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ELECTRON PARAMAGNETIC RESONANCE STUDIES
OF PHOTOSYNTHETIC SYSTEMS

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There are two main questions to be asked about the electron paramagnetic resonance signals observed in photosynthetic systems: first, are the signals directly related to photosynthesis; second, what is the molecular species responsible for the signal? Articles in the literature relevant to these questions are reviewed. Most of the experiments reported here were performed using an EPR spectrometer with superheterodyne detection and a digital averaging computer. Samples could be maintained at temperatures between 1.5°K and room temperature. The preparation of a chromium sample, which has many advantages as an internal standard for the EPR spectrometer, is described. Dried films of whole cell *R. rubrum* have a small EPR signal in the dark. Illumination at room temperature results in two reversible light-induced signals, one with a decay time of a few seconds, the other with a decay time of a few hours. Small reversible light-induced EPR signals are observed in *R. rubrum* down to temperatures below 2°K. The kinetics of the photochanges were measured at temperatures between 1.7°K and room temperature, and in most cases, the rise and decay curves could be fitted by two exponentials. The rise and decay rates of the EPR signal increase rapidly as the temperature is lowered from room temperature to 200°K; below 200°K the rates are approximately constant. Although EPR signals of some metals have been seen at low temperature, a search for signals due to triplet states was unsuccessful. At room temperature, illumination with saturating light
intensity produces one unpaired electron for every 55 bacterial chlorophyll molecules. Identifying the light-induced EPR signal in R. rubrum with the 433 nm absorbance change, I estimate the extinction coefficient at 433 nm of the molecule responsible for both the EPR and OD changes to be approximately $4 \times 10^4$ l/m cm. This calculation is combined with other evidence to suggest that a flavin is a likely candidate to be the molecule associated with the photo-induced EPR signal.
INTRODUCTION

General Aspects of Photosynthesis

Photosynthesis is the process by which living organisms, using light as an energy source, reduce carbon dioxide to form carbohydrates, amino acids, and other compounds which are needed for the maintainance and growth of the organism. Photosynthetic bacteria utilize various compounds in their environment, such as malate, hydrogen sulfide, or dissolved hydrogen gas, as hydrogen sources for the reduction of carbon dioxide. Algae and higher green plants are able to use water as the hydrogen source. While water is a poorer reducing agent than those used by the bacteria, it has the great advantage of widespread abundance, and green plants and algae were thus able to become by far the most abundant form of life on earth. A consequence of the ability to use water as the hydrogen source, which is somewhat incidental to the plant but rather important otherwise, is that oxygen is produced as a waste product. The increasing supplies of dead plant material and oxygen on the early earth formed a rich and convenient energy source which led to the rise of animal life.

Photosynthesis in living organisms can be conveniently divided into two parts: the light reactions or primary process, and the dark reactions or carbon fixation cycle. In the primary process, energy in the form of light is used to convert ADP to ATP and to reduce TPN$^+$ to TPNH.$^*$ These high energy chemical compounds are then used

$^*$The less common abbreviations used in this thesis are listed in Appendix C.
in the carbon fixation cycle to reduce carbon dioxide and incorporate it into various organic compounds. The detailed reaction sequence in the carbon fixation cycle is now fairly well understood,\textsuperscript{6,7} and the bulk of research effort is now centered on elucidating the mechanism by which light energy is converted into chemical energy.

One of the early proposals for the mechanism of the primary process was that light is absorbed by a chlorophyll molecule, raising it to an excited singlet state. The singlet state decays to an excited triplet state which in turn leads to the ionization of the Chl molecule and the production of a trapped electron and a "hole". The trapped electron enters the electron transport chain and eventually reduces TPN\textsuperscript{+} while the hole extracts electrons from water and evolves oxygen.\textsuperscript{11} It was suggested that electron paramagnetic resonance could be used to test this mechanism since it might be possible to detect the chlorophyll triplet state, the trapped electron, or the hole.\textsuperscript{55}

The first report of EPR studies of photosynthetic materials was published by Commoner, Townsend and Pake in 1954.\textsuperscript{17} They looked at lyophilized leaves from barley seedlings and showed that the EPR signal increased as the greening of the leaves progressed. They were, however, unable to detect any change in the EPR signal upon illumination of the leaves with light. In 1956 Commoner, Heise and Townsend\textsuperscript{16} reported reversible, light-induced EPR signals in an aqueous suspension of chloroplasts prepared from tobacco leaf. Sogo, Pon and Calvin\textsuperscript{55} obtained similar results with a variety of photosynthetic materials (leaves, whole spinach chloroplasts, and chloroplast fragments). In addition, they observed that lowering
the temperature from 25°C to -140°C had little effect on the rise time of the signal upon illumination, but greatly increased the decay time of the signal when the light was turned off.

These early experiments posed two questions which most of the work since that time has tried to answer. First, are the observed EPR signals directly related to photosynthesis and not just metabolic byproducts which have no function in the conversion of light energy into chemical energy? Second, exactly what is the molecular species on which the unpaired electron resides? Of course, the answers to these questions are not completely independent, e.g. if the species giving rise to the signal was chlorophyll positive ion, this would suggest that the EPR signal is directly related to the primary process in photosynthesis. We will first discuss approaches to these questions in general terms and then go on to examine evidence obtained by various recent workers in the field. After discussing my own experiments, we will take up these questions again in Section V.

One of the best ways to answer the question of whether the signals are directly related to photosynthesis is to measure the quantum yield of the EPR signal, i.e., the number of unpaired electrons produced for each quantum of light absorbed. If the unpaired electrons are on the direct pathway of energy conversion, then the quantum yield should be of order of magnitude 1. Also we would expect that the relative efficiency of light of different wavelengths in the production of unpaired electrons should correspond to that observed for normal measures of photosynthetic activity such as oxygen evolution or Hill activity. Chemicals which inhibit
or accelerate photosynthetic activity could influence the steady-state level and kinetics of unpaired electron production. The exact effect expected will depend on the assumed sequence of reactions and site of action of the chemical; if these are known, information can be obtained about the site of the unpaired electron. It is well established that the photosynthetic apparatus in most organisms actually consists of a large number of very small units (sometimes called quantaosomes) each of which is capable of carrying on the entire energy conversion process. These small units should also be capable of generating unpaired spins in the light. Also we might expect some small integral ratio between the maximum number of spins which can be produced in the light and the number of these photosynthetic units.

Now we will consider the question of the molecular species (one or more) giving rise to the EPR signal. EPR signals have been observed in transition metal ions, semiconductors, conductors, F-centers (electrons trapped at vacancies in a crystal lattice), free radicals, and triplet states. Transition metal ions, semiconductors, free radicals, and triplet states are the most likely possibilities in a biological system, and the prime suspects can be narrowed further when we consider the experimentally observed signals in detail.

The structure of an EPR signal can contain considerable information about environment of the unpaired electron. For example, the width of the line is related to the spin-spin relaxation time and to the inhomogeneity and anisotropy of the magnetic field in which the electron finds itself; the detailed structure of the line contains information about magnetic nuclei at which the spin density is not zero; the size of the signal is proportional to the number of unpaired
spins; the g value (a parameter proportional to the energy splitting of unpaired electrons in a given magnetic field) can tell us about the spin-orbit coupling of the electron. A major problem with photosynthetic systems has been that in general the signals observed are singularly indistinctive.

The usual approach has been to use the EPR signal as a label for the unknown species, perform various operations on the system, and follow the behavior of the signal. This is then combined with other types of information about the photosynthetic system to infer the identity of the unknown species. The types of operations which have been used can be divided into two classes: "internal" operations, such as fractionation of the system into component parts, use of mutant systems, or deuteration of the system; and "external" operations, such as the addition of inhibitors, altering the redox potential of the system, illumination of the system with light of various wavelengths and intensities, and altering the temperature of the system.

