CELLS
TIME STUDY OF D(O, A, pH 6) - D(pH 6)

A) D(10^-4 M O, A, pH 6) - D(pH 6)

B) D(5x10^-4 M O, A, pH 6) - D(pH 6)

C) D(10^-3 M O, A, pH 6) - D(pH 6)

D) D(3x10^-3 M O, A, pH 6) - D(pH 6)

E) D(10^-2 M O, A, pH 6) - D(pH 6)
Figure 3.121-8. R-26 *R. spheroides*, whole cells.

A) Absorption spectrum (near infrared)

B) Dark octanoic acid difference spectrum

[abbrev. D(O.A., pH 6) - D(pH 6)]

$10^{-4}M$ to $10^{-2}M$ in octanoic acid

Note: All samples under Helium.
Figure 3.121-9. R-26 R. spheroides, whole cells.

A) Absorption spectrum (visible to U.V.)

B) Dark octanoic acid difference spectrum (abbrev. D(OA, pH 6) - D(pH 6))

Note: All samples under Helium.
3.123 Conclusions

MO, pH 6, and OA at pH 6 cause absorbance changes in the B860 Bchl which has absorbance bands at 860nm, 590nm and 375nm. These absorbance changes have a time dependence and concentration dependence. A cytochrome b becomes oxidized in the presence of MO, pH 6, and OA at pH 6, and this condition intensifies with increased concentration. A cytochrome c becomes oxidized in addition to the cytochrome b with pH 6 and with OA at pH 6. Cytochrome c oxidation also intensifies at increased concentrations of OA at pH 6. MO, pH 7 and OA at pH 6 can be seen to alter the antennae Bchl at 860nm and block the reduction of one and two cytochromes respectively on the electron transport chain. These chemicals are changing structural relationships within the primary quantum conversion apparatus in the photosynthetic membranes.

3.2 Morphological Studies on Whole Cells

3.21 Membrane Anatomy in Cells

3.211 Techniques for Cell and Membrane Research

3.2111 The Freeze-Etch and Freeze-Fracture Technique (FET and FFT)

These methods are described by D. Branton (1966). His hypothesis that membranes split during the fracturing process so that regions of the inner part of the membrane become visible in the electron microscope is now generally accepted (P. Pinto da Silva and D. Branton, 1970; T. Tillack and V. Maschesi, 1970; H. W. Meyer and H. Winbelmann, 1969, 1970; E. Wehrli, K. Muhlethaler and H. Moor, 1970). It has generally been the case that all photosynthetic and electron transport membranes show internal membrane fracture faces embedded with particles (D. Branton,
1967). These particles are probably representative of proteins or some real structures in the membranes because hydrated and unhydrated artificial lipid membranes do not contain any particles, ice or otherwise, of the size range found in electron transport membranes (D. W. Deamer and D. Branton, 1967; D. W. Deamer, R. Leonard, A. Tardieu, and B. Dranton, 1970). It is for this reason that FET and FFT were used for studying membranes and their internal structure.

3.2.1.2 Membrane Identification

The presence of membranes in a freeze-etched cells are readily identifiable by two distinctive features. Membranes in *R. spheroides* show a characteristic fracture face containing particles averaging about 95Å in width or diameter. These surfaces can also show along their perimeter sharp elevation changes. Another distinct feature occurs when membranes are fractured in cross section. In this case continuous elevated ridges of about 80-150Å can be seen just within the cell wall. Circular membranes that reside within the cytoplasm are also visible as elevated ridges when cells are freeze-etched in distilled water. These identifying features of the membrane in freeze-etched wild type *R. spheroides* reveal the same anatomical features as the embedding and staining techniques of G. Drews and P. Giesbrecht (1963).

3.2.1.2 Anatomical Features

3.2.1.21 Correlation with Staining Techniques

Successful correlation of the anatomical membrane structure of *Rhodospseudomonas virides* by freeze-etching and staining techniques (of thin sections) has been achieved in two independent studies (R. H. Dilley, 1969; G. Drews and P. Giesbrecht, 1965).
Figure 3.212-1A. Wild type *R. spheroides* cell grown at
low light intensity (G. Drews and P. Giesbrecht, 1963),
which increases the number of vesicular membrane structures
(arrows) in comparison with cultures grown at high light
intensities.
The cell in Figure 3.212-1B was grown at a higher light
intensity than that in Figure 3.121-1A. (Magnification
80,000x)

Figure 3.212-1B. Wild type *R. spheroides* cell in distilled
water (FET).
This cell was fractured longitudinally and the ice (i) etched
(sublimed), showing the cell in relief. The circular structures
(arrows) are interpreted as membrane invaginations that have
been fractured in cross section. Storage vacuoles (V) have a
tendency to smear instead of fracture. (Magnification 54,000x)

Note: Membrane, membrane invaginations, and vacuoles are
revealed by embedding and sectioning techniques and FET with
equal ease.
Although membranes are readily visible as a "dark-light-dark" line of less than 100Å thickness by staining more care has to be taken in membrane identification in freeze-etching. This extra care is worthwhile because of the additional ability to see internal membrane fracture surfaces. Also cell features are less distorted. The cellular dehydration in the fixing, embedding stage prior to sectioning and poststaining probably distorts features at the membrane level. The cell wall in stained preparations usually has a much greater circumference than the cytoplasmic membrane just within its borders due to dehydration shrinkage. Sometimes the wall appears very thick and smeared as well. This kind of dehydration shrinkage is never seen with freeze-etched cells. The membranes and cell walls are usually appressed together in the osmotically swollen freeze-etched cells of *R. spheroides*, and together don't appear as thick as the wall in figure 3.212-1A. Figures 3.212 (1A and 1B) compare the two types of preparation techniques. Figure 3.212-1A is a longitudinal section of wild type *R. spheroides* that has been osmium fixed and poststained with uranyl acetate. Arrows indicate vesicles of stained circular membrane enclosing a white unstained area. "V" represents a storage vacuole. The cytoplasmic membrane is immediately proximal to the cell wall, surrounding the cytoplasm. The cell wall represented by the outermost stained layer, is somewhat diffuse in texture, probably because of harsh preparation conditions (F. S. Sjostromd, 1967). The cell membrane has apparently contracted slightly within the cell wall, under the dehydrating conditions employed.

The wild type cell in Figure 3.212-1B was prepared by FET; its appearance is similar to that of the cell shown in Figure 3.212-1A.
Figure 3.212-2A. Wild type *R. spheroides* (after G. Drews and P. Giesbrecht).

A tubular membrane invagination is sectioned longitudinally; its cytoplasmic membrane attachment site (arrow) is shown at the upper left-hand corner. At (X) a tubular invagination either bends perpendicular to the section face or terminates. (V) designates a storage vacuole. (Magnification 160,000x)

Figure 3.212-2B. Wild type *R. spheroides* (after G. Drews and P. Giesbrecht).

The small arrows indicate invaginations originating in the cytoplasmic membrane. (X) indicates an invagination that could be a tubular membrane invagination in cross section, similar to that in Figure 3.212-1A. (Magnification 160,000x)

Figure 3.212-2C. R-26 *R. spheroides* cell in distilled water.

This cell was fractured longitudinally, then etched. There is a system of membranes (M) at a pole of the cell. These membranes appear to be continuous with the circular membrane invagination section (X). The large arrow in the upper right-hand corner indicates shadow direction. (Magnification 140,000x)
In this and following electron micrographs, if no large arrow is shown in the upper right-hand corner, shadow direction is understood to emanate from the lower to the upper edge of the figure. Small arrows indicate the location of some representative vesicular membrane. The smeared structure marked "V" is a storage vacuole. During fracturing these vacuoles are either removed leaving a hole or deformed in situ. The cytoplasmic membrane and cell wall together are much thinner than in Figure 3.212-1A. These structures are contiguous because the cells were osmotically shocked by distilled water prior to freezing. (i) indicates surrounding ice that has been partially removed by sublimation, revealing the cell in relief. The important likenesses of Figure 3.212-(1A and 1B) are their similarities in the location of membranes.

Staining techniques have shown that vesicular membrane in R. rubrum and wild type R. spheroides is attached to the cytoplasmic membrane (S. C. Holt and A. B. Marr, 1965; G. Drews and P. Giesbrecht, 1963).

Figure 3.212-2A shows a longitudinally sectioned tubular invagination of the cytoplasmic membrane of wild type R. spheroides. At (X) this invagination either bends perpendicular to the section plane or terminates.

In Figure 3.212-2B, similar features are seen for another tubular membrane invagination (X). Other invaginations are marked with arrows. The numerous vesicles are most likely cross-sectioned tubular invaginations attached elsewhere. Figure 3.212-(3A and 3B) are drawings of these vesicular structures as they are presumed to exist in R. rubrum (S. C. Holt and A. G. Marr, 1965). Figure 3.212-3A was reconstructed from a number of serial sections; Figure 3.212-3B is an extrapolation of the features of Figure 3.212-3A. Wild type R. spheroides could very likely be
Figure 3.212-3A. This picture is a reconstruction from cell serial sections of *R. rubrum* (after S. C. Holt and A. G. Marr).

Figure 3.212-3B. This figure is an extension of the features seen in Figure 3.212-3A (after S. C. Holt and A. G. Marr).
represented by Figures 3.212-(3A and 3B).

Only in R-26 *R. spheroides* was membrane attachment to a vesicle convincingly demonstrated by FET (Figures 3.212-2C, 3.212-7B). The attachment (arrows) of a vesicle (X) to a small stack of three polar membranes (M) is shown. Attachment of vesicles to the peripheral cytoplasmic membrane of wild type *R. spheroides* cells was not unequivocally demonstrated by FET. Mere proximity of vesicles to the cytoplasmic membrane is not sufficient to prove attachment.

Another indication of the membrane origin of vesicles is a characteristic particulate fracture face. In Figures 3.212-(4A and 4B), circular vesicle cross-sections (X) show particulate fracture faces containing particles approximately the same size as those seen in cytoplasmic membrane. Particulate fractured vesicle surfaces are indicated by a (P). Because vesicles stain in a fashion characteristic of membranes and have been shown to be attached to cytoplasmic membrane, the presence of particulate fracture faces should not be surprising.

*R. spheroides* cells fracture variously with FET, depending on the method of preparation. In 5% glycerol, the cells normally fracture along a hydrophobic plane internal to the cytoplasmic membrane. However, cells frozen in distilled water frequently rupture and fracture randomly. Typical structures for wild type cells are seen in Figures 3.212-(5A and 5B). Numerous vesicles (X) are seen inside the cells.

The cell in Figure 3.212-5A is representative of cells frozen in distilled water. Note membrane fracture plane (Mf) and cell wall (W).

In Figure 3.212-5B the 5% glycerol formed a glycerol eutectic coating (G) on the cell wall. The thinness of the membrane fracture plane (Mf) is
Figure 3.212-4A. R-26 *R. spheroides* cell frozen in distilled water and fractured. Circular sections of vesicular invaginations (X) are seen. The fractured vesicle membrane shows particles (P) which resemble particles on the cytoplasmic membrane fracture surface (Mf). The ice (i) was etched, revealing the cell wall (W). The large arrow indicated shadow direction. (Magnification 80,000x)

Figure 3.212-4B. R-26 *R. spheroides* cell frozen in distilled water and fractured longitudinally. Again, circular sections of vesicular invaginations (X) are seen. The fractured vesicle membrane shows particles (P) that resemble particles seen on the cytoplasmic membrane fracture surface (Mf). This preparation was etched. The large arrow indicates shadow direction. (Magnification 80,000x)
Figure 3.212-5A. Wild type *R. spheroides* cell frozen in water.  
Note cytoplasmic membrane fracture plane (Mf), cell wall (W), vesicular membrane in cross section (X), and ice (i).  
(Magnification 100,000x)

Figure 3.212-5B. Wild type *R. spheroides* cell frozen in 5% glycerol.  
Note cytoplasmic membrane fracture plane (Mf), vesicular membrane in cross section (X), eutectic glycerol (G), and thinness of membrane fracture plane bordering the cytoplasm.  
(Magnification 80,000x)
Figure 3.212-6A. R-26 *R. spheroides* cell frozen in distilled water.

Freeze-fracturing has revealed the membrane fracture surface (Mf). Etching away the ice (i) has revealed the cell wall (W). This cell is in an intermediate stage of cell division. (Magnification 80,000x)

Figure 3.212-6B. R-26 *R. spheroides* cell frozen in distilled water.

Freeze-fracturing has revealed the membrane fracture surface (Mf). Etching away the ice (i) has revealed the cell wall (W). (Magnification 60,000x)
evident where its edge is exposed in cross section. A fracture plane through the cell interior rarely occurs in 5% glycerol.

All features except the polar stack of three membranes can be seen in Figures 3.212-(6A and 6B). The cell in Figure 3.212-6A is constricted at the center and is preparing to divide. The stack of three polar membranes seen in older mutant cells was not seen in young cells, and are probably formed at a later stage of cell development.

Figure 3.212-7A shows at least three particulate fracture planes corresponding to the three polar membranes. Arrows indicate the sharp elevation changes bordering the membrane fracture faces.

The stack of three polar membranes (M) is clearly recognized in Figure 3.212-7B. A vesicle (X) connected to this group of membranes resembles that seen in Figure 3.212-7B.

Normally in FET the cytoplasmic membrane is the only fracture plane visible. These particulate fracture faces are just below the cell wall surface and run parallel with it. The fracture plane only rarely travels through the cytoplasm underneath the cytoplasmic membrane.

3.2122 A New Fracture Plane

Just as rare as the fracture plane running through the cytoplasm is a fracture plane that travels within the cell wall but not internal to the cytoplasmic membrane. This cellular fracture plane is designated (fl). It is possible that (fl) could be the normally unobservable exterior of the cytoplasmic membrane. Although this is a reasonable and a logical interpretation of Figures 3.2122-(1A and 1B), there is possibly another interpretation because R. spheroides is a gram negative bacterium.

Since the composition of a gram negative bacterial cell wall somewhat
Figure 3.212-7A. R-26 *R. spheroides* frozen in water and freeze-fractured tangentially at one pole.

At least three particulate membrane fracture faces (Mf) are demonstrated. The preparation was etched revealing the cell wall (W). (Magnification 120,000x)

Figure 3.212-7B. R-26 *R. spheroides* cell frozen in water and freeze-fractured in a longitudinal section. A stack of at least three polar membranes lie close to the cell wall. A membrane invagination (X) is seen. Etching reveals the cell wall (W). (Magnification 100,000x)
resembles that of the cell membrane, the (f1) fracture plane could be occurring within the cell wall. A representative gram negative bacterial cell wall contains 10% mucopeptide, 20% extractable lipid, 20% lipopolysaccharide and 50% lipoprotein (H. J. Roberts and H. R. Perkins, 1968). It has been suggested that such a fracture plane may exist in *pseudomonas* sp. type IV, a gram negative bacterium (I. W. Devoe, J. W. Costerton, R. A. Macleod, 1971). In Figures 3.212-(IA and 1B) it is not clear whether the (f1) fracture plane represents the exterior of the cytoplasmic membrane or a cell wall internal fracture plane. Further experiments are required to establish the exact position of the fracture plane.

3.2123 Difference Between Wild Type and R-26 Mutant Cells

By means of FET, the location of membrane structure within both wild type and R-26 *R. spheroides* cells have been revealed. The R-26 mutant cells contain three polar membranes with clearly attached vesicles (Figures 3.212-2C and -7B). The polar membranes appear continuous with each other and with the cytoplasmic membrane. Though the stack of polar membranes is not present in young cells, it is present in the mature cells. This stack of three polar membranes is not present in the wild type cells. The circular vesicles are present in the R-26 mutant and are the only membrane present in the cytoplasm of the wild type cells. The identity of the cytoplasmic membrane and cytoplasmic membrane invagination (vesicles) fracture surface particles will be investigated in Section 3.5.

3.22 Morphological Studies on Whole Cells in The Presence of MO and OA

3.221 Studies on Centrifuged Wild Type Cells

These studies were made in conjunction with the chemical difference
Figure 3.2122-1A. R-26 *R. spheroides* cell frozen in distilled water.

Freeze-fracturing reveals a membrane fracture surface (Mf) and a second fracture surface (f1). This surface could be the exterior surface of the cytoplasmic membrane, or it could be a fracture plane within the cell wall (see text). The cell wall (W) has been exposed by etching away the ice (i). (Magnification 140,000x)

Figure 3.2122-1B. Wild type *R. spheroides* cell frozen in distilled water.

Freeze-fracturing reveals a membrane fracture surface (Mf) and a second fracture surface (f1). This surface could be the outside surface of the cytoplasmic membrane, or it could be a fracture plane within the cell wall (see text). The cell wall (W) has been exposed by etching away the ice (i). (Magnification 280,000x)
spectra in Section 3.11. At the time of these studies the effect of centrifugation on separation of MO and OA from the membranes had not been discovered or investigated. Since the cells are centrifuged at 20,000-40,000 x g for about thirty minutes instead of 300,000 x g for 90 minutes for chromatophores, it is assumed that centrifugation hasn't greatly reversed any of the chemically-induced absorbance changes.

These membrane fracture surfaces were complex because the membranes were always part of a greater cell structure. The internal membrane fracture surfaces were located and identified by their characteristic particulate fracture faces. The most prevalent fracture face occurred in the cytoplasmic membrane which lies next to and parallels the cell wall.