The EPR Signals Observed in Green Plant Materials

The commonly, although not universally, accepted outline for the primary process of photosynthesis in green plants is known as the Hill-Bendall scheme and is shown schematically in Figure I-1. A central feature of this scheme is the existence of two light absorbing systems, which have a different wavelength sensitivity to light and which successively provide the energy to move electrons along a redox chain. In System II, electrons from water reduce an unknown pigment system (perhaps connected with chlorophyll b) which is then oxidized in the light, transferring the electrons into a
Fig. I-1. The Hill-Bendall scheme for the primary process of photosynthesis in green plants.
coupled electron transport chain. This chain connects to System I terminating in chlorophyll \( a \), and the series of redox reactions is coupled to the production of ATP from ADP. The reduced chlorophyll \( a \) is oxidized by the light, and the electrons are transferred to another set of redox co-factors eventually resulting in the reduction of TPN\(^+\) to TPNH.

Another important feature of current thinking in photosynthesis is that not all the pigment molecules take part directly in the photochemical reactions. Accessory pigments such as phycoerythrin and phycocyanin absorb light and then transfer the energy to chlorophyll. In addition, the bulk of chlorophyll molecules serve only as antennae for gathering photons which are then transferred to "special chlorophyll molecules" at the photochemical reaction site. These special chlorophyll molecules are thought to be chemically identical with ordinary chlorophyll, but some of their absorption bands are shifted by the different environment in which they reside. In green plants there are approximately 300 ordinary chlorophyll molecules for each special chlorophyll molecule; in photosynthetic bacteria the relation is approximately 50 ordinary chlorophylls for each special chlorophyll.\(^{15}\)

Typical EPR signals observed in algae and higher green plants are shown in Figure I-2. A rather broad light-induced signal with some poorly resolved structure is observed centered around \( g = 2.005 \). Since this signal decays only very slowly after the light has been turned off, it is often referred to as the dark signal. Levine and Piette\(^ {36} \) have studied mutants of the green algae Chlamydomonas reinhardtii, which have no Hill activity (i.e. they do not evolve oxygen
Fig. I-2. EPR signals observed in whole spinach chloroplasts (after Androes, Singleton and Calvin\textsuperscript{2}). The magnetic field increases to the right.
in the presence of light and electron acceptors such as DCPIP). These mutants exhibit the normal light-induced signal, but the dark signal is completely absent. Weaver and Bishop\textsuperscript{62} have observed similar results with mutants of the green algae \textit{Scenedesmus obliquus} and these experiments indicate that the dark signal is connected with System II. Total deuteriation narrows the signal indicating that a part of the line width is due to proton splitting.\textsuperscript{32}

In green systems there is also a narrow light-induced signal at about \( g = 2.002 \) with rapid rise and decay kinetics. Androes, Singleton and Calvin\textsuperscript{2} were able to separate the slow and rapid light-induced signals by leaching and found the slow, broad signal in the water-soluble protein fraction, while the rapid, narrow signal remained in the green pigment fraction. For some time it has been suspected that the rapid light-induced EPR signal in green systems was due to an ionized form of chlorophyll \( a \). Similar signals have in fact been observed in pure crystals of chlorophyll \( a \).\textsuperscript{1} Tollin and Green\textsuperscript{57,58} have seen light-induced EPR signals in solutions containing chlorophyll \( a \) and various quinones. In all cases, however, the signals which they observed were due to semiquinone free radicals; they were never able to see the chlorophyll positive ion. The reason for this is not known. Beinert, Kok and Hoch\textsuperscript{10} have isolated from red algae a pigment complex containing "special chlorophyll" (P700) and a group of associated molecules. They found light-induced reversible EPR signals in this complex similar to those observed in intact systems. Weaver and Bishop\textsuperscript{62} studied a mutant of \textit{Scenedesmus obliquus} which had the normal concentrations of pigments but lacked the special chlorophyll P700. This mutant was unable to fix carbon dioxide and
displayed no rapid light-induced EPR signal. Thus it appears that the rapid narrow signal is closely associated with the System I photochemical reaction center.

EPR Signals Observed in Bacteria

In contrast to algae and green plants, photosynthetic bacteria have only one light absorbing system, and the general scheme for the primary process of photosynthesis in these organisms, shown in Figure I-3, resembles System I in green plants. Bacteria cannot reduce water and evolve oxygen. Instead they must use some other molecule in their environment, such as malate, succinate or bisulfide, as their electron source. These electrons are carried along an electron transport chain which is coupled to phosphorylation reactions to produce ATP as with green plants. The chain terminates in the reduction of bacterial chlorophyll, which is then oxidized in the light and eventually transfers the electrons through ferridoxin to TPN⁺.

Typical EPR signals seen in photosynthetic bacteria are shown in Figure I-4. These signals will be discussed in greater detail in Section IV; and for now I will mention some of the important observations which have appeared in the literature. Kohl et al. 32 found that when the red bacteria *Rhodospirillum rubrum* was grown in a totally deuteriated medium, the light-induced signal was narrowed by a factor of approximately 2, while the dark signal was unchanged. The dark signal is centered about \( g = 2.004 \), while the light-induced signal is centered about \( g = 2.002 \); and when the light-induced signal was narrowed by deuteriation, Kohl was able to observe the
Fig. I-3. The primary process of photosynthesis in bacteria.
Fig. I-4. EPR signals observed in *R. rubrum*. The magnetic field increases to the right.
height of the two signals independently. He reports that upon illum-
ination, the light-induced signal increased in size, but the dark
signal actually decreased and then increased to its former value
when the light was turned off. Loach\textsuperscript{37} has produced a signal in the
dark which is similar to the light-induced signal, by titrating with
redox reagents, and finds a midpoint potential of about 0.44 v. In
another experiment he found that the full light-inducible signal re-
mained after 95% of the pigments had been chemically bleached with
K\textsubscript{2}IrCl\textsubscript{6}, indicating that the majority of chlorophyll molecules are
not directly responsible for the EPR signal. Sistrom and Clayton\textsuperscript{52}
investigated a mutant of \textit{Rhodopseudomonas spheroides} which had the
normal compliment of bacterial chlorophyll and carotenoid pigments
but lacked the special chlorophyll (P870). This mutant gave neither
dark nor light-induced EPR signals. Ruby, Kuntz and Calvin\textsuperscript{49} tried
to correlate the decay kinetics of the light-induced signal in \textit{R.}
rubrum with the kinetics of the various light-induced optical den-
sity changes which have been observed. They found that the decay of
the EPR signal did not correlate with O.D change at 865 nm, corre-
sponding to the special form of bacterial chlorophyll, but instead
matched exactly the O.D. change at 433, which unfortunately has not
been assigned to any molecule.\textsuperscript{34}\* Thus we see that in the bacterial
systems the rapid light-induced signal seems to be associated with
the photochemical reaction center, but apparently is not directly
due to the special bacterial chlorophyll.

\*The relation between the optical density changes and the EPR signal.
will be discussed extensively in Section V.
INSTRUMENTATION

In electron paramagnetic resonance (or electron spin resonance, as it is sometimes called) the sample is placed in a magnetic field; any unpaired electrons present in the sample will have a net magnetic moment, and will thus tend to align in the magnetic field. For a particle such as an electron with spin 1/2 there are two possible orientations in a magnetic field, parallel and anti-parallel, which differ in energy, the parallel orientation being lowest in energy. The separation between these two energy levels is proportional to the external magnetic field and in this simple case is given by

$$\Delta E = g\beta H,$$

where $\beta$ is the Bohr magneton, $H$ the external magnetic field, and $g$ is the spectroscopic splitting factor (equal to 2.0023 for a free electron).

For convenient laboratory fields of a few thousand gauss the energy splitting turns out to be in the microwave region (about 10 GHz). One could imagine an EPR spectrometer where the sample was placed in a fixed magnetic field and the absorption of microwave power is measured as a function of frequency. In actual practice, in order to achieve high microwave field intensities, the sample is usually placed in a resonant microwave cavity. This restricts the microwave power to a single frequency, and therefore in order to scan a spectrum, the external magnetic
field is varied. Another small complication introduced for technical reasons is that usually a small amount of higher frequency modulation is added to the slowly varying external magnetic field. This has the effect of producing an a-c signal which is proportional to the slope of the absorption spectrum and which can be detected by a phase-sensitive lock-in amplifier. The net result of this is a considerable increase in instrument sensitivity, and for this reason most EPR spectra are recorded and presented as derivatives of the absorption.

Electron paramagnetic resonance spectra in these studies were measured on an X-band spectrometer operating at approximately 9.5 GHz with superheterodyne detection. This apparatus is shown schematically in Figure II-1. The microwave source is a klystron (Model VA-232, Varian Associates, Palo Alto, California). In the earlier experiments a Model V-58 klystron was used, but the Model VA-232 was found to have a significantly lower noise level.) The microwave power is transmitted by waveguide through a hybrid tee to the sample cavity, which has a Q of 2000. (The Q is the quality factor for a resonant cavity, and is given by $2 \pi$ times the stored energy divided by the energy losses per cycle.) Power reflected from the cavity is detected by a superheterodyne system (U.S. Navy surplus Transmitter-Receiver RT-100A/APS-19). In the superheterodyne receiver, power from the cavity is combined in a balanced mixer with microwave power from a local oscillator klystron operating at a frequency of 30 MHz off that of the signal klystron. The resulting 30 MHz difference frequency is then amplified by an IF amplifier and detected.
Figure II-1. Schematic diagram of the EPR spectrometer used in these studies.
The reflector voltage of the signal klystron is modulated at 60 KHz, which has the effect of moving the klystron frequency back and forth through the resonant frequency of the sample cavity. The resulting modulation of the output of the IF amplifier is used to lock the frequency of the signal klystron to that of the sample cavity. The magnetic field is modulated at 200 Hz and the resulting signal is then demodulated by a lock-in amplifier (Model JB-4, Princeton Applied Research, Princeton, New Jersey). The output of the lock-in amplifier is displayed directly on a strip chart recorder.

Biological samples often have relatively low concentrations of unpaired electrons, and in order to improve the signal-to-noise ratio a procedure was often used in which the spectrum is taken many times and then averaged. This can be done conveniently with one of the digital memory devices currently on the market. These are simple computers which convert a time varying signal to digital form and store the results successively in several hundred channels. On subsequent sweeps through the signal, the information is added in register, so that the total signal accumulated is proportional to the number of passes, while the noise tends to average out. In general, for random noise, the actual improvement in signal-to-noise ratio is proportional to the square root of the number of passes. I have used with good results two different instruments: The Computer of Average Transients (Model 400, Mnemotron Corporation, Pearl River, New York) and the Digital Memory Oscilloscope
(Model NS-544, Northern Scientific Inc., Madison, Wisconsin). Internal and external triggering circuits can be used to synchronize a transient signal with the digital averager. These instruments also have available a current which increases linearly as they step through the memory channels. I amplify this current and use it to energize a pair of magnet coils to sweep the magnetic field through the sample resonance. The maximum sweep width which can be obtained in this manner with my setup is about 60 gauss.

The sample is illuminated by a Sylvania DFA projection lamp. Light passes through a water bath and lens and into a light pipe. For the early experiments a lucite light pipe was used, but this was replaced by a quartz light pipe when it was discovered that lucite has an absorption band near 900 nm. The absorption spectrum of a block of lucite, 1.94 cm thick, is shown in Figure II-2. Most of my experiments were performed with R. rubrum, a photosynthetic bacteria which has an absorption maximum at 880 nm where the lucite light pipe had an OD of more than 3. Quartz is essentially transparent throughout this region.

The geometry of the light system and sample cavity is shown in Figures II-3 and II-4. The light emerging from the light pipe is reflected by two mirrors onto the sample which has been dried on a quartz slide and placed face down on the bottom of the microwave cavity. As indicated in Figure II-4, the bottom of the cavity has a series of slits
Figure II-2. The absorption spectrum of a block of lucite, 1.94 cm thick.
Figure II-3. Schematic diagram of the light system.
Figure II-4. The sample cavity.
which pass 50% of the impinging light. Approximately 1% of the light initially impinging on the light pipe reaches the sample. Although white illumination was used in most of the experiments, a monochromator and quartz-iodide lamp can be substituted for the tungsten lamp if desired.

The sample cavity was in a metal dewar of conventional design. With the use of liquid nitrogen and/or liquid helium the sample could be maintained at temperatures between 300° and 1.5°K. Temperature was measured with a copper-constantan thermocouple, carbon resistors, and, below 4.2°K, by measuring the vapor pressure above the liquid helium. Good thermal contact was assured since the sample was in direct contact with the microwave cavity which was made of gold-plated brass. The thermocouple and carbon resistor were also in direct contact with this cavity. At 4.2°K and below, the sample and microwave cavity were directly immersed in the liquid helium bath. For this reason much of the low temperature work was done below the lambda point of helium (2.2°K) to eliminate bubbling inside the cavity.
EXPERIMENTAL METHODS

Growth and Preparation of Samples

Rhodospirillum rubrum cultures used in these studies were originally obtained from R. Y. Stanier (Strain S 1). The bacteria were grown in 125 ml and 1 liter bottles on Modified Hutner's Medium. The medium was innoculated with 0.1 to 0.2% by volume of old R. rubrum culture and the cells harvested after three to five days, by which time they had attained their maximum density. Typical growth of the cultures is illustrated in Figure III-1, which shows the change in optical density at 650 nm where the absorption is a minimum and light scattering by the cells gives rise to most of the optical density. The cell doubling time was approximately six hours.

For whole cell preparations, the culture was centrifuged at 850 x g for 15 minutes. A small amount of the resulting thick suspension of cells was pipetted onto a quartz slide and dried for about 10 minutes under nitrogen gas; no effort was made to dry the sample thoroughly. The samples were normally almost opaque. Chromatophore fragments from R. rubrum were prepared by the method of Loach et al., and similarly dried on a quartz slide.

Nostoc muscorum cultures were originally obtained from M. B. Allen (Strain M 12.4.1). They were grown in continuously shaken 2.5 liter bottles. The growth medium used is given in Appendix A. Whole cell samples were prepared in the same manner as with R. rubrum.

Spinach chloroplasts were prepared from fresh commercially obtained spinach (Spinacia oleracea) according to the method of Park and Pon, and a thick suspension of these chloroplasts was pipetted onto a quartz slide and dried under nitrogen gas.
Fig. III-1. Growth curves for *Rhodospirillum rubrum*.
Quantitative Determination of Number of Unpaired Electrons

The number of unpaired electrons detected by an EPR spectrometer is directly proportional to the area under the electron paramagnetic resonance absorption curve. Since the curve normally obtained is the derivative of the absorption, we must effectively take the double integral of the spectrum we obtain. This process was relatively simple in my case since the spectra of interest had either Gaussian or Lorentzian line shapes, and it is a simple matter to calculate the area under a Gaussian or Lorentzian line from the height and width of the corresponding derivative curves.