The only treatments which affected the membrane fracture surfaces were pH 4.7 and OA at pH 4.7. Although, there are $10^{-3} M$ MO induced changes in the absorbance spectrum, significant changes in the FET cytoplasmic membrane fracture surface can not be detected. Chemical changes induced in the absorbance spectrum as a rule are a more sensitive measure of membrane changes than the changes seen by FET and FFT. A similar conclusion was reached by Bamberger and Park (1966). This general rule may not apply, however, to acid pH effects and perhaps osmotic strength change effects where absorbance changes are not great but the changes in the membrane substructure can be. The pH 4.7 changes in the wild type cell were performed in a 5% glycerol solution, not in modified Hutner's medium as in Figure 3.112-1 for the chemical difference spectrum. The changes in the membrane can be an acid pH effect or an osmotic strength change effect on the cell.
3.2211 Treated with $10^{-3}M$ MO, pH 7

Cells treated with $10^{-3}M$ MO at pH 7 look like the untreated cells in Figure 3.212-5 or Figure 3.221-2B. Although there are MO induced absorbance changes in Figures 3.111-3 and 3.111-4, there are no visible MO changes in the cytoplasmic membrane fracture surface. Only at much higher concentrations of $3\times10^{-2}M$ MO were changes in the particle population density found in R-26 chromatophores (Section 3.4121). No other type of changes could be seen in the R-26 chromatophores or in the cells.

. 3.2212 Treated with pH 4.7, 0.75% EtOH

A five percent glycerol, distilled water solution is used to prevent ice rupture of the frozen wild type cells. The cells are turgid (the cell wall specially limits swelling) giving a very smooth membrane fracture surface in Figure 3.221-2B. The effect of pH 4.7 in Figure 3.221-2A shows an undulating cytoplasmic membrane fracture surface. It is not clear whether this hilly fracture surface is due to pH 4.7 or to a total cell osmotic shrinkage caused by the acid pH. The detection of a true acid pH membrane effect is more satisfactorily answered with the studies on R-26 chromatophores at pH 4.75. There is a definite acid pH effect which changes the way the chromatophores freeze fracture. In distilled water, the pH 4.7, 0.75% EtOH changes in the absorbance spectrum are presumed to be slightly different from the changes at pH 5 in modified Hutner's media because there are no organic acids or buffers present (Figure 3.112-1).

3.2213 Treated with $1.5\times10^{-3}M$ OA pH 4.7, and 0.75% EtOH

Since the cell fracture plane sometimes travels through the membrane
Figure 3.221-1A. Wild type *R. spheroides* cell membrane fracture surface (Mf) prepared in 5% glycerol and covered with particles. (Magnification 510,000x) Large arrow indicates shadow direction.

Figure 3.221-1B. Wild type *R. spheroides* cell prepared in 5% glycerol showing membrane fracture surface (Mf), similar to that in Figure 3.221-1A, covered with particles. Eutectic Glycerol (G) and ice (i) surrounds the cell. (Magnification 102,000x) Large arrow indicates shadow direction.
Figure 3.221-2A. Wild type *R. spheroides* cell treated with pH 4.7 and 0.75% ethanol were frozen in an aqueous 5% glycerol solution. Freeze-etching reveals the membrane fracture surface (Mf), which shows particles (P). The low pH creates an undulating cytoplasmic membrane fracture surface that is rougher than normal. About 50% of the cells show this effect while the other 50% are like the untreated cells. Eutectic glycerol (G) and ice (i) surround the cell. (Magnification 102,000x) The large arrow in the upper right indicates shadow direction.

Figure 3.221-2B. Wild type *R. spheroides* cell frozen at pH 7.0 in an aqueous 5% glycerol solution. Freeze-etching reveals the membrane fracture surface (Mf) that shows particles (P). The indentations in the membrane fracture surface could very possibly be the vesicle or tubular openings in the cytoplasmic membrane. Eutectic glycerol (G) and ice (i) surround the cell. (Magnification 102,000x) The large arrow in the upper right indicates shadow direction.
Figure 3.221-3A. Wild type *R. spheroides* cell treated with $1.5 \times 10^{-3} M$ OA, pH 4.7, 0.75% ethanol, and frozen in an aqueous 5% glycerol solution.

The fracture plane travels within and without the cytoplasmic membrane. A few particles can be seen on the fracture surface. Approximately 65% of the cells in the preparation that were freeze-etched showed this behavior. Eutectic glycerol (G) and ice (i) surround the cell. (Magnification 90,000x)

Figure 3.221-3B. Wild type *R. spheroides* cell treated with $1.5 \times 10^{-3} M$ OA, pH 4.7, and 0.75% ethanol in an aqueous 5% glycerol solution.

The membrane fracture surface (Mf) shows particles (P); however, the surface is unusually rough. About 30% of the cells in this preparation showed this condition while about 5% look like the untreated cells. This preparation has been freeze-etched. Eutectic glycerol (G) has formed ridges around the cell bordered by ice (i). (Magnification 120,000x) The large arrow in the upper right indicates shadow direction.
and sometimes without, the presence of $1.5 \times 10^{-3} M \text{ OA}$ and pH 4.7 strongly affects the cell membrane (Figure 3.221-3A). As well, the fracture plane surface appears smeared. About 65% of the cells in the sample showed this kind of behavior. In Figure 3.221-3B the fracture plane has traveled through the membrane, exposing a rugged hilly, particulate, cytoplasmic, membrane fracture surface. This behavior is very much like the effect of pH 4.7 alone, and it occurs in about 30% of the cell population. The remaining 5% of the cells show cytoplasmic membrane fracture surfaces like those of untreated cells. The particle population density in these treated membranes may change, but there is no way of measuring that change when the freeze-fracture plane travels along a surface outside of the membranes or smears the fracture surface.

3.2214 Relationship of Spectrophotometric Pigment Denaturation Data with Cell Membrane Substructure Changes and Conclusions.

There are clearly absorbance changes and morphological changes induced by pH 4.7 and $1.5 \times 10^{-3} M \text{ OA}$, pH 4.7. However, the cell morphology is so complex that it is not possible to unambiguously assign the changes in the membrane fracture surface to denaturant induced changes in the membrane. Osmotic effects and new denaturant induced fracture surfaces through other cellular materials can not be rigorously excluded. It is only by doing similar experiments on chromatophores that these ambiguities can be eliminated. In fact, the experiments with chromatophores suggest that the pH 4.7 and OA, pH 4.7 membrane fracture face changes are due to changes in the membrane substructure.
3.3 Spectrophotometric Studies with Chromatophores

3.31 Wild Type Chromatophores

3.31.1 Studies with MO

3.31.1.1 Absorbance Difference Spectra

An MO concentration study (Figures 3.31.1-1 and 3.31.1-2) reveals a general blue shift of all the near infrared Bchl absorbance maxima and the carotenoid absorbance maxima. B800, B850 and B890 and the carotenoid absorbance maxima are slightly different in their sensitivity to MO at pH 7.5. The MO chemical difference spectra were done aerobically, so large changes in the oxidation state of the cytochromes do not interfere at about 422nm and 552nm and 560nm. These absorbance changes are present when the preparation is in a more reduced state and in an anaerobic environment. The aerobic conditions eliminate any interfering absorbance changes due to any redox changes in the electron transport intermediates. The chemically induced changes in the light harvesting pigment are isolated by these conditions.

3.31.1.2 Implications of Antenna Bchl Absorbance Changes

In Figure 3.31.1B the antennae Bchl absorbance bands at 800nm, 850nm and a 890nm band differ very slightly in their sensitivity to MO. A blue shift in the 850nm band is effected by 10^{-4} M MO, pH 7.5. Because the changes at 800nm and 890nm are not significant at this MO concentration, this is more evidence for the separate nature of B850 from B800 Bchl in the wild type (R. K. Clayton, 1962; C. Bril, 1964). Although the 800nm, 850nm and 890nm bands generally appear to be blue shifted with increasing MO concentration, there are other changes probably occurring as well.
Figure 3.311-1. Wild type *R. spheroides* chromatophores.

A) Absorption spectrum (near infrared)

B) Dark methyl octanoate difference spectra
(abbrev. D(M MO) - D) in \(10^{-4}\)M to \(6 \times 10^{-4}\)M MO,
pH 7.5 in 0.01 M tris

C) Dark methyl octanoate difference spectra
(abbrev. D(M MO) - D) in \(8 \times 10^{-4}\)M to \(2 \times 10^{-3}\)M MO,
pH 7.5 in 0.01 M tris

Note: All samples aerobic.
Figure 3.311–2.  Wild type *R. sphaeroides* chromatophores.

A) Absorption spectrum (visible)

B) Dark MO difference spectra (abbrev. D(M MO)–D)
in $10^{-4} M$ to $4 \times 10^{-4} M$ MO, pH 7.5 in $0.01 M$ tris

C) Dark MO difference spectra (abbrev. D(M MO)–D)
in $10^{-3} M$ to $4 \times 10^{-3} M$ MO, pH 7.5 in $0.01 M$ tris

Note: All samples aerobic.
In $3 \times 10^{-4} M$ MO the 890nm band loses absorbance without shifting. This type of change is not obvious at 800nm and 850 nm because of the complex nature of the absorbance difference spectrum. Nonetheless, it is probably occurring as well as the blue shifts because of the formation of a 770nm Bchl peak. This peak is the completely denatured condition of the antenna Bchl in a hydrophobic environment.

3.3.113 Implications of Carotenoid Absorbance Changes and Conclusions

It would, of course, be important if the effects of MO on the carotenoid absorbance indicate their membrane associations and structure. This is a tall order for a very complex effect. Since there are six or more individual carotenoids in the wild type cells, including spheroidenone, (see 1.221122), one would have to consider at least six carotenoids and their concentrations in order to begin to understand individual changes in the absorbance spectrum. Since this is much too difficult, we have to seek out qualitative information which might eliminate or select the molecular associations relisted below from Section 1.221122.

1. Carotenoid-carrier interactions
2. Carotenoid-carotenoid interactions
3. Carotenoid-Bchl interactions
4. Carotenoid monomers in a hydrophobic environment

Unfortunately the absorbance maxima of one of the more prevalent carotenoids, spheroidenone, can be blue shifted by 40nm by changing solvents from chloroform to petroleum ether. This change occurs in going from a less to a more polar solvent (T. W. Goodwin, D. G. Land, and M. E. Sissins, 1956). Spheroidenone absorbance in petroleum ether is similar
to the *in vivo* carotenoid spectrum. Since MO is a more polar compound than petroleum either, a 10nm blue shift in the carotenoid absorbance could be explained simply as a solvent effect. It is disappointing that the MO induced blue shift in the carotenoid absorbance could be rationalized as a solvent effect. Case number 4 is the least restrictive case and it can't be eliminated. Nonetheless through careful experimentation, alternatives on the list might be selected or eliminated. A circular dichroism study might be able to find carotenoid dimers or polymers if they exist. Thornber has found carotenoids in all of his isolated Bchl-protein complexes from *Chromatium*. Physical separation of the Bchl-protein complexes from carotenoids without the concomitant denaturation of the Bchl absorbance would eliminate the third possibility. A clean carotenoid-protein or carotenoid-lipid complex would probably have to be isolated to substantiate the first possibility. Since the carotenoids do not themselves fluoresce *in vivo*, titration of the carotenoid absorbance with increasing MO or OA concentration could possibly provoke fluorescent behavior. It should be clear that there is no good information on the structure of the carotenoids in the photosynthetic membranes and that there is much work to be done in this area.

3.32 Studies with R-26 Chromatophores

The chromatophores are the best system for the study of the effects of OA and MO since their light scattering properties are considerably less than those of cell systems and they do not readily settle out of solution like cells do. Nonetheless, it was believed that OA and MO would be more likely to damage the cell membrane fragments irreversibly at concentrations where the cell changes might be reversible. Although this may be true,
the fact that chromatophores are collected in a centrifuged pellet at very high g forces, conspired to make the effects of MO and OA greatly reversible. Forces of about 250,000 to 300,000 times gravity removed at least 80% of OA from the treated chromatophores. Both the density for MO \( \rho_{20^\circ C} = 0.88 \text{ gm/cc} \) and OA \( \rho_{20^\circ C} = 0.91 \text{ gm/cc} \) are less than the density of water and chromatophores.

By chance, it was found that OA at acid pH could denature the 860nm Bchl maxima completely shifting it to about 770nm. This effect on the membrane was studied by fluorescence and fluorescence polarization measurements, by circular dichroism, by FFT electron microscopy and by absorbance spectrophotometry.

The reversibility of the OA induced changes by centrifugation was studied by monitoring the hydrogens on the alpha carbon of OA in a Varian 220 NMR machine. In order to understand the effectiveness of OA at pH 4.7 in denaturing B860, the effects of acid pH and MO at acid pH on B860 were studied. Neither acid pH nor MO alone are effective denaturants of B860. Together they are very effective.

It was found that the RC is probably not denatured like B860, for upon its renaturation by centrifugation, RC is still active. Although RC could be denatured and renatured during the same process, it is quite unlikely because the RC Bchl are so readily photooxidized and pheophytinized upon denaturation. The resistance of RC to OA denaturation indicates that the OA induced absorbance changes are confined to the light harvesting pigments.
3.321 Studies with OA

3.3211 Absorbance Spectrum

The detection of OA effects on the photosynthetic apparatus in the chromatophores was accomplished through the documentation of the changes in pigment absorbance and fluorescence. These changes are recorded in the following subsections.

3.32111 Time-Dependent Changes

Time Study ($10^{-2}M$ OA at pH 4.75)

OA at $10^{-2}M$, pH 4.75, completely denatures the bacteriochlorophyll peak at 860nm (B860). The time course of this reaction is shown in Figure 3.32-1. Within 30 minutes more than 95% of B860 is shifted to about 770nm. For this reason, all effects of OA on R-26 *R. spheroides* chromatophores spectra were recorded after about a 30 minute waiting period.

A study of the time course of the reaction reveals that the denaturation of B860 occurs through the primary formation of bacteriochlorophyll (Bchl) absorbing at 770nm, 590nm and 375nm. Formation of bacteriopheophytin (Bph) absorbing at 760nm, 530nm and 360nm occurs as a secondary reaction of OA at pH 4.75. B860 is very likely a pigment-protein complex, which unfolds in $10^{-2}M$ OA, pH 4.75, separating the Bchl molecules so that energy transfer becomes negligible (see Section 3.3215). The Bchl is then probably exposed to the action of the carboxylic acid group, which can easily remove the Mg$^{++}$ ion converting Bchl to Bph.

3.32112 Concentration-dependent Changes

The changes in Figure 3.32-2 are not strictly concentration dependent because the solution pH also becomes increasingly more acidic in the 0.01
Figure 3.32-1. The time-dependent action of $10^{-2}\text{M OA}$, pH 4.75 on the absorbance spectrum of R-26 $R$. *spheroides* chromatophores.

Note: Samples not anaerobic.
with increasing OA concentration. Nonetheless, this does not detract from the complete denaturation of the 860nm Bchl peak transforming it into a 770nm Bchl peak. After the mixing of OA and a waiting period of about 30 minutes, the absorbance spectrum of the chromatophores was recorded. The effectiveness of OA at the various concentrations is recorded in Figure 3.32-3. It appears that $7.5 \times 10^{-3} M$ OA, pH 4.85 makes the most efficient use of each OA present.

3.3212 Fluorescence Spectrum

Taken simultaneously with the OA concentration changes in the absorbance spectrum were the changes in the fluorescence spectrum. There is normally only one fluorescent maximum in R-26 chromatophores just beyond the longest wavelength Bchl absorbance. This fluorescent maxima usually occurs at about 894nm to 900nm. A new fluorescent maxima at about 800nm appears with the formation of the 770nm peak. The changes in the fluorescence reflect the OA concentration effects on the absorbance spectrum.

It is also clear that the new 770nm Bchl can not transfer its energy to the 860nm Bchl peak which still remains undenatured.

3.3213 Reversal of the Effects of OA by Centrifugation

3.32131 Absorbance Spectrum and NMR Localization of OA

The extent to which OA interacts with chromatophores has been determined by NMR. By examining the proton spectrum of the $-\text{CH}_2-$ group adjacent to the carboxyl group in a $D_2O$ solution with and without the chromatophores present, some insights into the associations of OA in solution (Figure 3.32-5) have been gained. $10^{-2} M$ OA, pH 4.75, has an
Figure 3.32-2. Effects of OA concentrations of
2.5x10^{-3} M (pH 5.2), 7.5x10^{-3} M (pH 4.85) and
10^{-2} M (pH 4.75) on the absorbance spectrum of
R-26 R. spheroides chromatophores.

Note: Samples not anaerobic.
Figure 3.32-3. The number of OA molecules necessary to shift the absorption maxima of one Bchl molecule from 860nm to 770nm or 760nm.
Figure 3.32-4. Effects of OA concentrations of
2.5x10^{-3}M (pH 6.5), 5x10^{-3}M (pH 5.2),
7.5x10^{-3}M (pH 4.85) and 10^{-2}M (pH 4.75) on the
fluorescence spectrum of R-26 *R. spheroides*
chromatophores.

Note: Samples not anaerobic.
NMR spectrum with three absorption peaks in D$_2$O when the OA molecules are tumbling freely. Chromatophores do not have any sharp NMR bands in the OA region of 2.3 ppm.