A Gaussian curve can be represented by the equation

\[ G = A e^{-b^2x^2} \]

where \( A \) and \( b \) are arbitrary constants. The corresponding derivative is given by

\[ \frac{dG}{dx} = -2Ab^2x e^{-b^2x^2} \]

This curve is shown in Figure III-2. Note that the peak-to-peak width, \( W \), corresponds to the distance between points of maximum slope on the Gaussian curve, while the height, \( H \), of the derivative corresponds to the slope at those points. For a Gaussian derivative curve with width \( W \) and height \( H \), the area under the corresponding Gaussian curve is given by

\[ \text{Area}_G = 0.516 H W^2 \]

A Lorentzian curve can be represented by the equation

\[ L = \frac{A}{1 + b^2x^2} \]
Fig. III-2. Derivatives of Gaussian and Lorentzian curves.
where $A$ and $b$ are arbitrary constants. The corresponding derivative is given by
\[ \frac{dl}{dx} = -2Ab^2x(1 + b^2x^2)^{-2}. \]

This curve is also shown in Figure III-2, where its width and height have been adjusted to match those of the Gaussian derivative curve. Note that beyond the peaks the Gaussian derivative approaches zero much faster than does the Lorentzian derivative. For a Lorentzian derivative curve with width $W$ and height $H$, the area under the corresponding Lorentzian curve is given by
\[ \text{Area}_L = 1.814 H W^2. \]

Figure III-3 shows the Gaussian and Lorentzian curves corresponding to the derivative curves plotted in Figure III-2. As indicated above, for a given width and height of the derivative curve, the area under the Lorentzian curve is more than three times that under the Gaussian curve.

In order to determine the absolute number of spins in an unknown, the instrument must be calibrated with a sample containing a known number of spins. I used a sample containing about 0.1% chromium as an impurity in magnesium oxide. The spectrum of chromium\(^{40}\) in the cubic lattice of magnesium oxide consists of a single line at a $g$ value of 1.9800. There are also four lines of low intensity due to the Cr\(^{53}\) isotope with 9.5% natural abundance. The main chromium line is shifted from the free electron position to higher field by about thirty gauss. The EPR signals observed in photosynthetic systems normally have a $g$ value close to that of the free electron and can therefore be observed at the same time as the chromium with little overlap of the two signals.
Fig. III-3. Gaussian and Lorentzian curves corresponding to the derivative curves plotted in Fig. III-2.
In practice, therefore, the chromium sample was normally present in the microwave cavity and served as a continuous measure of the apparatus sensitivity. The number of unpaired electrons in the chromium sample was approximately known from its preparation, but the value used was obtained by calibration of the chromium sample against a sample of phosphorus-doped silicon, obtained from E. A. Gere of Bell Telephone Laboratories, which Gere had originally calibrated against copper sulfate.

Preparation of the Chromium Standard.

A 4 molar solution of magnesium chloride (250 ml) was scavenged by adding 12 g of magnesium metal turnings and allowing the mixture to react with stirring for two hours. The mixture was filtered, and magnesium hydroxide was precipitated from the filtrate by the addition of 100 ml of 30% ammonium hydroxide. The precipitate was centrifuged and washed with distilled water three times and dried at 400° for several hours to yield purified magnesium oxide. A weighed portion of the oxide was redissolved in dilute hydrochloric acid, and a known amount of chromium nitrate solution added. This solution was then precipitated with ammonium hydroxide, centrifuged, washed, and heated as before, yielding magnesium oxide which now contained chromium as a lattice impurity. This compound was then mixed with polyethylene powder in known proportions (approximately 1:10) and hot rolled between teflon sheets, producing a convenient sample which is stable and impervious to moisture, portions of which can be cut out and weighed as needed.

The EPR spectrum of this chromium sample is shown in Figure III-4. The line width is controlled by dipolar broadening; increasing the chromium concentration increases the line width. In this case the
Fig. III-4. The EPR spectra of Cr$^{3+}$ in MgO. The magnetic field increases to the right.
chromium-to-magnesium ratio is approximately $1 \text{ to } 10^4$ and the peak-to-peak line width is 1.66 oe. The hyperfine lines due to Cr$^{53}$ isotope each have 2.6% of the intensity of the main peak, and are separated by 17.3 gauss. In addition to serving as a standard for determination of the absolute number of unpaired electrons in a sample, the chromium hyperfine lines are convenient for the calibration of magnetic field sweeps and for the precise measurement of $g$ values of unknown samples.
RESULTS AND DISCUSSION

EPR Spectra of Rhodospirillum rubrum at Room Temperature

The EPR signals observed at room temperature in dried films of Rhodospirillum rubrum, a red photosynthetic bacteria, may be conveniently divided into three parts on the basis of kinetic response to light. The signal observed after the sample has been in the dark for several hours I call the dark signal (D), and let us for the present purposes give it a value of 1 for signal intensity (proportional to the number of unpaired electrons). As diagramed in Figure IV-1, upon illumination with light, the signal intensity increases to a value of about 10, with an exponential rise time of about 2 seconds. When the light is turned off the signal intensity decreases to a value of 5, with an exponential decay time of approximately 10 seconds. Subsequent illumination will return the signal to an intensity of 10; or if left in the dark, the signal intensity will return to its original value of 1, with an exponential decay time of about 150 minutes. Thus we see that the signal observed in the light consists of three components:

1) a fast light-induced signal (FL) with rapid rise and decay kinetics;
2) a slow light-induced signal (SL) with rapid rise but very slow decay kinetics; and 3) a dark signal (D) which is present without illumination.

*On experiments with R. rubrum grown on deuterated medium, Kohl reports that the dark signal decreases upon illumination with a time constant of less than 1 second. While it is not completely clear that our systems are comparable, I would not be able to distinguish between the presence or absence of the dark signal during illumination. Therefore it is possible that the signal observed in the light contains only the fast light and slow light components.
Fig. IV-1. The effects of light on the room temperature EPR signal in dried films of whole cell _R. rubrum_.

D + SL + FL

D + SL

dark 10 sec

light 2 sec

light 2 sec

light 2 sec

dark 150 min
IV-3

This behavior in dried films is different from that observed in aqueous suspensions of the bacteria, where only the dark and fast-light signals are observed. In addition, the room temperature kinetics of the fast-light signal in aqueous suspensions are faster by a factor of about four than the kinetics I have observed in dried films.

The decay of the slow light signal is shown in Figure IV-2. The signal height used in this plot is the experimentally measured peak-to-peak signal height minus the peak-to-peak height of the dark signal measured after the sample had been in the dark for twenty-four hours. (The height of the dark signal was 0.13 in these units.) The straight line has an exponential decay time of 147 minutes. The kinetics of the other signals will be discussed in a later section.

The peak-to-peak line widths and g values of the various signals were measured by comparison with a chromium sample which was also present in the cavity. Van Wieringen and Rensen\textsuperscript{64} report a g value for chromium in magnesium oxide of $1.9800 \pm 0.0005$. I have determined that the g value is $1.9804 \pm 0.0002$ by comparing the chromium sample with phosphorus-doped silicon, using Gere's value of $1.99875 \pm 0.0001$ for the g value of the phosphorus-doped silicon.

The dark signal is shown in Figure IV-3. It has a line width of $12.0 \pm 0.5$ oe, a g value of $2.0038 \pm 0.0003$, and an unsymmetrical line shape. If malate, the carbon source in the normal growth medium, is replaced by succinate, the dark signal has a similar shape and g value, but the width is narrowed significantly to $10.5 \pm 0.5$ oe. The slow light and fast light signals have the same line width ($9.7 \pm 0.2$ oe) and g value ($2.0025 \pm 0.0003$). Both signals have a Gaussian line shape; this is demonstrated in Figure IV-4, where the signal observed in the
Fig. IV-2. The decay of the slow-light signal at room temperature in dried films of whole cell R. rubrum.
Fig. IV-3. The dark EPR signal in dried films of whole cell *R. rubrum* at room temperature. The magnetic field increases to the right.
Fig. IV-4. The EPR signal, in the light, in dried films of whole cell *R. rubrum* at room temperature. The magnetic field increases to the right.
light is compared with calculated Gaussian and Lorentzian derivatives. Replacement of malate by succinate in the growth medium has no effect on the shape, line width, or g value of the light-induced signals. These signals can be saturated with microwave power quite easily, and it was necessary for this reason to work at rather low microwave power levels: about 0.5 mw incident on the cavity at room temperature and considerably lower power levels at lower temperatures.