When chromatophores and 10$^{-2}$M OA at pH 4.75 are mixed, the three line spectrum becomes broadened as would be expected if the majority of the OA is chromatophore associated and its movement is restricted. Upon centrifugation at 300,000 x g, the concentration of OA in the supernatant increases, while only a trace of OA remains in the chromatophores. Over 80% of the OA is separated from the membranes. The effect of 300,000 x g centrifugation on the absorption spectrum of R-26 R. spheroides chromatophores treated with 10$^{-2}$M OA, pH 4.75, is shown in Figure 3.32-6. The absorption spectrum of chromatophores centrifuged and resuspended in 0.01 M tris, pH 7.2, has returned 40-50% of the 765nm maxima to about 850nm. This effect suggests that just the release of 80% or more of the OA from the chromatophores alone is not sufficient to reverse the changes in the absorption spectrum and in the membrane at this concentration of OA. It would appear then that the majority of the OA can be removed from the chromatophores by centrifugation followed by pH neutralization. However, in the case of 10$^{-2}$M OA, pH 4.75, the continued presences of OA may not be necessary to maintain the OA induced absorbance changes. Nonetheless, the majority of the OA treated chromatophore preparations centrifuged and resuspended at neutral pH, probably experience removal of 80% or so of the OA.
Figure 3.32-5. NMR spectrum of $10^{-2}\text{M OA}$, R-26 chromatophores, $10^{-2}\text{M OA}$ with chromatophores, the centrifuged preparation and its supernatant all in a D$_2$O solution. The proton signals at 2.3 ppm are from the alpha carbon of OA. The ethanol is the carrier solution for OA.
Figure 3.32-6. Effect of centrifugation at 300,000 x g and resuspension in 0.01 M tris, pH 7.2, on the absorption spectrum of a chromatophore suspension treated with 10^{-2} M OA, pH 4.75, 4.8% EtOH.
3.3214 Denaturation of B860

3.32141 Formation of a Bchl Monomer at 860nm
   Studied by Circular Dichroism

Titrated with octanoic acid, the circular dichroism spectrum of R-26 chromatophores can be reduced to a single positive maximum in the region of 860nm for the 79% change in Figure 3.32-7. All other spectra have two or more maxima indicating Bchl-Bchl interactions (E. Dratz et al., 1966). The single maximum at 860nm suggests that a monomeric type of Bchl exists at the 860nm peak of R-26 chromatophores. Further evidence of a monomeric species absorbing around 860nm emerges from fluorescence polarization measurements at 900nm and from absorbed polarized light in the 860nm band. P-values as high as 0.5 were observed at high OA concentrations, proving that no energy transfer takes place. If there were still some aggregation at 860nm, energy transfer would occur depolarizing the 900nm fluorescence. It has been suggested that the probably major Bchl interaction causing Bchl to shift from about 770nm in vivo to 860nm in vivo is an interaction between the pi-electron system and certain functional groups in the protein with which it is associated in the biological system (H. Steffen and M. Calvin, 1970).

3.321411 Route of OA Denaturation and Its Implications

In effect this is the best case to discuss the interactions of OA with B860 because the effect is so dramatic and there is information available.

Direct proof is provided that OA at acid pH denatures the polymeric Bchl at 860nm through the loss of the complicated CD spectrum at 860nm. It is very likely that this effect occurs by the direct action of OA on the
Figure 3.32-7. R-26 R. spheroides chromatophores circular dichroism spectra of chromatophore suspensions titrated with octanoic acid.

Percent change refers to Figure 3.32-2 and is defined as the percent decrease of 860nm absorption

\[
\frac{100(\text{O.D.} (860\text{nm}) - \text{O.D.} (860\text{nm}))}{\text{O.D.} (860\text{nm})} \times M \text{ OA}
\]
associated Bchl and by the indirect action of denaturing its protein carrier. There would probably be no monomer at 860nm if OA didn't directly separate the associated Bchl. The question of whether part of the absorbance changes are caused by a direct association of OA and Bchl is still open to question. It is highly probable that OA is responsible for the removal of the Mg$^{++}$ in the pheophytinization of Bchl; however, it's not certain whether OA remains in direct association with Bchl. This question is of some value in understanding the effects of MO and OA at lower concentrations in the cell systems. The only clue that it could be is the slight blue shift of the 590nm Bchl band. Bchl at 770nm and at 860nm have a 590nm band. BPh at 760nm has a band at 530nm. It is very possible that the blue shift of 590nm may be caused by a direct association with OA's or MO's functional group.

3.3215 Uncoupling of Energy Transfer from B860 to Reaction Centers' P870

3.32151 Correlation of B860 Denaturation, with Fluorescence, Fluorescence Polarization Measurements and P870 Photoinduced Changes

The energy transfer mechanism in B860 is responsible for transferring energy of excitation to reaction center P870. Denaturation of B860 by OA disrupts this energy transfer process. Fluorescence polarization measurements are used as a direct measure of this energy transfer. If one B860 molecule absorbs light at 860nm and if the same molecule emits the excited energy in the form of fluorescence at 900nm, then the absorption and emitter oscillators are parallel and the resultant P-value should be 0.5. This is true in practice because the lifetime of the excited state of B860
is too short for molecular rotation. The P-value is defined as \( \frac{(I_{\parallel} - I_{\perp})}{(I_{\parallel} + I_{\perp})} \) where \( I_{\parallel} \) and \( I_{\perp} \) are the fluorescence intensities with the electric vector of the exciting and fluorescence light parallel and perpendicular respectively. If energy transfer occurs, then the P-value is less than 0.5. In chromatophores excitation energy is transferred to other molecules before fluorescence emission can occur. The depolarization of energy transfer in randomly oriented molecules can be represented mathematically as:

\[
\frac{1}{P_n} - \frac{1}{P_0} = \frac{1 - \frac{1}{n}}{(1-n)}
\]  

(Eq. 3.32-1)

Here \( P_0 \) is the intrinsic P-value for a molecule that does not rotate and does not transfer excited state energy, \( \bar{n} \) is the average number of energy transfer steps and \( P_n \) is the P-value after \( \bar{n} \) energy steps (G. Weber, 1966).

To compare B860 molecules with randomly oriented molecules is a rough approximation, but this approximation gives an idea of the order of the number of energy transfer steps.

In Figure 3.32-8 the P-value has been plotted as a function of OA concentration of changes in absorption, of changes in fluorescence and of changes in P870 bleaching. P-values of 0.5 are seldom observed. Such high values have been observed in reaction center preparations (T. G. Ebrey and R. K. Clayton, 1969).

In Figure 3.32-9 the number of energy transfer steps has been calculated using the equation of Weber (Eq. 3.32-1). The decrease in the number of energy transfer steps is positively correlated with the inhibitory effect of OA. This of course means that absorbed light is less efficiently transferred to the reaction center with increasing
Figure 3.32-8. R-26 *R. spheroides* chromatophores. The P-value of the 860 nm absorbance with fluorescence at 900 nm. The chromatophore suspension was treated with several OA concentrations (X M OA). The percentage change in the absorbance is

\[
100 \frac{(\text{O.D. (860nm)} - \text{O.D. (860nm)})}{\text{O.D. (860nm)}}\]

\[
= X M OA \quad X M OA
\]

The absorbance spectrum as a function of OA concentration is in Figure 3.32-2.
Figure 3.32-9. R-26 *R. spheroides* chromatophores. Average number of energy transfer steps in the 860nm Bchl peak compared with the percentage change of P870 bleaching as a function of OA concentration. The absorbance spectrum changes can be found in Figure 3.32-2.
OA concentration.

In Figure 3.32-10 the energy of light absorbed by BPh (760nm, 530nm and 360nm) and Bchl (770nm, 590nm, and 370nm) is not transferred to B860 (860nm, 590nm, and 375nm). The fluorescence of BPh and Bchl pigments at 800nm can be excited with 530 and 590 light. But the fluorescence of B860 at 900nm cannot be excited by 530nm light, clearly demonstrating that no energy transfer occurs from BPh (760nm) and Bchl (770nm) to B860.

P-value measurements in species absorbing at 760 and 770nm show that energy transfer between these species is not very efficient (H. Steffen and M. Calvin et al., 1971). Their inability to transfer energy indicates that these species are relatively well separated from each other as well as from B860 Bchl.

3.32152 OA Inhibition of P870 Photoinduced Absorbance Changes

Loss of P870 reaction center activity is a direct measure of OA inhibition of energy transfer in the chromatophores. The complete inhibition of photoinduced P870 occurs at 7.5x10^{-3}M OA, pH 4.85 but the P870 photoinduced activity can be restored by centrifugation. This observation will be of great importance to us later on because it indicates that P870 is not denatured. Although it would seem likely that P870 could undergo denaturation and then renaturation along with B860, it is most unlikely because denatured P870 Bchl is rapidly photooxidized or pheophytinized.

3.321521 Concentration-dependence

Since B860 supplies all the excitation energy to RC except the 1-3%, it absorbs directly, OA inhibition of the photoinduced RC absorbance changes is a good measure of the loss of energy transfer. The OA concentration
Figure 3.32-10. R-26 *R. spheroides* chromatophores. Fluorescence and fluorescence excitation spectra of a chromatophore suspension treated with OA so that BPh (760nm, 590nm, 360nm) Bchl (770nm, 590nm, 370nm) and B860 (860nm, 590nm, 375nm) are simultaneously present.
dependence of the photoinduced RC changes are given in Figure 3.32-11. These changes are correlated with OA induced absorbance changes. Fluorescence changes and fluorescence polarization changes are shown in Figure 3.32-10.

3.321522 Reversal by Centrifugation

It appears from the experiments that the only obstacle to restoration of the photoinduced RC absorbance changes is restoration of 770nm Bchl to B860. The greatest reversibility with a high OA concentration occurred with 6.25x10^{-3}M. There were indications that restoration of RC activity occurred at 10^{-2}M OA but reversal of the OA induced absorbance changes in the denatured B860 Bchl were generally poor. Restoration of RC activity in Figure 3.32-12 was achieved by centrifugation at 300,000 x g and resuspension in a neutral 0.01 M tris buffer solution.

3.322 Studies at Low pH in the Absence of OA

In order to understand the important factors in OA's ability to denature B860 in acid solution, two studies were made. In the first study, the effect of acid pH alone on the absorbance spectrum, fluorescence spectrum and on the photoinduced RC absorbance changes was measured. The acid pH's employed produced exceptionally small changes in all of these measurements compared to OA at these pHs.

The second study combines the effect of acid pH and MO. This study follows in Section 3.323.

3.3221 Absorbance Spectra and Fluorescence Spectra

In Figure 3.32-13, pH values between 7.4 and 4.05 do not greatly affect B860. The pH induced changes of most interest occur at pH 4.75.
Figure 3.32-11. Light-dark absorption difference spectra of chromatophore suspensions with different octanoic acid (OA) concentrations in 0.01 M tris. The recorded pH's are given with each concentration: 2.5x10^{-3} M (pH 6.55), 5x10^{-3} M (pH 5.2), 7.5x10^{-3} M (pH 4.85), 10^{-2} M (pH 4.75). Actinic light 590 nm, saturating.
Figure 3.32-12. Restoration of photoinduced reaction center activity of R-26 chromatophores treated with $6.25 \times 10^{-3} \text{M OA}$, occurs after centrifugation (300,000 x g) and resuspension in a neutral 0.01 M tris buffer. Actinic light, 590nm, saturating.
Figure 3.32-13. Absorbance spectra of R-26 chromatophores as a function of acid pH values.
Figure 3.32-14. Fluorescence spectra of R-16 chromatophores as a function of acid pH values.
Whereas pH alone only denatures about 1/8 of B860, $10^{-2}M$, pH 4.75 denatures it completely. The acid pH induced changes in the fluorescence spectrum are only slightly larger than the absorbance changes. The change at 900nm in the 900nm fluorescence for pH 4.75 is about 1/6 of the total. The increase in fluorescence at 800nm corresponds with the formation of a slight 770nm Bchl peak.

3.3222 Photoinduced Absorbance Changes in P870

In Figure 3.32-15, the relative photoinduced changes in P870 are little changed by the acid pHs employed in the OA studied. This study proves conclusively that it is the OA at low pH that is responsible for the inhibition of the P870 activity, not the low pH. It is also interesting that RC activity falls off dramatically at pHs below 4.75. The RC is subject to an acid pH effect at very acidic pH's, but within the bounds of this study it is not important.

3.323 Synergism of Acid pH and MO as B860 Denaturants

Since the effectiveness of MO as a denaturant is not strictly dependent on its protonated acid form like OA, it was the logical compound to use for this study. MO is structurally analogous to OA in every way except that its functional group is different. The likeness of their straight hydrocarbon chain in membrane solubility is emphasized as being of major importance. For this reason, MO represents a good estimate of OA's hydrocarbon nature. At pH 7, $2 \times 10^{-2}M$ MO can at very best denature $1/4 - 1/3$ of B860. It is about $1/4 - 1/3$ as effective as $10^{-2}M$ OA, at pH 4.75.

Since neither MO nor acid pH alone are effective denaturants of B860, it was felt that their combination might explain the effectiveness
Figure 3.32-15. Relative light induced absorbance changes in P870 (RC) in R-26 chromatophores as a function of pH.
of OA at acid pH. By holding the MO concentration constant at $8 \times 10^{-3} M$ MO and decreasing the pH, their combined effectiveness in B860 denaturation was studied. Their effect together was greater than the sum of their separated effects. MO and acid pH cooperate dramatically in denaturing B860.

3.3231 Absorbance Spectra

In Figure 3.32-16, the effects of $8 \times 10^{-3} M$ MO at pHs from 6.5 to 4 dramatically illustrate the cooperation between these two conditions in denaturing B860. Since this denaturation is greater than the sum of their separate effects on B860, their cooperation is termed a synergistic effect. This study serves to illustrate the reason for the great effectiveness of OA at low pH in denaturing B860. OA's excellent ability must also to a large degree depend on a cooperation between acid pH and its hydrocarbon membrane solubility.

3.3232 Reversal of Absorbance Changes by Centrifugation

Since MO-induced absorbance changes in acid solution are much greater than at pH 7, these systems lend themselves well to a centrifugation reversal of the absorbance changes. The extinction coefficient of denatured 775nm Bchl is approximately half the extinction coefficient of Bchl absorbing at 860nm. Scattering at these wavelengths is taken into consideration so the concentration of Bchl in the original sample and the centrifuged sample can be made equal. Figures 3.32-17 and 3.32-18 show that the effects of $8 \times 10^{-3} M$ MO are partially reversible at pH 6.5 and pH 4. A small peak at 530nm corresponding to bacteriopheophytin and a small peak at 680nm probably corresponding to an oxidized Bchl product.
Figure 3.32-16. Absorbance spectrum of R-26 *R. spheroides* chromatophores in 8x10^{-3} M M.O. as a function of acid pH.
Figure 3.32-17. Effect of 300,000 x g centrifugation and resuspension in 0.01 M tris, pH 7.2, on the absorption spectrum of R-26 R. spheroides chromatophore treated with pH 6.5, 8x10⁻³ M M.O.
Figure 3.32-18. Effect of 300,000 x g centrifugation and resuspension in 0.01 M tris, pH 7.2, on the absorption spectrum of R-26 R. spheroides chromatophores treated with pH 4, 8x10^{-3} M M.O.
appear in the pH 4 sample. An oxidized Bchl at 680nm is a reasonable interpretation since denatured Bchl in chromatophores absorbing at 770-775nm is slowly converted to a 683nm peak when exposed to air or oxidants (E. S. Gould, I. D. Kuntz, Jr., and M. Calvin, 1965). This irreversible product probably could be avoided if this procedure was carried out in an oxygen-free environment. Nonetheless, there is a partial reversibility of the effects of MO by 300,000 x g centrifugation and by resuspension in a neutral buffer of 0.01 M tris. The poor reversibility of these MO absorbance changes is probably the result of the two or so hours that the samples were subjected to MO at low pH. Reversibility is greatly affected by the time period the denaturants are in contact with the chromatophores.
3.4 Spectrophotometric and Structural Changes of Chemically Treated Chromatophores.

Since OA and MO, to a lesser degree, chemically induced absorbance changes in the light harvesting pigments, correlation of these changes with concomitant changes in the membrane substructure as revealed by FET, might furnish a greater understanding of the physical apparatus of the primary quantum conversion process.

The freeze-etch technique (FET) and the freeze-fracture technique (FFT) without etching are the only electron microscope techniques capable of directly viewing the inside of membranes. It is only recently that these techniques have been generally accepted as fracturing membranes along some internal hydrophobic surface (P. Pinto da Silva and D. Branton, 1970; D. Branton, 1966; H. W. Meyer et al., 1969, 1970; E. Wehrli et al., 1970).

The FET was used mostly for viewing cell membrane anatomy and internal membrane fracture faces of the cell. The FET was used on the isolated chromatophores because etching preparations of membrane chromatophores change their fracture surface relief by the rapid escape of subliming ice vapor.

Essentially the membrane fracture face we could see and/or measure were used as indicators of chemical changes in the *R. spheroides* membranes.

3.41 R-26 Chromatophores

3.411 Optical Properties of Chemically Treated Chromatophores Prepared for Electron Microscopy

3.4111 Uncentrifuged Preparations

Because centrifugation can reverse the chemically induced absorbance changes and this same centrifugation is a necessary step in the con-
centration of the chromatophore samples for FFT, the chemically induced changes in absorbance and fluorescence spectrum were taken at the beginning and after centrifugation and resuspension in neutral 0.01 M tris. The chemically induced changes in these FFT preparations are bracketed by these two extremes because the FFT preparations are centrifuged and are quickly frozen. They are not diluted and neutralized by resuspension at pH 7.5 in 0.01 M tris which is a method for looking at the reversal of the absorbance changes.

3.41111 $2 \times 10^{-2}$ M MO, pH 7.5, 4.8% EtOH Absorbance Changes

About 33% of the 860nm in vivo Bchl peak is denatured forming a new Bchl peak at about 775nm. From studies done in Section 3.32141 we know this is monomeric Bchl which is membrane associated (i.e., it is not left in the supernatant on centrifugation. This preparation was centrifuged at about 150,000 x g but it was never resuspended. It is doubtful that reversal of the MO-induced absorbance changes by centrifugation at the lower g value had much effect because of the 2-3 hours the chromatophores experienced a high MO concentration.