**EPR Spectra of Rhodospirillum rubrum at Low Temperature**

Table I summarizes a series of experiments in which a quantitative study was made of the number of unpaired electrons in the various signals as a function of illumination and temperature. These data were taken with a single sample. Similar results were obtained when the experiment was repeated with a different sample, but the relative proportions of the various signals can vary as much as ± 50% between samples. In Experiment I, the dark signal was observed at room temperature and then the sample was cooled to liquid nitrogen (LN) temperature while still in the dark, whereupon the same dark signal was observed. Upon illumination at LN temperature, a small reversible, light-induced signal was produced with rapid rise and decay kinetics.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>300ºK</th>
<th>81ºK</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>D</td>
<td>SL</td>
</tr>
<tr>
<td>I Cooled in dark</td>
<td>1.3</td>
<td>-</td>
</tr>
<tr>
<td>II Cooled after light</td>
<td>0.89</td>
<td>3.3</td>
</tr>
<tr>
<td>III Cooled in light</td>
<td>2.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>
In Experiment II, the sample was illuminated at room temperature, and after the light had been turned off for a few minutes leaving the dark and slow light signals, the sample was cooled to LN temperature, freezing in these signals. Again a small reversible light-induced signal was observed with similar kinetics and number of unpaired electrons as observed in Experiment I. In Experiment III, the sample was illuminated at room temperature and then cooled to LN temperature while the light was still on. In this case the entire light signal was frozen in, although a small reversible light-induced signal was again observed with similar kinetics and number of unpaired electrons as observed in Experiments I and II. Unfortunately the accuracy of these measurements is not sufficient to determine whether the reversible signal observed at LN temperatures represents a portion of the room temperature fast light signal or is in addition to it. What we do conclude, however, is that signals observed at room temperature are essentially frozen in (or frozen out) at LN temperatures, except for a relatively small light-induced signal which is completely reversible. The signals observed in *R. rubrum* at liquid helium temperature are identical in these respects to those observed at liquid nitrogen temperature.

Typical EPR spectra of *R. rubrum* at low temperature are shown in Figures IV-5 and IV-6. The spectra in Figure IV-5 were obtained by cooling the sample shortly after the light was turned off, so that most of the slow-light signal is frozen in. The "light off" spectrum was subtracted from the "light on" spectrum to obtain the bottom curve. A Gaussian derivative was adjusted by eye for best fit to the difference spectrum. The line width of the calculated curve is 10.0 oe. To obtain the spectra shown in Figure IV-6, the sample was kept in the dark
Fig. IV-5. Low temperature EPR signals in dried films of whole cell *R. rubrum*. The sample was cooled to 80°K after the light had been turned off. The magnetic field increases to the right.
Fig. IV-5. Low temperature EPR signals in dried films of whole cell *R. rubrum*. The sample was cooled to 1.7°K with the light on. The magnetic field increases to the right.
at room temperature for three hours before cooling to low temperature, thus freezing out the fast-light and most of the slow-light signals. Actually, the low temperature signal would have contained less of the slow-light signal than might have been expected based on the slow-light decay constant, since the sample was never exposed to bright illumination at room temperature and only a relatively small amount of light-induced signal was generated by the room lights during sample preparation. The "light on - light off" curve in Figure IV-6 was again obtained by subtraction and fitted by eye with a Gaussian derivative. The line width of the calculated curve is also 10.0 oe. It is not surprising that the room temperature signals have a similar shape and width when frozen in at low temperature. It is significant, however, that the small, light-induced signal observed at low temperature is essentially identical in shape and line width with the room temperature light-induced signal. This fact would suggest that the same molecular species is involved in both cases.

If the light-induced signals are reversible at room temperature and can be frozen in at liquid nitrogen temperature, there must be some intermediate temperature at which the light-induced signals "thaw out". To investigate this, a sample of R. rubrum was cooled in the light to liquid nitrogen temperature and the EPR signal was monitored as the sample was warmed to room temperature. During the warming period, the light was turned on and off at intervals of 100 seconds. The combined results of two such experiments are plotted in Figure IV-7. The signal height in the dark divided by the signal height in the light is plotted on the vertical axis; this function would be equal to unity if there were no light-induced change in the signal. Unfortunately the data are
Fig. IV-7. The ratio of light signal to dark signal in dried films of whole cell *R. rubrum* as a function of temperature.
very poor and all we can say is that the most rapid change occurs in
a region about forty degrees wide centered around 200°K.

**Triplet Search**

One of the early hypotheses in photosynthesis was that absorption
of a photon by a chlorophyll molecule resulted in the formation of a
chlorophyll triplet state, which might be detected by electron para-
magnetic resonance. In fact, Rikhireve et al.\textsuperscript{46} have reported a re-
versible light-induced signal with a g value of about 4 and a 13 oe
line width in alcoholic solutions of a mixture of chlorophyll a and b,
and also in alcoholic solutions of chlorophyll b alone. They attribute
this signal to the formation of a triplet state of chlorophyll b upon
illumination. In an effort to observe such triplet states in in vivo
systems, I have carefully searched the low field regions in the EPR
spectra of several photosynthetic organisms (bacteria, blue-green
algae and green plants) at a temperature of approximately 2°K. No
signals were observed in the region of g values of 3 to 5 in samples
of *R. rubrum* or *Nostoc muscorum* in either dark or light. A sample of
spinach chloroplasts had a moderate sized signal at g equals 4 in the
dark, but this signal was unaffected by illumination.

Assuming a line width of 10 oe and a g value of 4, I estimate
that I would be able to detect $1.3 \times 10^{13}$ unpaired electrons with a
signal-to-noise ratio of 1. An additional factor is that the transi-
tion probability for the resonance at g equals 4 ($\Delta m = 2$) is lower
than the transition probability for the resonance at g equals 2 by a
factor of roughly 10.\textsuperscript{61} Therefore the minimum number of unpaired
electrons which I could expect to see is approximately $10^{14}$. The ob-
served light-induced signal at g equals 2 in these samples represents
about $10^{15}$ unpaired electrons. Thus if triplet states are produced in similar numbers, I would expect to see them.

Other EPR Signals Observed

In addition to the dark and light-induced signals at $g = 2$, I have observed various other EPR signals at liquid helium temperatures in *R. rubrum*. An asymmetric signal at $g = 6.5$, probably due to iron, can be seen at $2^\circ K$. There is also a very broad asymmetric signal around $g = 30$. Illumination does not cause any reversible changes in these low field signals.