3.41112 pH 4.7, 4.8% EtOH Absorbance Changes

A number of spectra are recorded along with the pH 4.7, 4.8% EtOH absorbance spectrum. The reason that the pH 4.7 spectrum was recorded is because 4.8% EtOH was forgotten. Afterwards it was remembered and belatedly added and its absorbance recorded. If the chromatophores had experienced both 4.8% EtOH and pH 4.7 simultaneously the absorbance changes would be a lot less than the 30% absorbance decrease at 860nm in Figure 3.41-2. The timing and the sequence of mixing the chemicals has a big effect on the absorbance spectrum of R-26 chromatophores.
Figure 3.41-1. Absorbance spectrum of R-26 R. spheroides chromatophores treated with 2x10^{-2}M MO, 4.8% EtOH and pH 7.5 in 0.01 M tris before centrifugation.
Figure 3.41-2. Influence of pH 4.7 and 4.8% ethanol on the absorption spectrum of isolated R-26 *R. spheroides* membrane in 0.01 M tris uncentrifuged.
10^{-2} M OA, pH 4.7, 4.8% EtOH and 7.5 \times 10^{-3} M OA pH 4.85, 4.8% EtOH Absorbance Changes

10^{-2} M OA, pH 4.7, 4.8% EtOH completely denatures the B860 Bchl peak forming a denatured BPh and Bchl peak at 760 nm. 7.5 \times 10^{-3} M OA, pH 4.85, 4.8% EtOH has denatured about 75% of the B860 Bchl peak forming a new Bchl monomer peak at 770 nm. The pH 4.7, 4.8% EtOH and pH 7.2, 4.8% EtOH effects on the absorbance spectrum are included for comparison. Section 3.321 can be referred to for detailed understanding of the changes in the absorbance spectrum.

The fluorescence spectra of these preparations reflect the effect of the various conditions on B860. 10^{-2} M OA, pH 4.7, 4.8% EtOH has converted all of B860 to a BPh peak (760 nm, 530 nm and 360 nm). BPh fluoresces at about 780 nm. 7.5 \times 10^{-3} M OA, pH 4.85, 4.8% EtOH has converted about 75% of the B860 Bchl peak to Bchl (770 nm, 590 nm and 360 nm). The 770 nm Bchl fluoresces at about 802 nm and the small amount of undenatured B860 shows some fluorescence at around 900 nm. The pH 4.7, 4.8% EtOH which depress the 860 nm absorbance peak only shows a slight decrease in fluorescence. The pH 7.2, 4.8% EtOH and pH 4.7 are included for comparison. The chemically induced changes in the fluorescence spectrum reflect the changes in the absorbance spectrum.

Centrifuged Preparations Resuspended in 0.01 M tris at pH 7.2

Centrifugation at 300,000 x g for 90 minutes followed by resuspension
in 0.01 M tris at pH 7.2 has reversed some of the changes in the absorbance spectrum. The $10^{-2} M$ OA, pH 4.7, 4.8% EtOH effects are important because they are greatly irreversible. The B860 Bchl is still about 90% or more denatured after centrifugation and resuspension. The $7.5 \times 10^{-3} M$ OA pH 4.85, 4.8% EtOH preparation is more reversible than the higher concentration of OA. An 850nm peak is formed which is probably the renatured protein complex containing BPh instead of Bchl. The large 530nm peak indicates that the majority of the Bchl has been converted to BPh. A B860 like complex containing BPh is probably 60% renatured.

Because of the exposure of these preparations to air, an oxidized Bchl peak at 680nm is formed in all of the chemically treated preparations. Even the pH 4.7, 4.8% EtOH preparation has formed a small amount of the 680nm compound. The effects of concentrations less than $10^{-2} M$ OA are, nevertheless, greatly reversible.

The fluorescence spectrum reflects the complexity of the absorbance peaks in centrifuged preparations and the presence of the new absorbance peak at 680nm.

$$3.41122 \text{ pH 4.7, 4.8% EtOH and } 7.5 \times 10^{-3} M \text{ OA, pH 4.85, 4.8% EtOH, and } 10^{-2} M \text{ OA, pH 4.7, 4.8\%}$$

EtOH Fluorescence Changes and Conclusions

There are a number of new fluorescent emission peaks that were not present at the beginning of the experiment. The 710nm peak is a reflection of the 680nm peak which has formed in the centrifuged preparations. As was stated before, the 680nm peak probably is an oxidized Bchl product (Gould et al., 1965). A fluorescent peak at about 780nm is the result of a BPh peak at 760nm in the centrifuged preparations. There is practically no Bchl absorbing at 770nm in these preparations which correlates with the
Figure 3.41-3. Absorbance spectrum of R-26 *R. spheroides* chromatophores.

Influence of $10^{-2} M$ OA, pH 4.7, 4.85% EtOH and $7.5 \times 10^{-3} M$ OA, pH 4.85, 4.85% EtOH on the absorbance in 0.01 M tris, uncentrifuged.
**Figure 3.41-4.** Fluorescence of R-26 *R. spheroides* chromatophores.

Influence of $7.5 \times 10^{-3} M$ OA, pH 4.85, 4.8% EtOH and $10^{-2} M$ OA, pH 4.7, 4.8% EtOH on the fluorescence spectrum in 0.01 M tris, uncentrifuged.
absence of an 800nm emission peak. The small 885nm shoulder on the
7.5x10^{-3} M OA, preparation corresponds to the 850nm absorbance peak.

These results clearly demonstrate that the FFT preparation with 10^{-2} M OA,
pH 4.7, 4.8% EtOH has more than 90% of its B860 Bchl peak denatured. In
the FFT preparation of 7.5x10^{-3} M OA, pH 4.85, 4.8% EtOH about 40-75% of
the B860 is denatured. The FET preparation of pH 4.7, 4.8% EtOH only has
10-33% of the B860 Bchl peak denatured. The same could probably be said
of the 2x10^{-2} M MO, pH 7.5, 4.8% EtOH preparation. These effects will help
in the interpretation of the effect of these chemicals on the substructure
of the R-26 chromatophores.

3.412 Morphology of Chemically Treated, Centrifuge Chromatophores

The FFT preparations were treated chemically and then centrifuged for
90 minutes at 300,000 x g. Small samples from the pellets were frozen
prior to their freeze-fracturing and their platinum-carbon replication.

3.4121 Treated with 2x10^{-2} M MO
pH 7.5, 4.8% EtOH

Chromatophores treated with 2x10^{-2} M MO were different from the un-
treated chromatophores in one way. The particle population density on the
membrane fracture surfaces were greatly decreased. In Figure 3.41-7B,
large areas of the chromatophore fracture surface are bare of particles.
Figure 3.41-7A and Figure 3.41-8B show untreated chromatophores which have
a much higher frequency of particles on their fracture surfaces. There
was no change in the treated chromatophore fracture plane although there
was one rare event recorded in Figure 3.41-9A. This figure is particu-
larly interesting because it shows a membrane fracture surface (Mf) like
that in Figure 3.41-7B, as well as another surface with particles and bare
Figure 3.41-5. Absorbance spectrum of R-26 *R. spheroides* chromatophores. Preparations treated with pH 4.7, 4.8% EtOH and 7.5x10^{-3} M OA, pH 4.85 4.8% EtOH and 10^{-2} M OA, pH 4.7, 4.8% EtOH that have been centrifuged for 90 minutes at 300,000 x g and resuspended in 0.01 M tris at pH 7.2.
Figure 3.41-6. Fluorescence of R-26 *R. spheroides* chromatophores.

Preparations treated with pH 4.7, 4.8% EtOH and 7.5x10^{-3} M OA, pH 4.85, 4.8% EtOH and 10^{-2} M OA, pH 4.7, 4.8% EtOH that have been centrifuged for 90 minutes at 300,000 x g and resuspended in 0.01 M tris at pH 7.2.
Figure 3.41-7A. R-26 *R. spheroides* chromatophores frozen in aqueous 0.01 M tris, pH 7.5, and 4.8% ethanol. The membrane fracture plane surface (Mf) with no etching contains a greater number of particles (P) per unit area [ice (i)]. (Magnification 140,000x)

Figure 3.41-7B. R-26 *R. spheroides* chromatophores frozen in aqueous 0.01 M tris, pH 7.5, 2x10^{-2} M MO, and 4.8% ethanol. Membrane fracture plane surface (Mf) with no etching treated with 2x10^{-2} M MO and centrifugation at 150,000 x g for 60 minutes. There are fewer particles (P) per unit area on the fracture surface than in the untreated membrane. (Magnification 140,000x)

The large arrow indicates shadow direction.
patches at a lower elevation. In this unusual fracturing process, particles have been strewn around on the ice surface. This is the only time such an event has been witnessed, which is important to the interpretation of the particle loss mechanism from the membranes. In most all observations, strong chemical treatment does not cause the fractured chromatophores to drop their particle contents on the ice surface. It is unlikely that particles are lost by this mechanism. There is no reason either that the particles should be removed with the upper longitudinally cleaved half of the membrane. If the attachment of particles in the membrane is greatly weakened, then there should be probably no increased preference of leaving the membrane with the upper cleaved half but the particles might fall out on the ice. It is, therefore, preferable to choose in situ particle denaturation in the membranes for the decrease in the particle population density. Evidence will be presented that the particles' dimensions do not change significantly upon chemical treatment even with harsher chemicals than MO. By analogy to other results, MO probably does not change the size of the membrane fracture surface particles.

3.4122 Treated with pH 4.7, 4.8% EtOH

It is assumed that pH 4.7, 4.8% EtOH has no measurable effect on the particle dimensions because $10^{-2} M$ OA, pH 4.7, 4.8% EtOH has no effect. It does, however, change the associations in the membrane by creating a new fracture plane route through the membrane. This new membrane fracture surface is very uneven where the fracture plane doesn't even always travel within the membrane. Even though $2 \times 10^{-2} M$ MO, pH 7.5, 4.8% EtOH and pH 4.7, 4.8% EtOH can denature about the same fraction of the B860 Bchl
Figure 3.41-8A. R-26 *R. spheroides* chromatophores frozen in aqueous 0.01 M tris and treated with pH 4.7 and 4.8% ethanol.

This preparation shows a membrane fracture face (Mf) without etching. The (Mf) surface shows a steep transition to an upper plateau of chromatophore outside surface (OM). Particles (P) can be seen in the rugged Mf surface. (Magnification 100,000x)

Figure 3.41-8B. R-26 *R. spheroides* chromatophores frozen in aqueous 0.01 M tris, pH 7.5, and 4.8% ethanol.

The membrane fracture face (Mf) with no etching is very regular in comparison to Fig. 3.41-8A and its particles (P) appear in patches next to blank areas. This patchy effect usually occurs in older preparations of chromatophores. (Magnification 100,000x)

The arrow in the upper right-hand corner indicates shadow direction.
peak, their effects on the membrane fracture plane are quite different. Unlike the MO-treated chromatophores the fracture plane texture in the pH 4.7, 4.8% EtOH preparations is very rough and irregular. It suggests that the extent of denaturation of B860 is not enough in accessing membrane structural changes. Because of this pH 4.7 induced fracture plane effect, it was impossible to access the change in particle population density. The membrane changes described can be assessed by comparison with untreated chromatophores in Figure 3.41-8B. In this preparation, the membrane fracture plane has a more even surface which does not travel outside of the membrane.

3.4123 Treated with $7.5 \times 10^{-3} M$ OA, pH 4.85, 4.8% EtOH and $10^{-7} M$ OA, pH 4.7, 4.8% EtOH

There are two major chemically induced changes in the membranes in these preparations. A large fraction of the chromatophores with OA at pH 4.85 and pH 4.7 fracture in the same manner as the pH 4.7 preparation without OA (Figure 3.41.9B). Again, there were no measurable changes in the particle dimensions. These results are explained in Section 3.413. Changes in the particle population density are difficult to observe, because fracture surfaces look similar to those in the pH 4.7, 4.8% EtOH preparation. It was necessary to search out fracture surfaces so this change could be evaluated for there are fractured chromatophore surfaces which aren't so uneven. These chromatophore fracture surfaces show a large decrease in the particle population density for both of the OA treated preparations (Figure 3.41-10A, Figure 3.41-10B).

3.4124 Conclusions

Each of the chemical treatments used caused changes in the chromato-
Figure 3.41-9A. R-26 *R. spheroides* chromatophores frozen in aqueous 0.01 M tris, pH 7.5, and 4.8% ethanol, and treated with 2x10^{-2} M MO before centrifugation at 300,000 x g for 90 minutes. The normal membrane fracture plane (Mf) appears to be elevated above another surface area which shows an alternately smooth and particulate surface. In the fracturing process, particles (P) loosened by 2x10^{-2} M MO which are normally held in the membrane have been scattered onto the ice (i) surface in the freeze-fracture process without etching. (Magnification 140,000x)

Figure 3.41-9B. R-26 *R. spheroides* chromatophores frozen in aqueous 0.01 M tris, pH 4.7, and 4.8% ethanol, and treated with 10^{-2} M OA and centrifuged at 300,000 x g for 90 minutes. The membrane fracture plane (Mf) is very irregular. The outside of the membrane (OM), which is sharply elevated above the membrane fracture surface (Mf), can be seen occasionally. There are particles (P) on the fracture surface, but they are difficult to locate because of its rugged relief. (Magnification 100,000x)
Figure 3.41-10A. R-26 *R. spheroides* chromatophores frozen in aqueous 0.01 M tris, pH 4.85 and 4.8% ethanol, and treated with $7.5 \times 10^{-3} M$ OA prior to centrifugation at 300,000 x g for 90 minutes. Membrane fracture face (Mf) without etching shows very few particles (P) on its surface.

(Magnification 120,000x)

Figure 3.41-10B. R-26 *R. spheroides* chromatophores frozen in aqueous 0.01 M tris, pH 4.7, and 4.8% ethanol, and treated with $10^{-2} M$ OA before centrifugation with 300,000 x g for 90 minutes. The membrane fracture face (Mf) shown without etching is similar to the fracture surface in Figure 3.41-10A because it contains very few particles (P) [ice (i)].

(Magnification 120,000x)
phores prepared for FFT. R-26 chromatophores treated with $2 \times 10^{-2} \text{M MO}$, pH 7.5 and 4.8% EtOH showed a diminished particle frequency on their fracture faces. R-26 chromatophores treated with pH 4.7 and 4.8% EtOH fractured differently from chromatophores at normal pH. The texture of the fracture plane was very uneven and there were places where the fracture plane traveled along a surface exterior to the membrane.

$10^{-2} \text{M OA}$, pH 4.7 and 4.8% EtOH and $7.5 \times 10^{-3} \text{M OA}$, pH 4.85 and 4.8% EtOH treated chromatophores both show a rough membrane fracture face texture which is the effect of acid pH. Since it's not possible to access changes in particle frequency on the very rough chromatophore fracture faces, this information was taken from a few fracture faces that were not so rough (Figure 341-10A, 10B). It appears that the particle frequency is decreased by the OA treatments just as it was with $2 \times 10^{-2} \text{M MO}$ treatment.

Within the precision of measurement of the particle widths, the fracture face particles do not change size. That is, the changes in width could still be less than 5 Å.

It was never clear in the earlier experiments performed that there were large variations in the platinum-carbon shadow densities from experiment to experiment and that these variations greatly influenced the size of the measured membrane fracture face particles. This effect on particle measurement biased my results so that a pattern was never evident. When these treated chromatophore preparations were freeze-fractured a specimen holder designed by D. Branton was available so I could simultaneously replicate as many as four frozen samples. The samples replicated simultaneously are designated with the same letter.
in Table 3.4.124. The differences in the mean particle width fall well within their uncertainty which is about plus or minus 5 Å. Within the precision of this experiment, the particle widths don't change. The close correspondence between the means of the 4.8% EtOH, chromatophore replicas in A and B is fortuitous. Section 3.5.23 shows the spread of the means due to variations in platinum-carbon shadowing. The triton X-100 treated chromatophores were included because this is the first step in the extraction of reaction center. The size of the RC particles will be compared to these membrane particles in Section 3.5.23. Before this comparison can be useful, it will be demonstrated that a homogeneous preparation of RC was obtained. This is not easy since only recently have G. Feher and coworkers (1971) been able to accomplish it.

3.4.13 Relationship of Chemically Induced Spectrophotometric Changes with the Changes in Chromatophore Substructure

pH 4.7 treatment and 1.5x10⁻³ M OA, pH 4.7 treatment of wild type whole cells show an effect on the absorbance spectrum and the cell substructure (Section 3.2.214). The absorbance and substructure changes could not be related unambiguously in these experiments. The R-26 chromatophores, are easier to relate because they are far less complicated than cells and because the absorbance changes can be interpreted (Section 3.3.2).

Quantization of the results of the chemically induced absorbance changes in B860 Bchl are recorded in Table 3.4.13-1. This data suggests that the B860, which is probably a Bchl-protein complex particle, is denatured in situ and is no longer seen on the membrane fracture surfaces. This could explain the loss of particles from the membrane fracture
Influence of Chemicals on the Membrane Particle Widths in Isolated R-26 R. spheroides Membrane

<table>
<thead>
<tr>
<th>Replica</th>
<th>Treatment</th>
<th>Mean particle width (Å)</th>
<th>Std. dev. (Å)</th>
<th>Total no. of measured particle widths</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4.8% EtOH</td>
<td>88</td>
<td>15</td>
<td>511</td>
</tr>
<tr>
<td></td>
<td>4.8% EtOH</td>
<td>86</td>
<td>14</td>
<td>715</td>
</tr>
<tr>
<td></td>
<td>10^{-2}M O.A. pH 4.7</td>
<td>86</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>4.8% EtOH</td>
<td>82</td>
<td>15</td>
<td>398</td>
</tr>
<tr>
<td></td>
<td>4.8% EtOH</td>
<td>86</td>
<td>15</td>
<td>322</td>
</tr>
<tr>
<td></td>
<td>7.5x10^{-3}M O.A. pH 4.85</td>
<td>86</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>3% Triton</td>
<td>86</td>
<td>14</td>
<td>417</td>
</tr>
<tr>
<td></td>
<td>X-100, 1 hr 0°C centrifugate</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.4124. Influence of chemicals on the membrane mean particle widths in isolated R-26 R. spheroides membrane. Replicas of the same letter were shadowed at the same time. Different letters indicate that the replicas were made in separate experiments.
surfaces. Although the alternate interpretations of particles leaving with the upper fracture face or falling out in the fracturing process are probably of low probability, they cannot be entirely excluded.

Since a fair number of particles remain in the case where about 100% of the B860 (10^{-2} M OA, pH 4.7 and 4.8% EtOH) is denatured, these particles could be RC because high concentrations of OA can leave them undenatured (Section 3.32152). These conclusions will be discussed in more detail in Section 3.525.