For some experiments, *R. rubrum* was grown in a medium in which malate, the normal hydrogen source, had been replaced by ascorbate. The bacteria were grown by inoculating 125 ml of ascorbate medium with 0.25 ml of *R. rubrum* suspension which had reached steady-state growth level in the normal malate medium. After 15 days the bacteria were spun down and resuspended in 125 ml of fresh ascorbate medium; after 3 days of growth, this culture was harvested in the normal manner. The EPR spectra of these bacteria is shown in Figure IV-8. A small asymmetric signal was observed in the dark. Illumination of the sample resulted in a much larger asymmetric signal, which did not decay significantly during a subsequent period of about 30 minutes in the dark. The signals have a $g$ value of $2.0055 \pm 0.0003$. (The uncertainty in the $g$ value for the dark signal is about twice this value.) The small signal on the right of the spectra shown in Figure IV-8 is the low field hyperfine line of the chromium standard which was also present in the cavity.
Fig. IV-8. The EPR signals observed in dried films of whole cell
*R. rubrum* at room temperature. These cells were grown
in medium in which malate had been replaced by ascorbic acid. The magnetic field increases to the right.
I have also done some studies with dried films of Nostoc muscorum, a blue-green algae. EPR spectra of Nostoc are shown in Figure IV-9. In the dark there is a small signal comparable in intensity with the dark signal observed in *R. rubrum*. Illumination of the sample produces a rather small increase in signal intensity. The rise and decay kinetics are approximately exponential with time constants of about 5 seconds at room temperature. The signals are symmetrical, approximately Gaussian in shape, with a peak-to-peak line width of about 8.5 oe. The center of the resonance corresponds to a $g$ value of $2.0030 \pm 0.0005$. The small signal on the right of the spectra shown in Figure IV-9 again is the low field hyperfine line of the chromium standard.

**Kinetics**

Rise and decay kinetics of the light-induced EPR signal were measured by setting the dc magnetic field to one of the peaks of the resonance line and then following the increase and decrease of this peak as the light was turned on and off. Since short instrument time constants must be used in this work to avoid distortion of the kinetic response of the signal, it was normally necessary to average a large number of repeated responses to light and dark in order to obtain a reasonable signal-to-noise ratio. This was conveniently done with one of the small digital memory averagers which were described in Section II. The light is turned on and off by interrupting the light path with a metal leaf attached to a small stepping motor (see Figure II-3). With this system the light goes from fully off to fully on in approximately 5 msec.
Fig. IV-9. The EPR signals observed in dried films of *Nostoc muscorum* at room temperature. The magnetic field increases to the right.
Figure IV-10 shows typical rise and decay kinetics for whole cell *R. rubrum* at room temperature. Figure IV-11 shows the kinetics of the same system at 2°K. In general, the whole cell rise and decay curves at various temperatures could be fitted quite well by the sum of two exponentials. (In one case only one exponential was required; in another case, three.) The room temperature rise and decay kinetics for chromatophores required only one exponential; at lower temperatures two exponentials were required for a good fit. Thus, most curves can be characterized by three independent parameters: a time constant for each of the two exponentials and the ratio of their contribution to the initial signal. The values measured for these parameters are tabulated in Appendix B.

For purposes of comparing various curves, it was found most useful to combine the two exponential time constants according to the ratio of their contributions to the initial signal. For example, if a given kinetic curve can be approximated by the following equation,

\[ s = s_0 \left( 0.25 e^{-t/0.1} + 0.75 e^{-t/1.0} \right) \]

we would calculate an "effective time constant" as

\[ 0.25(0.1) + 0.75(1.0) = 0.775 \]

The individual measured time constants and the ratio of their contributions to the observed kinetic curve had a rather large variation from sample to sample at a given temperature. The "effective time constant", when calculated in this manner was more reproducible between samples and also had a smoother variation with temperature. In addition, there were a few cases where the kinetic
Fig. IV-10. Room temperature rise (top) and decay (bottom) curves of a dried film of whole cell *R. rubrum*. 

\[ S = 1 - (0.77e^{-t/59} + 0.23e^{-t/3.5}) \]

\[ S = 0.19e^{-t/9.4} + 0.81e^{-t/9.1} \]
Fig. IV-11. Rise and decay curves for a dried film of whole cell *R. rubrum* at 2°K.
curve resolved into two exponentials with time constants differing by a factor of five or less. In such cases, there is considerable leeway in the choice of exponentials to obtain a good fit; the various choices yield closely the same effective time constant, however, and much of their arbitrary nature is eliminated.

Figure IV-12 shows the "effective time constant" calculated for a series of rise curves as a function of temperature. Where more than one point is plotted for a given temperature, this represents the results of independent experiments. All kinetic data were taken using a light intensity sufficient to saturate the steady-state intensity of the light-induced signal. While the decay kinetics were essentially independent of light intensity, a few preliminary experiments indicated that the rise kinetics became faster as the light intensity was increased at low intensity-levels and then became constant as the light intensity reached a value sufficient to saturate the steady-state signal.*

Figure IV-13 shows the "effective time constant" for a series of decay curves as a function of temperature. The kinetics of chromatophore fragments are essentially identical with those of whole cells. The only significant difference is in the rise kinetics at low temperature, and this is most probably due to a difference in illumination intensity.

*All kinetic experiments through Run 87 were performed using the same light intensity and the lucite light pipe. A constant light intensity, approximately one third the earlier value, and the quartz light pipe were used for Run 161 and all subsequent kinetic experiments. The lower light intensity was still sufficient to saturate the steady-state signal because of the greater transparency of the quartz light pipe. The spectral quality of the light reaching the sample was somewhat different in the two cases due to the different lamp voltages and the red absorption of the lucite light pipe. The actual light intensity reaching the sample was approximately $10^{16}$ quanta/cm² sec.
Fig. IV-12. The effective rise times of the light-induced EPR signal in dried films of R. rubrum.
Fig. IV-13. The effective decay times of the light-induced EPR signal in dried films of R. rubrum.
There is a surprising correspondence between the rise and decay times. In Figure IV-14 the rise times for whole cells have been multiplied by 3.5 and plotted on the same graph with the decay times. The close correlation between the rise and decay times as a function of temperature suggests that the rise and decay processes are quite similar. Perhaps the decay process is simply the reverse of the process giving rise to the light-induced signal.

The most striking aspect of these kinetic data is that the time constants decrease (that is, the reaction rates increase) as the temperature is decreased. We normally expect chemical reaction rates to decrease as the temperature is lowered. There are, however, a few chemical reactions known, where the rate increases with a decrease in temperature. A number of factors have been cited to account for this behavior: 1) the reactants are localized in liquid pools as the solvent freezes out; 2) ice crystals act as catalytic surfaces; 3) many of the reactions studied involve transfer of protons and the mobility of protons is known to be about two orders of magnitude greater in ice than in water; 4) in the case of some enzyme reactions the enzyme is thought to be in a more active or "native" state at lower temperatures.

In all reactions of this sort which I have found in the literature, the reaction rate, while increasing at first as the temperature is lowered, goes through a maximum and then decreases as the temperature is lowered further. In my experiments with R. rubrum the rate of appearance and disappearance of the EPR signal increases sharply down to about 200°K and then remains approximately constant as the temperature is lowered to 2°K. At no time did the reaction rate decrease with decreasing temperature. Androes, Singleton and Calvin measured the
Fig. IV-14. A comparison of the effective rise and decay times of the light-induced EPR signal in dried films of whole cell R. rubrum.
kinetics of light-induced EPR signal in *R. rubrum* and found that the reaction rates increased as the temperature was lowered. The lowest temperature at which they worked was 115°K; the slow time response of their instrument did not allow them to obtain any detailed data.

I was not able to observe any reversible light-induced changes in the EPR signals of the blue-green algae *Nostoc* at 77°K. When a dried film of spinach chloroplasts was cooled to 77°K in the dark, upon illumination I observed an increase in the EPR signal. The change was irreversible, however, since the signal did not decrease when the light was turned off, and subsequent changes in illumination had no effect on the signal. Similar results with spinach chloroplasts have been reported in the literature.55

Some optical density changes have been found in photosynthetic bacteria at low temperatures. The irreversible light-induced oxidation of a cytochrome in *Chromatium* at low temperature, associated with a bleaching at 422 nm, has been studied in Chance's group.13,20 The reaction rate decreases as the temperature is lowered to about 100°K, and is relatively constant from there to 30°K, the lowest temperature investigated.20 Chance and Nishimura13 were unable to detect any evidence of cytochrome oxidation in *R. rubrum* at 80°K. Arnold and Clayton3 observed a reversible light-induced increase in optical density at 420 nm in *Rhodopseudomonas spheroides* at low temperature. They found that the decay of the optical density change, when compared with the room temperature decay, is somewhat slower at 250°K and considerably faster at 77 and 1°K.