Table 3.413-1

R-26 R. spheroides Chromatophores

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent B860 denatured in FFT preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x10^{-2} M MO, pH 7.5, 4.8% EtOH</td>
<td>10 - 33% more like 33%</td>
</tr>
<tr>
<td>pH 4.7, 4.8% EtOH</td>
<td>10 - 30% more like 30%</td>
</tr>
<tr>
<td>7.5x10^{-3} M OA, pH 4.85, 4.8% EtOH</td>
<td>45 - 75% more like 75%</td>
</tr>
<tr>
<td>10^{-2} M OA, pH 4.7, 4.8% EtOH</td>
<td>90 - 100% more like 100%</td>
</tr>
</tbody>
</table>

Chemically induced changes in the fracture plane are not readily related to changes in the absorbance spectrum. 2x10^{-2} M MO, pH 7.5, 4.8% EtOH and pH 4.7, 4.8% EtOH both denature about 30% of the B860 absorbance peak. However their effect on the membrane fracture plane are very different. The MO treated chromatophores fracture with a normal looking fracture face except for a lower particle frequency (figure 3.41-7A, 7B).
The pH 4.7, 4.8% EtOH treated chromatophores have a very uneven fracture plane in figure 3.41-8A compared to the normal case in figure 3.41-8B. Because treatment with $2 \times 10^{-2} M$ MO and pH 4.7 have about the same effect on B860 but different affects on the fracture plane, the absorbance effects are probably unrelated to the changes in the fracture plane.

Nevertheless, the pH 4.7, 4.8% EtOH induced changes in the chromatophore fracture plane is very interesting. It appears that pH 4.7, 4.8% EtOH has created new planes of weak association which the fracture plane has followed. Acid pH should affect the proteins and phospholipids in the membranes to some degree. Perhaps this is the reason for the acid pH induced irregular fracture plane surfaces; accordingly, the B860 absorbance changes could only be an indicator of an acid pH effect on the proteins. This suggests that osmotic salt effects and changing effects might also have similar effects on membrane protein and on the membrane fracture surfaces.
3.5 Studies on Isolated P870 Reaction Center

3.51 Failure of MO to Denature

Although RC is isolated by triton X-100 extraction from isolated R-26 chromatophores, it was necessary to show that RC is denaturation resistant to high concentrations of MO. Showing that the RC Bchl absorbance spectrum is unaltered by MO, unequivocally establishes the MO-induced absorbance changes are in the light harvesting antenna pigment system of wild type and R-26 R. spheroides.

As proof that the RC's P870 is not inactivated by high concentration of MO, three tests of this hypothesis were performed. Isolated reaction center subjected to a concentration of $2 \times 10^{-2} M$, MO pH 7.5 tested the resistance of the Bchl absorbance spectrum to chemically induced absorbance changes. Circular dichroism was used to analyze for changes in the Bchl-Bchl interactions in the RC. Light reaction activity in RC observed by the steady state light induced bleaching of P870 was also not significantly affected by the high concentration of MO.

The conclusion of these experiments is that the RC morphological entity which contains P870 and P800 is not structurally altered by concentrations of $2 \times 10^{-2} M$ MO or less at pH 7.5.

3.511 Absorbance Difference Spectrum

In Figure 3.51-1, the addition of $2 \times 10^{-2} M$ MO and 4.8% ethanol has a very slight effect on the RC absorption spectrum. The loss of absorbance at 865nm due to MO is about 3% of the absorption at that wavelength. The absorption changes at 793nm and 808nm are less than 3% of their absorbance. It is not unexpected that RC absorption spectrum is not significantly affected by very high concentration of MO, since RC is extracted with 1 - 3% triton X-100.
Figure 3.51-1. (upper) Absorption spectrum of R-26 *R. spheroides* reaction center. (lower) Chemical absorbance difference spectrum of R-26 *R. spheroides* reaction center treated with $2 \times 10^{-2} M$ MO, pH 7.5 and 4.8% EtOH, $D(2 \times 10^{-2} M$ MO)$-D$. 
3.512 Circular Dichroism

In order to check that the changes in the Bchl-Bchl associations in the RC were not significantly changed, the CD spectrum of RC in the presence and absence of 2x10^{-2}M MO, pH 7.5 was run. The previously recorded CD spectrum of RC is similar to the untreated one recorded in Figure 3.51-2 (Kenneth Sauer, Edward A. Dratz and Lelia Coyne, 1968). In the same figure the MO-treated RC circular dichroism spectrum is very slightly different from the untreated RC. Experimental reproducibility of the control is not much better than the difference between these spectra. The changes in the circular dichroism spectrum are not considered significant.

3.513 Photoinduced Changes in the RC Absorbance Spectrum

The reversible light-induced bleaching of the 865nm maximum and the blue shift of the 800nm band are unimpaired by the presence of 3 or 20x10^{-3}M MO. IR I in Figure 3.51-3 is the normal absorption spectrum in monochromatic light. The IR II mode passes high intensity white light directly through the preparation then through a monochromator to a phototube. The strong bleaching action of the IR II mode on P870 normally destroys a small percentage of freshly isolated RC. The MO treated preparation is only slightly more susceptible to irreversible photodestruction of the RC Bchl pigments; however the steady state P800 and P870 light-induced changes are unaffected by 2x10^{-2}M MO at pH 7.5.

3.514 Conclusions

These experiments confirm the resistance of the RC absorbance spectrum RC Bchl-Bchl associations and the RC light-induced absorption
Figure 3.51-2. Circular dichroism of R-26 R. spheroides reaction centers in the presence and absence of $2 \times 10^{-2} M$ Methyl Octanoate (MO), pH 7.5 and 4.8% EtOH.
Figure 3.51-3. *R.* *spheroides* reaction centers RC

A) Absorbance spectrum in 4.8% ethanol in the unbleached (IR I mode) and bleached condition (IR II mode)

B) RC in $2 \times 10^{-2} \text{M MO}$, pH 7.5, 4.8% EtOH in the unbleached (IR I mode) and bleached condition (IR II mode)
changes to high concentrations of MO. The RC morphological entity is resistant to structural alteration at concentrations less than or equal to $2 \times 10^{-2} M$ MO, pH 7.5. Therefore, the chemically induced absorbance changes in the visible and near infrared absorbance spectrum of $R. spheroides$ due to MO are not absorbance changes in the RC Bchl. The large changes in the visible and near IR absorbance spectrum induced by MO in the wild type and R-26 cells are due to changes in the light harvesting Bchl and in the carotenoid pigment system.

3.52 Are P870 Reaction Center Particles the Same as Membrane Fracture Face Particles?

This is a difficult question to answer in a direct experimental fashion. The evidence presented in the following section is of the indirect kind, since at present direct observation of inner membrane structure has to be made by replicating the fractured membrane surfaces and by digesting away membrane material adhering to the replica. For this reason, structurally marking the inner membrane particles by Ferritin-coupled RC specific antigens or biosynthetic radioisotope incorporation and autoradiography techniques probably can't succeed. The method chosen for lack of a better approach was the size comparison of RC to the cell and chromatophore fracture face particles.

In order to measure the size of RC particles they first had to be isolated and purified. In the last few steps of the procedure, elution fractions were collected from a Bio-gel agarose 1.5M 100-200 mesh column. A first pigmented fraction, fraction 1, contained particles of about $108^\circ$ and large pieces of chromatophore like material. Separation of the particles from the larger material was accomplished by centrifugation.
The size of these photoactive particles was compared to the R-26 chromatophore and cell membrane fracture face particles.

A second pigmented fraction, fraction 2, from the Agarose Column was studied. Its rate of elution indicates it's of lower molecular weight. Gel electrophoresis indicated the presence of a number of denatured proteins and free pigment. This kind of heterogeneity cannot be eliminated by ultracentrifugation. By analogy to Fraction 1, the particles were assumed to be RC. These were the smallest particles measured. It is possible that they are the same 44,000 molecular weight RC that G. Feher (1971) has isolated.

Certain chemical effects on the membrane were used to advantage in the investigation of the nature of the membrane particles. Since B860 Bchl in R-26 chromatophores can be denatured in situ, effects on the fracture face particles were studied. Under treatment, the particles' sizes remain the same, but the membrane particle frequency is greatly decreased. This kind of behavior is consistent with a RC membrane particle. Chromatophores treated with triton X-100 in the same manner as in the RC isolation were examined by FFT. The membrane fracture surfaces showed unchanged particle sizes and a greatly diminished particle population density. This particle loss behavior is consistent with the extraction of RC from the membranes. These results will be explained in more detail in the following subsections.
3.521 Agarose Gel Column Chromatography of R-26 Reaction Centers

The isolation of R-26 *R. spheroides* reaction center (RC) was accomplished by the method of D. Reed and R. K. Clayton (1968). Two major agarose gel chromatographic fractions, designated fractions I and II, were examined. Only fraction I could be purified by centrifugation.

3.5211 Characterization and Purification of Fraction I (F1)

In the chromatographic separation of RC by Bio-gel A 1.5 m agarose 100-200 mesh, the first 16 effluent fractions contained the most reaction center pigment peaks (800nm, 860nm) to pigment contaminate peaks (757nm, 683nm). These samples were combined into fraction I because the RC yield was so low. Preliminary electron microscope experiments on this sample indicated that the RC preparation was a heterogenous mixture of particles and large membrane fragments. The heterogenous nature of the preparation did not interfere with the MO chemical difference spectra and photoinduced changes with MO present. This homogeneity made it impossible to localize the RC.

3.52111 Ultracentrifugation

To determine whether RC was associated only with large membrane fragments and/or small particles, sedimentation velocity studies were made on the crude preparation. When the RC preparation was tested in the presence of 0.5-2.0% triton X-100 in 0.01 M tris at pH 7.5, a high molecular weight material sedimented immediately followed by two species with sedimentation coefficients of *S*_{20\,^\circ C} = 5.9 S and *S*_{20\,^\circ C} = 2.8 S. When the RC preparation was diluted 10 fold with 0.01 M tris pH 7.5, all the material sedimented, leaving only a trace of contaminant pigments, absorbing
at 757 nm and 683 nm. When the RC preparation was diluted 10 fold with 0.5% triton X-100 in 0.01 M tris pH 7.5 and precentrifuged in the preparative centrifuge for 1 hour at 150,000 x g, ultracentrifugation of this sample gave a single pigmented and sedimenting band of $S_{20\,\text{w}} = 2.3\,\text{S}$. Since green light between 510 and 555 nm in the schlieren optical system was absorbed by the sedimenting band, we know it was pigmented. The $S$ value of 2.3 S for an RC particle can be used to set an upper and lower bound on the RC's molecular weight. If one uses the equation

$$\frac{\text{NS} \left[ n \right]^{1/3} n}{1 - \bar{V}_2 p_o} = (100)^{1/3} B' M^{2/3}$$

(C. Tanford, 1961) where $B' = 2.12 \times 10^6$ for solid spheres and picks $\bar{V}_2 = .83\,\text{cc/gm}$ for micelles of 50% triton and 50% RC and $\bar{V}_2 = .91\,\text{cc/gm}$ for micelles of 90% triton and 10% RC which is almost pure triton X-100 ($\bar{V} = .93\,\text{cc/gm}$). One can bracket the range of possible molecular weights.

<table>
<thead>
<tr>
<th>Partial Specific Volume $\bar{V}$ cc/gm</th>
<th>Molecular Weight gms</th>
</tr>
</thead>
<tbody>
<tr>
<td>.76, $\bar{V}_{\text{RC}}$</td>
<td>33,000</td>
</tr>
<tr>
<td>.83, .5 $\bar{V}_{\text{RC}} + .5,\bar{V}_T$</td>
<td>45,000</td>
</tr>
<tr>
<td>.91, .1 $\bar{V}_{\text{RC}} + .9,\bar{V}_T$</td>
<td>102,000</td>
</tr>
<tr>
<td>.93, $\bar{V}_T$</td>
<td>166,000</td>
</tr>
</tbody>
</table>

A reasonable estimate of the upper and lower limits of RC's molecular weight are given in Table 3.5211. A range of 33,000 to 102,000 is not very different from the values estimated by G. Feher (1971) from gel electrophoresis with SDS.
3.521111 Photoinduced Absorbance Changes

The ultracentrifuged sample which showed the one $S_{20^oC} = 2.3$ S material was irradiated for P800 and P870 reaction center activity. The entire 865nm band was reversibly photo-bleachable and the coupled 800nm peak was reversibly blue shifted (Figure 3.52-1). If the preparation was exposed to high intensity light for about 10-30 minutes, RC pigments at 800nm and 865nm were destroyed and converted into 757nm and 683nm peaks. Upon ultracentrifugation, this photoconverted material displayed a pigmented sedimentation peak (green light absorbing) of $S_{20^oC} = 2.3$ S. These results suggest that the contaminant peaks at 757nm and 683nm can be associated with a particle of about the same shape and molecular weight as the reaction center. Although, the reaction center bacteriochlorophyll pigments have been chemically changed, the gross shape and weight of RC protein shell remains unchanged.

3.521112 Gel Electrophoresis

The centrifuged sample which showed one sedimentation band of 2.3 S was checked for homogeneity by polyacrylamide gel electrophoresis using the techniques of Davis and Clarke (1964). In Figure 3.52-2A, the purified F1 preparation gave only one brown colored band which also stained as protein. There was no other pigment band or protein band. This result confirms the ultracentrifuge results. The photoactive RC and photooxidized RC are not distinguishable in the ultracentrifuge. Their electrophoretic mobilities in polyacrylamide gel are also indistinguishable. Even though these two species are chemically different, there should be no difference in their size and shape.
Figure 3.52-1. Centrifuged R-26 *R. spheroides* Reaction Center, F1.

IR I - The absorption spectrum using low intensity monochromatic light

IR II - The absorption spectrum in the presence of high intensity white light which bleaches P870 and blue shifts P800 in the RC
Figure 3.52-2A. Isolated and purified R-26 *R. sphaeroides* reaction center centrifuged F1 gives a single band B that contains the pigment and stains black because it also contains all the protein. There is very little material left at the origin (0). Not all the blue protein stain has been removed. 0.5% triton X-100 is present in the sample. A buffer of 0.01 M tris, pH 8.6 was used during electrophoresis.

Figure 3.52-2B. The last R-26 *R. sphaeroides* reaction center fraction F2 taken from the Bio-gel A 1.5 M agarose column. There are two unstained brown-colored bands C. There are at least seven protein bands in the stained column where at least five are associated with the colored bands. Some material is left at the origin O. This is the low molecular weight effluent fraction from the agarose column. 2-5% triton X-100 is present in the sample. A buffer of 0.01 M tris, pH 8.6 was used during electrophoresis.
3.521113 Electron Microscopy

The crude F1 Agrose column fraction was prepared for electron microscopy by shadowing it on a carbon substrate and by FFT. In Figure 3.52-3A, a large piece of membrane like material surrounded by smaller less distinct particles can be seen. It was necessary to purify this crude fraction in order to identify the RC.

A shadowed preparation of the centrifuged RC is shown in Figure 3.52-3B. Since the RC dried in small groups of particles and individually, this supposedly homogeneous preparation looked heterogeneous. FFT proved useful in overcoming this difficulty. Before FFT of the centrifuged RC is discussed; FFT of the crude F1 RC preparation should be examined. The triton X-100 present in about 0.5-2% concentration forms an eutectic structure similar to glycerol. This eutectic triton X-100 (T) freezes out of solution in interconnected sheets, rods, and larger globs. These eutectic triton X-100 (T) structures fracture in a manner different from membranes and different from triton X-100 treated membranes (Figure 3.52-7B). The eutectic triton X-100 structures normally fracture along an ice-T interface. If the fracture plane travels within the eutectic triton X-100 material, it does so along a randomly chosen surface. However, the fracture plane normally travels along an ice-T interface. Unfortunately the sheet of eutectic triton X-100 in Figure 3.52-3C is about the thickness of a membrane. This picture demonstrates the fracture surface behavior of a sheet of triton (T) and an adjoining rod of triton (T). The same behavior is seen in fraction 2 which contains even more triton X-100. The particles collect in the T layers since it is the triton that keeps them suspended in solution. According to a recent finding very fast freezing of the
Figure 3.52-3A. R-26 *R. spheroides* crude reaction center preparation.
The first 16 fractions isolated from a Bio-gel agarose A 1.5 M column contained pieces of membrane like material (M) as well as small particles (P) that can be seen near the membrane like material. The preparation was put on a carbon film and was shadowed with platinum.
(Magnification 80,000x)

Figure 3.52-3B. R-26 *R. spheroides* purified reaction center dried on a carbon film and shadowed with platinum. Some of the particles (P) appear to have clumped, destroying the homogeneous appearance. The majority of particles appear to be the same size as those in Figure 3.52-4C.
(Magnification 200,000x)

Figure 3.52-3C. R-26 *R. spheroides* crude reaction center prepared by FFT without etching. Particles (P) are embedded in the eutectic triton X-100 (T). Unfortunately, the concentration of the eutectic triton X-100 forms sheets about the thickness of a membrane. Eutectic triton normally fractures along an interface between ice and triton, or it sharply fractures through the triton X-100 to the opposite ice-triton X-100 interface.
(Magnification 200,000x)
Figure 3.52-4A. 0.5% triton X-100 and aqueous 0.01 M tris, pH 7.5.
This picture shows an internal freeze-fractured face without etching. Ice (i) surrounds the eutectic triton X-100 (T) fracture surface. There are no particles formed by fracturing triton X-100.
(Magnification 400,000x)

Figure 3.52-4B. 0.5% triton X-100 and 0.01 M tris, pH 7.5.
This picture shows a fracture face without etching. Ice (i) surrounds the eutectic triton X-100 (T) fracture surface. There are no particles formed by triton X-100 alone.
(Magnification 400,000x)
The large arrow indicates the shadow direction.