In 1941 Szent-Gyorgyi56 suggested that the pigment system in photosynthetic organisms may have an electronic energy band structure similar to that of inorganic crystals. Later Katz27 proposed, on the basis of
fluorescence studies, that the chlorophyll and its associated protein could form a system analogous to a two-dimensional crystal, with the early processes of photosynthesis taking place in this "solid-state" matrix. Similar ideas were also developed independently by Calvin, based in part on studies of model systems. Since then, many workers have found support for the solid-state or semiconductor model with measurements on various photosynthetic systems of such properties as glow curves, EPR and optical density changes at low temperature, delayed light, and photoconductivity.

The rapid rates which I have observed at low temperature with the light-induced EPR signals in *R. rubrum* are also suggestive of a solid-state or semiconductor model. In a pure crystal lattice, where phonon scattering is the main interaction, the electron mobility increases as the temperature is decreased. Relatively simple theory predicts that the mobility will be proportional to \( T^{-3/2} \). If impurity atoms are present, they also can scatter electrons, and at high enough concentrations the electron mobility may decrease at low temperature. This behavior has been experimentally confirmed. The electron mobility as a function of temperature for a series of arsenic-doped germanium crystals, as measured by Debye and Conwell, is shown in Figure IV-15. The numbers correspond to different samples with varying concentrations of arsenic impurity: sample 55 contains the lowest impurity concentration and sample 58, the highest. The electron mobility of the purest sample follows quite well the \( T^{-3/2} \) behavior predicted by theory. Samples with higher impurity concentrations deviate more and more from the pure crystal prediction.
Fig. IV-15. The electron mobility as a function of temperature for a series of arsenic-doped germanium crystals, as measured by Debye and Conwell.21 The effective decay rate constants of the light-induced EPR signal in dried films of whole cell R. rubrum are also plotted. The two triangular points are the decay rate constants for the EPR signal in films of phthalocyanine and ortho-chloranil.28
I have also plotted on the same graph the effective decay rate constant of the light-induced EPR signal in whole cell *R. rubrum*. The effective decay rate constant is the reciprocal of the effective decay time constant; rates calculated from the individual measured decay times or from the rise times show the same general behavior with temperature. If the movement of an electron through a solid matrix is the rate-limiting step in the formation and decay of the EPR signal, then the rise and decay rates should be proportional to the electron mobility. Further, if this solid matrix in the bacteria has characteristics similar to those of a semiconductor, then a plot of the decay rates in *R. rubrum* should be similar to a plot of the electron mobility in a semiconductor. The comparison between the curves in Figure IV-15 is not very encouraging for the semiconductor model in *R. rubrum*. The most difficult feature to swallow is the rapid increase in rates between 300 and 200°K, although it is possible to envisage mechanisms which could show this behavior, in which electron migration is the rate-controlling step.

For reasons which will be brought out later, the following is a quite reasonable picture for the production of the EPR signal: light photo-oxidizes the special bacterial chlorophyll at the photochemical reaction site and an electron is then transferred from a neighboring molecule to the chlorophyll, creating the observed unpaired electron on the neighboring molecule. If the relative orientation of the chlorophyll and its neighboring molecule changes on cooling, this could result in a striking increase in the electron mobility. We hope to test the possibility of changing orientation in the near future, by studying the circular dichroism of the special chlorophyll in bacterial systems.
as a function of temperature. In any event, we are still left with the fact that the rates do not show a decrease with temperature to below 2°K, and it seems clear that electron (or hole) migration must be involved in the production and decay of the EPR signal even if it is not the rate-controlling step.

Some work has been done with organic semiconductors as photosynthetic model systems. Kearns, Tollin and Calvin\textsuperscript{28} have studied films of phthalocyanine with ortho-chloranil deposited on one surface. These samples show an EPR signal which reversibly decreases in the light. The two triangular points connected by a short dashed line in Figure IV-15 are proportional to the rate constants for the decay of the photochange when the light is turned off. Although we have only two points, they seem to be similar to the inorganic semiconductors in that their slope is less steep than that of the pure crystal theoretical prediction.

In addition to the scheme in which electron migration is the rate-controlling step, I have considered several other models which might predict the observed kinetic results.

In the electron-hole trap model, we postulate that the light-induced EPR signal resides on a positively charged molecule embedded in a solid state matrix. The signal is created by a photoionization of this molecule and decays by recombination with electrons from the bulk matrix. Throughout the bulk matrix there are electrons in quite shallow traps and holes in relatively deep traps. At low temperatures, the holes remain trapped and the electrons neutralize the EPR signals. At higher temperatures, however, the holes begin to escape from their traps and compete with the EPR species for the electrons. Thus fewer electrons are available to neutralize the EPR signal at higher
temperature, and the decay rate becomes slower. I have made quantitative calculations based on this model and find that with appropriate selection of the hole and electron trap depths and their relative number it is possible to reproduce the observed temperature dependence of the decay rate. Unfortunately, the choices of parameters which predict an increase in decay rate with decreasing temperature also predict a decrease in rise rate with decreasing temperature, contrary to what is observed.

In another model which I have considered, the kinetics are determined by the rate of transfer of an electron from a donor molecule to an acceptor molecule. We assume that this transfer is intrinsically rapid, but the donor molecule has a certain amount of thermal motion, and the electron transfer is possible only in one of its orientations. At high temperature, the rise and decay rates of the EPR signal are determined by the rate of movement of the donor molecule (relatively slow). At low temperature, however, the donor molecule is frozen into its various possible configurations. Most of the donor molecules are in configurations where they are unable to transfer an electron and their light-induced signals will be either frozen in or frozen out. A small proportion of the donor molecules will be in a position where electron transfer is possible and for these we will observe the rapid intrinsic rate. This picture corresponds fairly well to the observed behavior.

As was stated earlier, most of my kinetic data could be fit quite well by the sum of two exponentials. This would suggest that we have two independent first-order reactions. On the other hand, the observation that the individual time constants are not completely reproducible suggests that the sum of exponentials is simply approximating
a more complex kinetic curve. In an effort to gain more insight into the reaction mechanism, I have also tried other methods for analyzing the data. Figure IV-16 is a second-order plot of the rise curve shown in Figure IV-10. With second-order kinetics, the points should fall on a straight line. Although this rise curve obviously does not follow second-order kinetics, it is actually typical of the best fits which I obtained; most of the rise and decay curves deviated much further from second-order behavior.

Cope\textsuperscript{18} has stated that the decay of the light-induced EPR signal in an aqueous suspension of \textit{R. rubrum} chromatophores measured by Ruby, Kuntz and Calvin\textsuperscript{49} could be described by the Elovich equation. The Elovich equation is a theoretical prediction for reactions which are rate-limited by electron transport across an activation energy barrier at a liquid-solid interface. I have plotted many of my kinetic curves to test for conformity to the Elovich equation, which may be written as

$$-\frac{dx}{dt} = m e^{nx},$$

where \(x\) is the substrate concentration, \(t\) is the time, and \(m\) and \(n\) are constants. If a set of data can be described by the Elovich equation, a plot of \(\log(-dx/dt)\) against \(x\) should form a straight line.\textsuperscript{18} Such a plot is shown in Figure IV-17; the set of data plotted here is the decay curve shown in Figure IV-10. The points fit a straight line quite well over most of the range, but deviate strongly at low substrate concentrations. Ruby's data actually show a similar behavior at low concentrations, although this was not indicated in Cope's paper.
Fig. IV-16. A second-order plot of the rise curve shown in Fig. IV-10.
Second-order kinetics would be indicated by a straight line.
Fig. IV-17. An Elovich plot of the decay curve shown in Fig. IV-10.