Figure 3.52-4C. Purified R-26 R. spheroides reaction centers frozen in 0.5% triton X-100 and 0.01 M tris, pH 7.5.
The particles (P) are not an artifact of triton fracturing, and they are the same size as the ones seen in the crude reaction center preparation. This preparation shows an internal freeze-fractured face without etching [ice (i)].
(Magnification 400,000x)
samples could eliminate the eutectic triton X-100. Fast freezing can produce a homogeneous glass in which embedded particles can be seen dispersed evenly throughout (L. Bachman and A. B. Schmitt, 1971).

Nonetheless, the particles in the eutectic triton X-100 preparation appear to be homogeneous and are easy to measure.

The centrifuged F1 RC preparation of 0.5% triton X-100 was frozen forming very thin triton sheets, rods and isolated spherical globules.

It is shown in Figure 3.52-4 that fractured 0.5% triton X-100 does not create its own fracture face particles. The particles in Figure 3.52-4C are genuine RC particles. The particle width versus frequency distributions of these preparations are included in Section 3.523, where they can be compared with the Fraction II particles.

3.5212 Characterization of Fraction 2 (F2)

A second major RC fraction was examined from the Biogel A-1.5 m agarose column. It was the 18th and last pigmented fraction eluted from the column. Fraction 2 (F2) was richer in contaminant absorption peaks (757nm and 683nm) to RC absorption peaks (800nm, 860nm) than was fraction I (F1). This fraction could not be purified by centrifugation.

3.52121 Ultracentrifugation and Photoinduced Absorbance Changes

Ultracentrifugation of F2 at the highest speed and gravity did not reveal any sedimenting material. Probably because the triton X-100 (ρ = 1.08 cc/gm) concentration was 4 to 10 times greater than in F1 and its greater association can decrease the density of the particles preventing their sedimentation. The RC reversible photoreactions were still present even though there was no sedimenting material (Figure 3.52-5).
Figure 3.52-5. Absorption spectrum of R-26 *R. spheroides* RC, F2.

IR I Absorbance under low intensity monochromatic light

IR II Absorbance under high intensity white light which bleaches the P870 and blue shifts P800 in RC
3.52122 Gel Electrophoresis

Since ultracentrifugation didn't improve our understanding of the homogeneity of this preparation, the F2 preparation was examined by electrophoresis. The F2 preparation is a mixture of seven or more proteins. There are two light brown bands in the unstained preparation marked C. The upper one contains free pigment and also numerous protein bands. The lower pigmented band only has one protein band. Since denatured protein can't move as quickly in polyacrylamide gel as a globular protein not differing greatly in charge, it is reasonable to assume that the lowest pigment-protein band is probably reaction center. This can easily be substantiated by cutting the band out of the gel. This could prove very convenient in purifying the RC in the future.

3.52123 FFT Electron Microscopy

The F2 fraction was prepared by FFT. The concentration of triton X-100 in F2 was 4 to 10 times greater than in the purified RC preparation, which was 0.5% triton X-100. This fraction was concentrated by pressure dialysis. The eutectic triton X-100 fractured just as it did in Figure 3.52-3C, i.e., along an ice eutectic triton X-100 interface or in cross section (Figure 3.52-6A). Particles (P) can be seen embedded in the eutectic triton X-100 in Figure 3.52-6B. It seems reasonable to identify the particles in F2 as RC by analogy to F1, even though F2 has other protein and pigmented materials present. The particle sizes in this preparation will be compared with the F1 RC in the following section.
Figure 3.52-6A. R-26 *R. spheroides* RC in the last colored fraction taken from the Bio-gel A 1.5 m agarose column. E. M. shows there is more eutectic triton X-100 (T) present than in the crude or the purified F1 RC preparation. The eutectic triton X-100 rarely fractures extensively along an internal plane, but the fracture plane normally follows the eutectic ice-triton X-100 interface. This preparation has been freeze-fractured without etching [ice (i)].

(Magnification 40,000x)

Figure 3.52-6B. R-26 *R. spheroides* RC in the last colored fraction taken from the Bio-gel A 1.5 m agarose column. Particles (P), probably RC, are embedded in the surface of a eutectic triton X-100 layer. This preparation has been freeze-fractured without etching [ice (i)]. There is about 2 to 5% triton X-100.

(Magnification 200,000x)
3.522 Similar response of R-26 Chromatophore Fracture Face Particles and Reaction Center to Chemical Treatment

3.5221 Treatment with $2 \times 10^{-2} \text{M} \ MO$, pH 7.5, 4.8% EtOH

$2 \times 10^{-2} \text{M} \ MO$, pH 7.5, 4.8% EtOH does not denature RC (Section 3.51). This MO treatment does, however, denature about 30% of the B860 Bchl in the R-26 chromatophores. The chromatophore fracture faces show a diminished particle population density, an assumed unchanging particle size and the continued presence of inner membrane particles which is consistent with RC's presence there.

3.5222 Treatment with $10^{-2} \text{M} \ OA$, pH 4.7, 4.8% EtOH and $7.5 \times 10^{-3} \text{M} \ OA$, pH 4.85, 4.8% EtOH

In Section 3.321522 there is evidence that the chromatophore associated RC is undenatured by these concentrations of OA. The highest concentration of OA denatures about 100% of the B860 Bchl. The lower OA concentration denatures about 75% of B860. The membranes fracture in an abnormal fashion: the membrane fracture face particle population density decreases, and the particles remain unchanged in size. That particles remain on the chromatophore fracture faces and that they are unchanged in size is consistent with an inner membrane RC particle.
3.5223 Treatment with 3% Triton X-100

R-26 chromatophores were treated with 3% triton X-100 under the same condition in which RC is solubilized from the chromatophores. The treated and centrifuged chromatophores were prepared by FFT. (Figure 3.52-7). This preparation showed a dramatic decrease in the fracture face particle population densities and no change in the particle sizes (Section 3.4124). Of incidental interest is the change in the membrane fracture face texture. The unchanging particle size and the tremendous decrease in the frequency of the fracture face particles correlates with the loss of RC from the chromatophores by extraction. Their unchanged particle sizes is to be expected if RC is undenatured by triton X-100. Again the results correlate with the behavior of an inner membrane RC particle.

3.523 Size Comparison of Reaction Centers with Particles on the Chromatophore Fracture Faces and with Particles on the Cell Membrane Fracture Faces

It should be remembered that the RC and membrane particles are measured orthogonally to their shadowed direction. The uneven surfaces of the fractured membranes made other types of measurements such as shadow lengths and particle widths parallel to the shadow direction unreliable. This orthogonal measurement of width has its own peculiarities which will be made clear in Appendix 5.2.

It was shown, quite convincingly in Section 3.4124, Table 3.4124 that OA and triton X-100 treatment do not change the size of the membrane
Figure 3.52-7A. Isolated R-26 *R. spheroides* chromatophores fracture surface (Mf) frozen in aqueous 0.01 M tris at pH 7.5, and 4.8% ethanol.

There was no etching of the ice (i) and there are no more particles (P) per unit area than in Figure 3.52-7B (Magnification 120,000x)

The large arrow indicates shadow direction.

Figure 3.52-7B. Isolated R-26 *R. spheroides* chromatophores fracture surface (Mf) frozen in aqueous 0.01 M tris pH 7.2 without etching and treated with 3% triton X-100 and stirring at 0°C for 1 hour.

The chromatophores were separated from excess triton X-100 by centrifugation at 250,000 x g for 90 minutes. There are fewer particles (P) per unit area in B than in Part A. (ice (i)).

(Magnification 100,000x)

The large arrow indicates shadow direction.
particles. Based upon this evidence, FET and FFT preparations of each system whether chemically treated or not were given equal importance in a histogram (Figure 3.52-8). Except for the chromatophore results listed in Table 3.4.124 each FFT or FET preparation was replicated separately. The spread of the mean particle widths is surprising. For the membrane fracture face particles of cells the means vary from 85Å to 107Å. For chromatophores and RC the means vary from 82Å to 108Å and 80Å to 108Å respectively. The spread in the means can be 22Å to 28Å. The extremes in the mean sizes are a result of shadowing variations. The larger means are found in the heavily shadowed, contrasty replicas and the smaller means are found in the lightly shadowed, uncontrasty replicas.

The average mean of the means taken from Figure 3.52-8 are given in the following table along with their uncertainties.

<table>
<thead>
<tr>
<th></th>
<th>Average Mean</th>
<th>Uncertainty</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-26 R. spheroides RC</td>
<td>99 Å</td>
<td>± 19 Å</td>
</tr>
<tr>
<td>R-26 R. spheroides chromatophores (isolated membrane)</td>
<td>92 Å</td>
<td>± 16 Å</td>
</tr>
<tr>
<td>R-26 and wild type R. spheroides cells</td>
<td>98 Å</td>
<td>± 13 Å</td>
</tr>
</tbody>
</table>

Within their precision, the average mean for each type of preparation are the same. This similarity of mean sizes is not inconsistent with the hypothesis that RC is an internal membrane particle. There is additional support for this hypothesis presented in the next section.
Figure 3.52.8. Histogram of particle width means for preparations measured from individual replicas.

Particle widths were measured perpendicular to shadow direction either on a membrane fracture face or on the fracture face of eutectic triton X-100.
3.5231 Comparison of Particle Widths Measured by Other Methods and Conclusions

G. Fehers (1971) isolated and characterized RC from R-26 R. spheroides. His smallest room temperature photoactive RC had a molecular weight of 44,000 whereas he estimated a larger complex containing RC could have a molecular weight of 70,000 with an upper most molecular weight of 100,000.

If $\bar{V} = .76$ cc/gm, estimated for the Bchl-protein complex in C. ethylicium is used, a RC spherical diameter can be estimated. Values of the diameter for $\bar{V} = 1$ cc/gm are included as an upper size limit and a demonstration of how weak a function the diameter is with respect to its partial specific volume, $\bar{V}$ or density.

<table>
<thead>
<tr>
<th>RC, Molecular weight, gms.</th>
<th>$\bar{V} = .76$ cc/gm</th>
<th>$\bar{V} = 1$ cc/gm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>diameter Å</td>
<td>diameter Å</td>
</tr>
<tr>
<td>44,000</td>
<td>48</td>
<td>52.5</td>
</tr>
<tr>
<td>70,000</td>
<td>55</td>
<td>60</td>
</tr>
<tr>
<td>100,000</td>
<td>64</td>
<td>70</td>
</tr>
</tbody>
</table>

An acceptable theoretical estimate of the size of RC would be as low as $48\text{Å}$ and as high as $55\text{Å}$.

Negative staining of R. rubrum chromatophores has indicated the presence of a $50\text{Å}$ membrane particles (Low and Afzelius, 1964; S. C. Holt and A. G. Marr, 1965).

X-ray diffraction studies of R. spheroides chromatophores show a diffuse reflection in the neighborhood of $50\text{Å}$, which may be due to a substructure of about that size (R. Langridge, P. D. Barron and W. R. Sistrom, 1964).
Now the shadowed preparations gave particle widths generally much larger than the estimated RC diameter of 48Å-55Å and the negative stained membrane particles of 50Å. The difference between the shadowed preparations and a 50Å particle compute the estimated increase in particle widths due to shadowing. Using the average shadowed particle means in Section 3.523, the increase in the RC size is 49Å ± 19Å. For the shadowed membrane fracture face particles, the increase in width is 42Å ± 16Å.

These values are in agreement with C. F. Hall's (1960) findings. He calculated the increase in widths of shadowed globular proteins by comparing particle dimensions computed from ultracentrifugation results and particle heights calculated from shadow lengths. He found in increase in the particle shadow width of 68Å ± 12Å for his shadowing geometry and for his particular experimental conditions. The distortion in particle widths due to platinum-carbon shadowing is considerable in his experiments as well as ones included here. Also in support of the shadowing size increase of RC is the fact that RC can be shown to occur as monomers in the RC preparation by polarized fluorescence measurements (T. G. Ebrey and R. K. Clayton, 1969). Under normal platinum-shadowing conditions the particles probably experience an approximate doubling of size, and for this reason the membrane particles and the RC particles are very likely about 50Å in size.

3.524 Relation of Reaction Center to the Membrane Fracture Face Particles

There are two pieces of indirect evidence which relate to the reaction centers to inner membrane particles. The first is the size similarity between reaction centers and chromatophore fracture face particles and cellular cytoplasmic membrane fracture face particles.
RC is 99Å±19Å and the others are 92Å±13Å, respectively. These particles are the same width within the precision of measurement. By comparison to measurements other than by EM in Section 3.5231, the mean particle widths have been increased by about 42-49Å by shadowing. By subtracting the shadowing increase the RC and the membrane fracture face particles are about 50Å in size. This particle size could easily fit within a membrane of less than 100Å. The response of the chromatophore fracture face particles to MO, triton x-100 and especially 10⁻² M OA, pH 4.7 indicate these treatments do not denature all the membrane particles. Since 10⁻² M OA pH 4.7 and 4.8% EtOH denatures all of the B860 and since it does not readily denature chromatophore bound RC, the continued presence of chromatophore fracture face particles and its unchanged size are consistent with RC as an internal membrane particle. This is reasonable because RC are isolated from chromatophores and because RC is very resistant to denaturation by chemical oxidants (T. Kuntz et al. 1964) and triton x-100 extraction. An inner membrane location would protect RC from denaturants and it would allow space for the electron transport intermediates to connect it through the membrane.
4 Discussion and Conclusions

4.1 Analysis of Results

4.1.1 Efficacy of OA and MO as Denaturants and as Reversible Denaturants

The efficacy of OA and MO as denaturants will be judged in these studies by the solution denaturant concentration and by induced changes in some observable function or structure. Treating cell and subcellular systems as black boxes with an input and an output allows a level of comparison without assessing the denaturant concentration within the cells or subcellular systems. Assessment difficulties arise because OA and MO are only slightly water soluble. Their system concentration is enhanced by their membrane solubility and diminished by their ability to self aggregate. Denaturant permeability into cells should be mentioned as well, because it can greatly reduce or enhance the denaturant concentration acting in a whole cell. The efficacy of OA and MO as denaturants has to be qualified in terms of their solution concentration and the sensitivity of some observable structure or function to this concentration.

T. A. Pederson, M. Kirk and J. A. Bassham (1966) evaluated the effects of MO and OA on the CO₂ fixation cycle and its specific enzymatic machinery in *Chlorella pyrenoidosa*. 3x10⁻⁴ M OA, pH 5 inhibited 86% of O₂ evolution and CO₂ fixation and these effects were about 60% reversible. 6x10⁻⁴ M MO, pH 5 inhibited photosynthesis; nonetheless centrifugation followed by resuspension returned about 60% of the photosynthetic activity. Another study in this vain was the study of MO inhibition of photosynthetic growth in the wild type *R. spheroides* (Section 3.1111). A concentration of 5x10⁻⁴ M MO partially inhibited cell growth (100 MO/Bchl) but 1x10⁻³ M MO was 100%
effective in cell growth inhibition (200 MO/Bchl). These experiments attempted to gauge the value of MO as a denaturant by measuring inhibition in the complex cellular enzymatic system for cell growth.

The next type of experiments monitored the membrane structure by measuring the effects of MO and OA on the spectral properties of the photosynthetic pigments and by monitoring the FFT membrane fracture face substructure. Pigment changes were very sensitive to MO and OA ($10^{-4}M$). The FFT membrane changes required high concentrations of MO and OA for their effects to become visible.

A number of experiments were performed on the effects of MO and OA on the wild type cells and chromatophores. Two new variables showed themselves in these experiments. It was found that a fixed concentration of MO could have a weak or strong effect depending on whether the concentration of cells or chromatophores was large or small respectively. Essentially all the OA or MO is membrane associated (Section 3.32131). The proper measure of MO's or OA's effectiveness is a mole ratio of the compounds to the hydrocarbon content of the chromatophore or cell system. In the absence of such a measurement the ratio of these denaturants to Bchl content was used as a relative measure of hydrocarbon content. To achieve the same effect, this ratio of course is greater for cells than for chromatophores. Second, there was a time dependent effect. Normally within half an hour MO and OA effects had completed their rapid phase of formation. Unfortunately, this long wait for the denaturant induced pigment changes also creates irreversible changes. There is an upper time limit on the reversibility of the denaturant-induced pigment changes. This was the case with the $1.5 \times 10^{-3} M$ OA, pH 5 experiment with wild type cells in
modified Hutner's. If a time period longer than twenty minutes was exceeded, the absorbance changes were not reversible by pH neutralization. If the experiment was performed rapidly, the absorbance changes could be reversed and the cells could be used as inoculum for new cultures.

When experiments with R-26 chromatophores were being investigated it became clear that $10^{-2}$ M OA at pH 4.7 could denature B860 Bchl completely. However, $2 \times 10^{-2}$ M MO at pH 7.5 could only at best denature 1/3 of the B860 Bchl. This difference in effect made it imperative to investigate the effect of acid pH. Acid pH by itself is an ineffective denaturant of B860. Acid pH combined with MO proved to be almost as effective as OA at acid pH. There is definitely a cooperative effect between acid pH and MO or OA in denaturing B860 (Section 3.3.23).

Reversibility experiments with the denaturants and chromatophores met with better success than any other system. It was found by chance that centrifugation at 250,000-300,000 x g followed with resuspension at normal pH could remove as much as 80% or more of OA (Section 3.3.21) or reverse the B860 absorbance changes by as much as 65-75% at lower OA concentrations. It seems clear that the denaturants can be removed by 80% or more. Their effects, however, may remain behind in greater or lesser degree depending on denaturant concentration, pH, and the length of denaturant-system contact time.

The effect of denaturants on the electron transport intermediates was limited to a cytochrome b and a cytochrome c. These effects began to occur with concentrations in the range of $10^{-3}$ M. Again low pH and OA were more effective than MO at normal pH. Even acid pH alone had an effect on cytochrome b.
Because changes on a molecular level occur long before the FFT changes in the membrane are evident, the effect of the denaturants on the FFT of chromatophores was only observable at high concentrations in about $7.5 \times 10^{-3} \text{M}$ range. As a rule of thumb it was assumed that changes in the pigments were a more sensitive measure of membrane changes. This, however, may not be the case with acid pH. The absorbance changes are small, but the membranes fracture in a greatly changed manner. Very likely, FFT may be a more sensitive measure of pH changes on the membrane than the pigment changes.

The effectiveness of OA and MO as denaturants is very great under the proper conditions. Although the reversibility of their effects is not 100%, this may not be important. That they can be made reversible during the time scale of the experiment allows the membrane changes to be related to the original *in vivo* membrane. Reversible denaturants are the bridge over which structural alterations can be related to the unchanged parent structure.

4.12 Bacteriochlorophyll at 860nm, Revealed by B860 Denaturation

The CD study by E. Dratz, A. Schultz and K. Sauer (1966) on Bchl and Chl dimers in photosynthetic organisms indicated that a very large part of these pigments are aggregated *in vivo*. In the chromatophores this aggregation is probably more complex than just the dimer form. The authors interpreted their results in terms of the presence of little to no Bchl monomer in *R. sphaeroides* chromatophores. Even though Chl monomers may exist in the chloroplasts, Chl doesn't undergo as large an *in vivo* red shift as the Bchl does. Because large aggregates of Chl can be red shifted 80nm,
it's possible that aggregates of Bchl are responsible for the 30nm-120nm red shifts in *R. spheroides* Bchl. Even though Bchl aggregates may cause large red shifts, this explanation was insufficient in explaining the diversity of the near IR Bchl absorbance peaks or their differential susceptibility to OA denaturation. This kind of behavior points to the existence of perhaps large Bchl-protein interactions. Certainly the variety of the cellular Bchl peaks could be explained by assuming a variety of protein associations.

The titration of the B860 Bchl peak in the R-26 chromatophores with OA and pH proved very interesting. At 79% denaturation of B860 a Bchl monomer was found at 860nm in the CD spectrum. Definitely other interactions than Bchl-Bchl type produced the monomer absorbance at 860nm. H. Steffan and M. Calvin (1970) interpreted the presence of the monomer at 860nm as a Bchl-protein interaction because the Bchl could be reaggregated by centrifugation and pH neutralization. Perhaps the Bchl-protein interactions is responsible for the large *in vivo* infrared shifts seen in all photosynthetic bacteria.

4.13 Structure of the Wild Type and R-26
*R. spheroides* Cells

A comparison is made between the conventional fixing, plastic embedding, sectioning and post-staining technique and the freeze-etch technique. The general structural features visible by these two techniques are similar. Freeze-etch is less likely to distort the cell wall or the membrane microstructure than the conventional techniques. Using freeze-etch, the membrane anatomy of cells can be identified by their characteristic ridges of 80-150Å and the characteristic inner membrane fracture surfaces. Although, the membranes are more evident in preparations using
the conventional technique, cells frozen in distilled water can show all the same features. Freeze-etch should show these features with less distortion, in addition to new information about inner surfaces of fractured membranes.

The freeze-etch technique has shown that the R-26 mutant of *R. spheroides* differs in cell structure from the wild type, in addition to lacking carotenoids and having a different near infrared Bchl absorbance spectrum. The mature R-26 cells contain a polar stack of three membranes, which don't exist in the wild type. The young R-26 cells don't show these membranes and they do not have much vesicular membrane.

R-26 *R. spheroides* is not a point mutant, it must differ from the wild type in at least three or more genes.

4.14 Correlation of MO and OA induced Absorbance Changes with FFT and FET changes in *R. spheroides*.

Correlation of MO and OA induced absorbance changes with FFT and FET changes in *R. spheroides* was the primary objective of this study at its inception. Correlation of $10^{-3} M$ MO and $1.5 \times 10^{-3} M$ OA, pH 5 absorbance changes in wild type cell with FET changes met with uncertain success. The normal membrane fracture planes in untreated and pH 5 treated cells were rarely found in the $1.5 \times 10^{-3} M$ OA, pH 5 Treated Cells. No changes in the $10^{-3} M$ MO treated cells were detected. $1.5 \times 10^{-3} M$ OA, pH 5 treated cells increased the frequency of the cellular fracture planes traveling outside of the membranes. Conclusions about denaturant induced changes in the membrane were uncertain. The possibility that the denaturants were creating nonmembranous fracture planes without changing the membranes could not be ruled out. Chromatophore systems were then tried because
their FFT structure was easier to interpret. At the same time experiments were begun on the R-26 mutant cells because its simpler absorbance spectrum made absorbance changes easier to interpret. R-26 chromatophores were used exclusively in the following experiments. It was found that centrifugation at about 150,000 x g and greater was necessary to concentrate the chromatophores for the FFT but this step also removed MO and OA. This potential difficulty could not be resolved by eliminating the centrifugation step because in the FFT process an MO eutetic froze out of solution allowing no membrane fracture faces to be seen. It was felt that correlation experiments should only be carried out at very high denaturant concentrations where reversibility of absorbance changes was slight. Using 2x10^{-2}M MO only about 1/3 of B860 was denatured. The only change that could be found was a diminished particle frequency on the chromatophore fracture faces.

The most successful experiment was done with 10^{-2}M OA, pH 4.7 and 7.5x10^{-3}M pH 4.85 and pH 4.7 treated chromatophores. Under these conditions the B860 absorbance was 100%, 75% and 30% respectively denatured. Upon concentration by centrifugation, and resuspension at neutral pH the denaturation of B860 was slightly reversed. B860 denaturation probably remained about 100%, 75% and 30% respectively. Acid pH of 4.7 had a big effect on the texture of the membrane fracture face. The fracture plane traveled not only within the membrane but also appeared to travel along its outside surface at times. When a 10^{-2}M OA pH 4.7 and 7.5x10^{-3}M OA, pH 4.85 treated membrane could be found with an internal fracture plane, there was a big loss in the fracture face particle frequency. The significance of these results is presented in Section 4.15.
4.15 Changes in the Association of Membrane Substructure Observable by Electron Microscopy

There are three parameters which can be observed in freeze-fractured chromatophores. Although these parameters are explained in detail in Appendix 5, it's worth mentioning them again.

1) The location and texture of the membrane fracture surface
2) The particle population density on the membrane fracture surface
3) The particle widths orthogonal to the platinum shadow direction

No changes in particle sizes were evident in all the experiments.

Particle frequencies did decrease on the fracture surfaces under chemical treatment. These changes in the particle population are tricky to interpret because biological variability such as age and prior treatment, also influences them. Particle loss from the membranes might occur by a mechanical mechanism because of the violence of the fracturing process. Although it doesn't seem too likely, it is possible that particles lost from the chemically treated membranes may be carried away with the upper fracture surface. Normally, particles don't fall out of the membranes onto the ice (Figure 3.41-9A). This behavior has only been seen once!

A more satisfactory interpretation is based on the evidence that B860 is denatured \textit{in situ} by MO and OA. Denaturation of the particles is a more satisfactory explanation of particle loss mechanism, although the other interpretations can not be rigorously excluded.

The location and the relief texture of the membrane fracture plane is very sensitive to low pH. pH 4.7 causes the chromatophores to fracture randomly within the membranes and sometimes along their outer surface. Acid pH should very strongly affect the proteins. If indeed the normal
fracture plane is through the lipid region of the membrane, then weakening the protein interactions with acid pH should shift the fracture planes through protein regions as well. The composition of the chromatophores is about 66% protein and 33% lipid (A. Gorehein, A. Neuberger and G. H. Tait, 1967; R. Schmitz, 1967).

U. Goodenough et al (1971) observed a salt effect on the lamellar system of chloroplasts. The chromatophores are also probably sensitive to ionic strength. The high salts may allow the fracture plane to travel through a more proteinacious part of the membrane. This effect did show a change in particle sizes in the lamellar system membrane fracture faces.

FET and FFT are very useful for observing chemically induced membrane changes. The freeze fracturing properties of the membranes are sensitive to changes in the intermolecular association properties of the membrane substructure.

4.16 Significance of Membrane Fracture Face Particles

It has been well established that membrane fracture face particles are not artifacts caused by external or internal ice crystals associated with hydrated fracture surfaces (D. W. Deamer and D. Branton, 1967; D. W. Deamer et al, 1970). Therefore it is worth reviewing the candidates for membrane particles and the evidence for them. In R-26 R. spheroides the candidates are:

1) Reaction center
2) Antennae Bchl-protein complex, B860
3) Cytochrome aggregates in the photosynthetic electron transport system
4) Cytochrome aggregates in the oxidative electron transport system

5) Unknown particles

The wild type membranes probably contain the three antennae Bchl-protein complexes, B800, B850, and B890. The best understood of all of these are the RC and the B860 complex; the least understood candidates will be discussed first and the better understood will be treated last.

A cytochrome aggregate particle size in the photosynthetic electron transport chain depends on the concentration of the cytochromes and their position on the transport chain. A Cyt c should be closely associated with the RC because it feeds electrons directly to P870+. The cytochrome b is probably on a membrane surface location because it is more readily changed by MO and various oxidants (O₂, DMSO, etc.) than the Cyt c. If there is more than one cytochrome closely associated in either location, they could make a particle of 60Å to 80Å in at least one dimension because most cytochromes are about 35Å in diameter.

The oxidative metabolism system is characteristic of most of the Athiorhodacae but not of the Thiorhodacae. For a long time it was believed that the photosynthetic electron transport system and the oxidative metabolism system shared a common electron transport system (Van Niel, 1941). This theory no longer has the support that it did have (J. Oelze and G. Drews, 1972), because of the presence of all the nonphotosynthetic component cytochromes for the oxidative system (see Section 1.22311). A system of cytochromes such as are present in the oxidative system could be constructed as a linear aggregate of cytochromes which function as a membrane crossing electron pump. This kind of construction fits the
molecular model of the oxidative electron transport system and it also explains the ubiquitous presence of electron transport membrane fracture face particles.

4.161 Case for a B860 Particle and Its Possible Location

An antenna Bchl-protein, B860, particle seems highly certain. B860 Bchl denaturation by acid and OA and its renaturation by base neutralization and centrifugation are consistent with a Bchl-protein complexes. The complex can also explain the Bchl monomer at 860nm in the presence of OA at acid pH.

D. Reed et al (1970) claim to have seen a negative stained 50Å particle in a concentrated heterogeneous preparation of B860. This particle would have about the right size for an inner membrane particle. D. Reed's B860 preparation aggregated if the detergent concentrations fell below a certain minimum value. This could indicate low water solubility for B860. It's not unlikely that B860 is not as water soluble as the Bchl-protein complex isolated from C. ethylicum (J. Olson 1966, 1969). This complex is about 80Å in diameter and is easily washed from the membranes. There are no particles of about the right size seen on the outside edges of the cytoplasmic or vesicular membranes fractured in cross section.

The antennae Bchl in wild type R. spheroides chromatophores, as well as other types of chromatophores, can be greatly oxidized without destruction of RC Bchl (Kuntz et al., 1964). Since B860 can be denatured in a similar manner with $10^{-2}$ M OA, pH 4.7 without denaturation of RC Bchl, a B860 location near the membrane surface seems probable. The denaturation of B860 in situ with $10^{-2}$ M OA, pH 4.7 shows a dramatic loss of inner membrane particles in FFT. A particle with about 50Å diameter adjacent to
a membrane surface could also be seen as a membrane fracture face particle. Denaturation of such a particle could also account for its absence on denaturant treated membrane fracture faces. It is very possible that B860 is a inner membrane particle located in a position adjacent to a surface as opposed to a less vulnerable position at the center of the membrane. The presence of reaction center in the R-26 membranes was investigated in great detail. The results of these investigations are indicated below.

4.162 A P870 Reaction Center Particle as a Membrane Fracture Face Particle

P870 reaction center was isolated and purified in order to compare its size to the particles measured on the R-26 mutant membrane fracture faces. The RC has also been characterized recently by G. Feher and coworkers (1971) who found a minimum reaction center of 44,000 molecular weight, although a larger isolated unit containing the RC could be 70,000 but no higher than 100,000 molecular weight. Using a \( \bar{\nu} = 0.76 \) cc/gm, the theoretical diameters of these three protein molecular weights were computed as 48 Å, 55 Å and 64 Å. In the unlikely circumstance that \( \bar{\nu} \) is the same partial specific volume as that of water (\( \bar{\nu} = 1 \) cc/gm), then the calculated diameter increased by 10%. The diameters are not greatly influenced by small changes in \( \bar{\nu} \). Feher's shadowed RC preparation showed particle sizes of about 80 Å. The RC particles in the fraction 1 preparation showed shadowed particles of 108 Å. In fraction II, the particles were about 80 Å in width. The later preparation very possibly contained the minimum RC particle because of the relative elution position of this fraction coming off the Agarose molecular sieve column.
Since the replicas of these two preparations were shadowed with about the same contrast a real difference in particle size between fractions 1 and 2 is believable, in spite of large shadowing variations in the particle width means of 22-26Å (Sections 3.523) for the membrane fracture faces.

The chromatophore fracture face particles show a mean particle size of 92Å ± 16Å. The *R. spheroides* cytoplasmic fracture faces show particles of 98Å ± 13Å. If the results for the fractions 1 and 2 preparations are averaged, then a mean reaction center could be 99Å ± 19Å. On the basis of these measurements it is suggested that RC is a membrane fracture face particle in *R. spheroides*. Since it has been shown by fluorescence polarization measurements that the RC particles are separate entities in their isolated state, it is reasonable that they can occur singly on the membrane fracture surfaces. Denaturation of B860 does not change the size of the R-26 chromatophores fracture face particle so it is unlikely that RC and B860 are complexed side by side on the membrane either. Instead, the particle sizes on the membrane are attributed to functionally separate particles that have been increased in size in the shadowing process (Section 3.5231, 5.212). There is good evidence by negative staining and x-ray work that particles of about 50Å occur in the membrane (Section 3.4231). By this reasoning it appears that shadowing has increased the particle's shadowed width by about a factor of two. (Section 3.5231). The shadow widths minus the shadow increase correspond nicely with the theoretical diameter of 48-55Å for RC.

The chemical treatments which denature B860 and do not denature RC still retain some of the membrane fracture face particles (Section
Triton X-100 treatment of the chromatophores diminishes the membrane fracture face particles to a few. This is the same treatment used in the extraction of RC.

Added proof that RC is on the inside of the membrane comes from the fact that RC is essentially hydrophobic in nature. When triton X-100 is decreased below a minimum concentration, the RC can aggregate and precipitate out of solution. G. Feher and coworkers (1971) have analyzed the hydrophilic and hydrophobic protein content of RC (44,000 M. Wt.). Their results are compared to the C. ethylicum Bchl-protein complex which can be washed free of cells and Bovine serum albumin, which is highly water soluble.

<table>
<thead>
<tr>
<th></th>
<th>Reaction center protein from R-26 R. spheroides</th>
<th>Bchl-protein complex from C. ethylicum</th>
<th>Bovine serum albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>polar residues</td>
<td>32%</td>
<td>45%</td>
<td>50%</td>
</tr>
<tr>
<td>non-polar residues</td>
<td>68%</td>
<td>55%</td>
<td>50%</td>
</tr>
</tbody>
</table>

The ratio of nonpolar to polar amino acids is consistent with a hydrophobic RC particle. The evidence in toto, indicates that RC particles of about 50Å in diameter are located within the photosynthetic membranes of R-26 R. spheroides.

4.2 Future of Reversible Denaturants in Membrane Research

The proper reversible denaturant for any one kind of membrane may have to be tailored to the molecular components of that membrane. This must be done to maximize its denaturant and reversible properties. MO and OA are excellent reversible denaturants although their changes cannot always be totally reversed. Nevertheless, the use of reversible
denaturants allows a controlled alteration of the structural parameter of a membrane and makes it possible to interpret the membrane changes in relation to the original structures. The reversibility of the denaturant's effects acts as a bridge between the original membrane system and the changed system. This bridge allows the membrane changes to be given biological significance. This approach of *in situ* reversible denaturation of membrane structure opens up a whole new and productive dimension in membrane research.
5. Appendix

5.1 Indicators of Substructure Association
Changes in R. spheroides Membrane

Since FET and FFT both employ a mechanical freeze-fracturing mechanism, the internal membrane fracture surface relief is a result of where the fracture plane has to travel. This fracturing mechanism is not a gentle one because it has been found that the two membrane fracture faces cannot be exactly fitted back together (R. Park, private communication). For example, it has been found that the small plastic electron microscope spheres are badly distorted and can move position in the fracturing process. This type of behavior is not eliminated even at liquid Helium temperatures (D. Branton, unpublished experiments). It should be realized that membrane fracture faces are not exactly like they are in the unfractured membrane, but they are probably representative of the membrane's internal contents and they are reproducible from one preparation to another. The type of substructure which we can observe is explained briefly in the following subsections.

5.11 Location and Relief Texture of the Membrane Fracture Surface

In these experiments, as well as those of others in electron transport membranes, the membrane fracture surface normally appears very even and smooth with randomly located and embedded particles protruding from it. Examples of this behavior can be seen in Figure 3.221 and Figure 3.41-8B. Any large variation from this standard membrane fracture surface in R. spheroides cells and chromatophores upon chemical treatment is interpreted as a chemically-induced change in membrane internal organization.
5.12 Population Density of Particles Embedded in the Membrane Fracture Surface

Although this property can be quantitatively measured, it was found by experience that the number and area of patches without particles on the membrane fracture surfaces varied sufficiently with cell age and prior treatment such that only very large changes in the number of particles per area had real significance. Variations in the untreated membrane fracture surface particle population densities can be seen in Figures 3.41-7A and 3.41-8B. The particle population densities always decrease, they never increase with the chemical treatments used in this study.

5.13 Width of Particles Embedded in the Membrane Fracture Surface

It is very tedious, indeed, to measure the width of the platinum-carbon shadowed particles embedded in the membrane fracture faces. This job is made even more difficult because these particles are not normally located on flat smooth surfaces but are embedded in hilly surfaces. The only particle parameter that could be measured was the particle widths taken in a direction orthogonal to the platinum shadow direction (Figure 5.13-1 and Figure 3.221-1A). The results of the particle measurements are recorded as particle width versus frequency distributions in Appendix 5.211. Each width is measurable within a precision of ±20%. The mean width in each distribution was computed and compared to the other mean widths in Section 3.523. Unfortunately, the uncertainty in the particle width means due to platinum shadowing variations may mask any chemically-induced changes in the particle widths. If there are any changes in the particle widths, they would have to be as large as 5Å.
Figure 5.13-1. In this model the shadowing of an ideal spherical particle does not change its width or shape. In reality the width and the shape of the spheres change greatly in the shadowing process because the platinum shadow stacks up against the front side of the sphere expanding its lateral width.
to be significant in replicas made simultaneously. Any changes in the particle widths must be smaller than $5\AA$, if they exist.

5.2 Results and Discussion of Particle Width Measurements

5.21 The Precision and Accuracy of Mean Particle Width Measurements by Electron Microscopy

5.211 The Particle Width Distributions and the Significance of Their Distribution Characteristics

In principle, the particle width measurements and their distribution characteristics can reveal the presence of one or more sizes of membrane fracture face particle, if particle measurements are not distorted greatly by the platinum-carbon shadowing. In theory, a small amount of platinum-carbon shadowing distortion could mask particle size differences, leave them unchanged, or magnify them—the latter two cases being the most interesting. The distribution shape and its calculated parameters could choose between these possible alternatives.

In order to determine the approximate size of the particles on the membrane, it was necessary to estimate their increase of size due to the shadowing process. The approximate particle size is very important because it can discriminate against particles that are too large or are of too high a molecular weight or confirm whether a membrane particle candidate is about the right molecular weight and size.

The particle width versus frequency distributions were subjected to a variety of statistical measurements. The distribution shapes were compared by the chi square test to a theoretical Gaussian curve. Since none of the distributions even appear to be Gaussian, its not surprising that all of the curves are significantly different statistically from a Gaussian. Some information can be extracted from this non-Gaussian behavior,
namely whether the distribution curves are a sum of more than one
Gaussian. The characterization of the curves is also important because
it creates a basis for their comparison. Some of the nonGaussian meas-
urements are explained below.

The two parameters which were measured are Kurtosis and skewness.
The distribution asymmetry or the skewness, indicates that the tails of
the distribution curves are not similar. This behavior is a symptom of
noncoincidence between the mean and median. Distribution curves are
skewed to the right or the left, depending on whether their right or left
tail is drawn out. All of the experimental distributions are right-
skewed as indicated by the positive numerical values of skewness in
Figure 5.21-1, Figure 5.21-3, and Figure 5.21-5. The right skewness is
so pronounced it can be seen in all the distributions.

Kurtosis is a more interesting measure of nonGaussian behavior. It
is a measure of the narrowness of a distribution curve. There can be
two kinds of Kurtosis, platy-Kurtosis and lepto-Kurtosis. A platy-Kurtic
curve has fewer items at the mean and at the tails than the normal
distribution curve and it has more items in the intermediate regions. A
bimodal distribution with two kinds of particles is an extreme platy-Kurtic
distribution. A poly-modal distribution could probably also be detected
by this measurement. A lepto-Kurtic distribution curve has more items
near the mean and at the tails, and fewer items in the intermediate
regions relative to a normal distribution with the same mean and variance.
This kind of behavior would, however, support the existence of only one
particle size in the membranes. All of the distributions are lepto-Kurtic.

There is reason to believe there is more than one type of particle in
Particle Width Distributions Measured on the Cytoplasmic Membrane Fracture Surface of *R. Spheroides* Cells

<table>
<thead>
<tr>
<th>Date</th>
<th>Type</th>
<th>Treatment*</th>
<th>Mean particle width (Å)</th>
<th>Mean particle width of cell (Å)</th>
<th>Std. dev. of particle means</th>
<th>No. of cells</th>
<th>Total no. of particle widths</th>
<th>Student t values for chi sq. fit of the distribution to a theoretical gaussian</th>
<th>Fit</th>
<th>Skewness</th>
<th>Kurtosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>9/67</td>
<td>W.T.</td>
<td>107</td>
<td>18</td>
<td>107 Å</td>
<td>3</td>
<td>262</td>
<td>---</td>
<td>None</td>
<td>+.27</td>
<td>+3.0</td>
<td></td>
</tr>
<tr>
<td>9/67</td>
<td>&quot;</td>
<td>10⁻³ M.O.</td>
<td>94</td>
<td>18</td>
<td>94 Å</td>
<td>7</td>
<td>423</td>
<td>---</td>
<td>&quot;</td>
<td>+.43</td>
<td>+3.0</td>
</tr>
<tr>
<td>1/68</td>
<td>&quot;</td>
<td>95</td>
<td>16</td>
<td>95 Å</td>
<td>3</td>
<td>163</td>
<td>4</td>
<td>+.63</td>
<td>+4.2</td>
<td>+3.6</td>
<td></td>
</tr>
<tr>
<td>1/68</td>
<td>&quot;</td>
<td>10⁻³ M.O.</td>
<td>101</td>
<td>15</td>
<td>100 Å</td>
<td>3</td>
<td>339</td>
<td>9.4</td>
<td>&quot;</td>
<td>+.23</td>
<td>+3.6</td>
</tr>
<tr>
<td>2/68</td>
<td>&quot;</td>
<td>pH 4.7</td>
<td>90</td>
<td>15</td>
<td>90 Å</td>
<td>4</td>
<td>818</td>
<td>67.0</td>
<td>&quot;</td>
<td>+.56</td>
<td>+3.3</td>
</tr>
<tr>
<td>2/68</td>
<td>&quot;</td>
<td>0.7% EtOH</td>
<td>91</td>
<td>15</td>
<td>91 Å</td>
<td>4</td>
<td>561</td>
<td>30.3</td>
<td>&quot;</td>
<td>+.39</td>
<td>+3.1</td>
</tr>
<tr>
<td>2/68</td>
<td>&quot;</td>
<td>pH 4.7</td>
<td>100</td>
<td>15</td>
<td>99 Å</td>
<td>5</td>
<td>205</td>
<td>27.1</td>
<td>&quot;</td>
<td>+.31</td>
<td>+3.1</td>
</tr>
<tr>
<td>2/68</td>
<td>&quot;</td>
<td>1.5x10⁻³ M.O. 0.A.</td>
<td>94</td>
<td>13</td>
<td>93 Å</td>
<td>5</td>
<td>754</td>
<td>34</td>
<td>&quot;</td>
<td>+.46</td>
<td>+3.1</td>
</tr>
<tr>
<td>2/68</td>
<td>&quot;</td>
<td>pH 4.7 → 7.5</td>
<td>85</td>
<td>14</td>
<td>85 Å</td>
<td>5</td>
<td>584</td>
<td>50.5</td>
<td>&quot;</td>
<td>+.60</td>
<td>+3.3</td>
</tr>
<tr>
<td>3/68</td>
<td>&quot;</td>
<td>1.5x10⁻³ M.O. 0.A.</td>
<td>103</td>
<td>15</td>
<td>103 Å</td>
<td>3</td>
<td>149</td>
<td>4.0</td>
<td>&quot;</td>
<td>+.87</td>
<td>+4.6</td>
</tr>
<tr>
<td>3/68</td>
<td>&quot;</td>
<td>pH 5</td>
<td>102</td>
<td>18</td>
<td>103 Å</td>
<td>6</td>
<td>560</td>
<td>56</td>
<td>&quot;</td>
<td>+.43</td>
<td>+3.4</td>
</tr>
<tr>
<td>3/68</td>
<td>&quot;</td>
<td>1.5x10⁻³ M.O. 0.A.</td>
<td>104</td>
<td>17</td>
<td>105 Å</td>
<td>13</td>
<td>1046</td>
<td>---</td>
<td>&quot;</td>
<td>+.37</td>
<td>+2.6</td>
</tr>
<tr>
<td>7/68</td>
<td>&quot;</td>
<td>pH 5 → 7.5</td>
<td>99</td>
<td>16</td>
<td>99 Å</td>
<td>6</td>
<td>833</td>
<td>---</td>
<td>&quot;</td>
<td>+.87</td>
<td>+4.6</td>
</tr>
<tr>
<td>7/68</td>
<td>&quot;</td>
<td>10⁻³ M.O.</td>
<td>104</td>
<td>16</td>
<td>104 Å</td>
<td>6</td>
<td>647</td>
<td>43.2</td>
<td>&quot;</td>
<td>+.71</td>
<td>+3.9</td>
</tr>
<tr>
<td>11/69</td>
<td>R-26</td>
<td>No glycerol</td>
<td>96</td>
<td>17</td>
<td>95 Å</td>
<td>8</td>
<td>940</td>
<td>140</td>
<td>&quot;</td>
<td>+.71</td>
<td>+4.3</td>
</tr>
</tbody>
</table>

*Unless otherwise noted, each contains 5% glycerol, at pH 7, in distilled water.*

Figure 5.21-1
Figure 5.21-2. Particle widths on the cytoplasmic membrane fracture surface of R-26 *R. spheroides* cells as a function of particle widths versus population frequency.
Particle Width Distributions Measured on the Membrane Fracture Surface of R-26 R. Sphaeroides Isolated Membrane

<table>
<thead>
<tr>
<th>Date</th>
<th>Replica</th>
<th>Treatment</th>
<th>Mean particle width (Å)</th>
<th>Std. dev. of particle widths</th>
<th>Total no. of particle widths</th>
<th>Student t values for chi sq.fit of the distribution to a theoretical gaussian</th>
<th>Fit</th>
<th>Skewness</th>
<th>Kurtosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>11/69</td>
<td>---</td>
<td>4.8% EtOH</td>
<td>108</td>
<td>21</td>
<td>299</td>
<td>16.6</td>
<td>None</td>
<td>+.21</td>
<td>+2.3</td>
</tr>
<tr>
<td>11/69</td>
<td>---</td>
<td>2x10^{-2} M M.O. 4.8% EtOH</td>
<td>106</td>
<td>21</td>
<td>766</td>
<td>87</td>
<td>&quot;</td>
<td>+.59</td>
<td>+3.2</td>
</tr>
<tr>
<td>8/70</td>
<td>A</td>
<td>4.8% EtOH</td>
<td>88</td>
<td>15</td>
<td>511</td>
<td>42.5</td>
<td>&quot;</td>
<td>+.48</td>
<td>+3.0</td>
</tr>
<tr>
<td>8/70</td>
<td>B</td>
<td>4.8% EtOH</td>
<td>82</td>
<td>15</td>
<td>398</td>
<td>21.2</td>
<td>&quot;</td>
<td>+.70</td>
<td>+4.0</td>
</tr>
<tr>
<td>8/70</td>
<td>C</td>
<td>4.8% EtOH pH 4.7</td>
<td>92</td>
<td>15</td>
<td>888</td>
<td>[--]</td>
<td>&quot;</td>
<td>+.72</td>
<td>+3.8</td>
</tr>
<tr>
<td>8/70</td>
<td>A</td>
<td>10^{-2} M O.A. pH 4.7 4.8% EtOH</td>
<td>86</td>
<td>14</td>
<td>715</td>
<td>121</td>
<td>&quot;</td>
<td>+.83</td>
<td>+4.1</td>
</tr>
<tr>
<td>8/70</td>
<td>B</td>
<td>7.5x10^{-3} M O.A. pH 4.85 4.8% EtOH</td>
<td>86</td>
<td>15</td>
<td>322</td>
<td>44</td>
<td>&quot;</td>
<td>+.74</td>
<td>+3.7</td>
</tr>
<tr>
<td>8/70</td>
<td>B</td>
<td>3% triton X-100 for 1 hr, 0°C, centrifugate</td>
<td>86</td>
<td>14</td>
<td>417</td>
<td>44</td>
<td>&quot;</td>
<td>+.69</td>
<td>+3.4</td>
</tr>
</tbody>
</table>

Figure 5.21-3
Figure 5.21-4. Particle widths on the membrane fracture surface of R-26 *R. spheroides* chromatophores as a function of particle width versus frequency. These same untreated chromatophores were replicated on two separate occasions. Their distributions are compared.
Particle Width Distributions Measured on the Eutectic Triton X-100 Fracture Surface Containing R-26 R. spheroides Reaction Center

<table>
<thead>
<tr>
<th>Date</th>
<th>Replica</th>
<th>Treatment</th>
<th>Mean particle width (Å)</th>
<th>Std. dev. particle width (Å)</th>
<th>Total no. of particle widths</th>
<th>Student t values for chi sq. fit of the distribution to a theoretical gaussian</th>
<th>Fit</th>
<th>Skewness</th>
<th>Kurtosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>11/69</td>
<td>---</td>
<td>Crude fraction 1</td>
<td>108</td>
<td>18</td>
<td>686</td>
<td>73.3</td>
<td>None</td>
<td>+.72</td>
<td>+3.7</td>
</tr>
<tr>
<td>11/69</td>
<td>---</td>
<td>Purified fraction 1</td>
<td>108</td>
<td>20</td>
<td>244</td>
<td>18</td>
<td>None</td>
<td>+.44</td>
<td>+3.7</td>
</tr>
<tr>
<td>12/69</td>
<td>---</td>
<td>Fraction 2</td>
<td>80</td>
<td>14.6</td>
<td>301</td>
<td>9.8</td>
<td>None</td>
<td>+.57</td>
<td>+3.8</td>
</tr>
</tbody>
</table>

Figure 5.21-5
Figure 5.21-6. Particle width distribution.

A) Crude fraction 1 reaction center before purification
B) Reaction center, Fraction 1 after purification
C) Reaction center, Fraction 2
the cytoplasmic membrane of R-26 *R. sphaeroides*. Not only can this membrane perform the oxidative metabolism reactions, but it can also perform the light reactions of photosynthesis. There is probably some kind of oxidative metabolism membrane particle similar to that found in the membranes of the nonphotosynthetic bacteria and there is probably a reaction center particle in the photosynthetic membranes. There are probably still more membrane particles, but the point is that the particle width distributions do not demonstrate their existence. Because the spherical diameter of a particle varies as the cube root of the molecular weight, particles that differ by a factor of two in molecular weight differ in diameter by a factor of 1.26. Particles of about the same molecular weight could not be distinguished and very likely particles that differ by a factor of two in molecular weight may be indistinguishable as well (Section 5.213).

The distributions in Figures 5.21-2, 5.21-4, and 5.21-6 all show about the same distribution shape, although one of the distributions is supposed to contain a homogeneous preparation of Reaction Center. This same general distribution shape can be found with isolated homogeneous particle preparations shadowed with platinum on a carbon substrate. (C. E. Hall, 1961; D. N. Misra and N. N. Das Gupta, 1965). The distribution shapes are independent of FFT or FET and seem to be a function of the platinum-carbon shadowing process. It is unlikely that any hard information about the variety of membrane particles can be taken from the particle width distributions if the molecular weight of the particles are similar.

The characters of the particle width distributions and a few
representative distributions are presented in Figures 5.21-1 through 5.21-6. As a word of explanation for Figure 5.21-1 the populations of particle widths were recorded for fracture faces on individual cells. Thus, the heading number of cells indicates the number of cells whose fracture face particle populations were measured. A positive skew value indicates a right-skewed curve. A positive Kurtosis value indicates leptokurtic behavior. In the absence of chi square fit data to the Gaussian, the skewness and Kurtosis were sufficient to indicate non-Gaussian behavior.

5.2.12 Variations in Platinum-Carbon Shadowing

From Section 3.5.23, it should be clear that shadowing variations influence the particle width means. This can be seen in Figure 5.21-4 in which the same chromatophore preparation was replicated twice. The mean widths are 88Å and 82Å, respectively, which is better than should be normally expected from separate platinum-carbon replicas.

What has not been worried about until now is the lack of uniform shadowing within an individual replica. Most investigators seek out the more contrasty areas in a replica for their pictures. This kind of bias has an effect on the particle mean widths on the fracture surfaces of individual cells.

Within one replica, eight different cells with particulate membrane fracture surfaces were selected. Each of the eight cells was considered as a separate particle width distribution. Each of these particle width distributions were compared to the summation of these particle width distributions by the chi square test. Each of the eight distributions was significantly different from the overall distribution (Figures 5.21-2 and 5.21-7).
Although the particle width means aren't too different for the eight-cell fracture face particles, the distribution shapes are. It would appear that the overall shape of the particle width distribution may not be very significant unless a more uniform shadowing process is used.
Figure 5.21-7. R-26 *R. spheroides* Cells, November 1969.

<table>
<thead>
<tr>
<th>Cell Number</th>
<th>Mean Width</th>
<th>Standard Deviation</th>
<th>Number of Particles</th>
<th>t Value</th>
<th>Fit</th>
<th>Degrees Freedom</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>97 Å</td>
<td>20 Å</td>
<td>125</td>
<td>32.3</td>
<td>none</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>99 Å</td>
<td>17 Å</td>
<td>378</td>
<td>15.8</td>
<td>none</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>98 Å</td>
<td>19 Å</td>
<td>40</td>
<td>15.6</td>
<td>none</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>99 Å</td>
<td>16 Å</td>
<td>72</td>
<td>26.8</td>
<td>none</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>95 Å</td>
<td>16 Å</td>
<td>49</td>
<td>9.1</td>
<td>none</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>88 Å</td>
<td>14 Å</td>
<td>31</td>
<td>25.0</td>
<td>none</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>95 Å</td>
<td>17 Å</td>
<td>43</td>
<td>28.82</td>
<td>none</td>
<td>8</td>
</tr>
<tr>
<td>8</td>
<td>93 Å</td>
<td>16 Å</td>
<td>202</td>
<td>25.4</td>
<td>none</td>
<td>12</td>
</tr>
</tbody>
</table>

Note: The overall distribution is given in Figure 5.21-2.
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