Conformity to the Elovich equation would be indicated by a straight line.
Bacterial Chlorophyll Molecules per Unpaired Electron

At low light intensities, the intensity of the light-induced signal in *R. rubrum* is proportional to light intensity, but at high light intensities (about $10^{16}$ quanta/cm$^2$ sec in my case) light saturation is observed and a maximum number of unpaired electrons is produced. It is of interest to inquire whether this maximum number of unpaired electrons corresponds to any other quantity in the sample, such as the number of bacterial chlorophyll molecules. Beinert and Kok$^9$ have reported such a comparison, and obtain a value of 63 bacterial chlorophylls per light-induced unpaired electron. Unfortunately, their accuracy was uncertain (the authors state that their results may be in error by a factor of 2 or 3).

In order to make this comparison, it is of course necessary to obtain good values for both the absolute number of unpaired electrons and the absolute number of chlorophyll molecules in the sample. The method used to determine the absolute number of unpaired electrons in the sample is described in Section III. The number of chlorophyll molecules was determined from optical density measurements. The molar extinction coefficient of bacterial chlorophyll in acetone has recently been determined by Sauer, Lindsay Smith and Schultz$^{50}$ to be $6.92 \times 10^4$ l/m cm at 770 nm. Using this value, I determined the molar extinction coefficient of bacterial chlorophyll in 4:1 acetone:water ($6.63 \times 10^4$ l/m cm at 770 nm). By measuring the optical density of chlorophyll in the *in vivo* system and then extracting all of the chlorophyll with 4:1 acetone-water and again measuring the optical density, I obtained a value of $1.16 \times 10^5$ l/m cm for the molar extinction coefficient of
bacterial chlorophyll in \textit{R. rubrum} at the 880 nm absorption maximum.\footnote{The 880 nm absorption band is considerably narrower than the 770 nm band in acetone-water. This accounts for much of the increase of the extinction coefficient in the \textit{in vivo} system. Rough measurements indicate that the oscillator strength is 30\% higher in the \textit{in vivo} system compared with acetone-water. This is quite interesting in the light of Sauer, Lindsay Smith and Schultz's\textsuperscript{50} finding that the oscillator strength of the red band in bacterial chlorophyll increases by 14.5\% in going from the monomer to the dimer.} This value assumes that all of the \textit{in vivo} chlorophyll is equally represented in the 880 nm band. If this assumption is not completely valid it will not affect the accuracy of my final calculation, however, since any deviation will cancel out when the extinction coefficient is used to calculate the concentration of chlorophyll in the experimental sample.

To obtain a sample with a known number of bacterial chlorophyll molecules the optical density of a suspension of \textit{R. rubrum} was measured and a known weight of this suspension was pipetted onto a quartz slide and dried. Care was taken to obtain a uniform thin film of \textit{rubrum} on the slide. (O.D. about 0.1 at 880 nm). The number of chlorophyll molecules actually used in the calculation was one half the number on the slide, since the grid on the bottom of the cavity prevented light from reaching half of the sample. (It was assumed that the excitation energy cannot travel distances comparable to the width of the grid, about 0.5 mm.) Table II shows the results of two experiments. The last column is based on the total number of unpaired electrons observed in the light. The fast-light signal corresponds to about 55\% of the total observed signal intensity; assuming this value and taking the average value of 30 bacterial chlorophyll molecules per total unpaired electron, I calculate that the number of bacterial chlorophyll molecules per fast-
light unpaired electron is $55 \pm 15$. Most of the uncertainty in this value is due to possible systematic errors, especially those related to the calibration of the number of unpaired electrons. Assuming a typical value of 40% for the contribution of the slow-light signal yields approximately 75 bacterial chlorophyll molecules per slow-light unpaired electron. Similarly assuming a typical value of 5% for the contribution of the dark signal yields approximately 600 bacterial chlorophyll molecules per dark unpaired electron.

Table II

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<th>Expt.</th>
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<td>$6.7 \times 10^{14}$</td>
<td>$2.08 \times 10^{13}$</td>
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</tbody>
</table>

The value of 55 bacterial chlorophylls per fast-light unpaired electron is particularly interesting with regard to the picture of a "special chlorophyll" molecule in a photochemical reaction site that was discussed in Section I. In photosynthetic bacteria this special chlorophyll normally represents 2 to 5% of the total chlorophyll. In *R. rubrum* the special chlorophyll is commonly referred to as P890; analogous pigments, P890 and P870, are found in the bacteria *Chromatium* and *Rhodopseudomonas spheroides*, respectively. The relative proportions of the special and ordinary chlorophylls are often measured spectroscopically, assuming that the peak extinction coefficients of the two pigments are equal. Using this method, the number of bacterial chlorophyll molecules per P890 has been determined variously as 40 and 50. Nishimura has studied the
production of ATP in \textit{R. rubrum} by light flashes of varying intensities and found that there is one reactive center for every 43 bacterial chlorophylls. The close correspondence between the number of fast-light unpaired electrons and the number of P890 molecules supports the idea that these electrons are closely associated with the photochemical reaction center.

There is another interesting calculation which can be made using the data of these experiments. It was mentioned earlier that Ruby, Kuntz and Calvin\textsuperscript{49} found that the decay kinetics of the light-induced EPR signal were identical with the decay kinetics of the O.D. change at 433 nm in \textit{R. rubrum}. Assuming that the same molecule is responsible for the EPR signal and the O.D. change at 433 nm, and that there is one spin per molecule, we can estimate the extinction coefficient of that molecule at 433 nm. Kuntz, Loach and Calvin\textsuperscript{35} report an O.D. change at 433 nm of 0.0135 for a sample of \textit{R. rubrum} chromatophores with an O.D. at 880 nm of 2.2 (see Figure V-1). The sample in Experiment I of Table II was 0.0093 gm of a suspension with an O.D. at 880 nm of 16.85. Using these data and assumptions, I calculate that our molecule has an extinction coefficient of $4 \times 10^4$ l/m cm. This estimate is probably good within an order of magnitude. The O.D. change at 433 nm in whole cells is considerably smaller than in chromatophores\textsuperscript{34} and leads to a correspondingly smaller estimate for the value of the extinction coefficient ($1 \times 10^4$). I preferred not to use the whole cell data since the shape of the difference spectra suggests that there may be considerable cancellation of the 433 nm peak in the whole cell difference spectrum.
CONCLUDING DISCUSSION

We will now take up again the questions which were raised in the Introduction: Are the EPR signals directly related to photosynthesis, and what is the molecular species responsible for them?

Quantum yields of the order of 1 would indicate that the EPR signal is on the mainstream of photosynthesis, but these measurements are difficult to make. Sogo, Carter, and Calvin\textsuperscript{54} report a quantum yield of 0.03 in spinach chloroplasts. Ruby\textsuperscript{47} has obtained a value of order of magnitude 1 with \textit{R. rubrum} chromatophores. In both cases the uncertainty is quite large. Action spectra, which measure the dependence of the EPR signal intensity on the wavelength of exciting light, indicate that production of the EPR signal parallels general photosynthetic activity.\textsuperscript{36,48,63} My determination that there is one rapidly reversible unpaired electron per photochemical reaction center also strongly suggests that the EPR signal is directly related to the primary process in photosynthesis.

The evidence pinpointing the molecular species responsible for the EPR signal is not so clear. Experiments, described in the Introduction, on mutants lacking the special chlorophyll, and on pigment extracts containing the photochemical reaction center, indicate that the light-induced signal is associated with the reaction center. The fact that I can observe reversible light-induced EPR signals at 2\textdegree K also implies a close association with the site where the light energy is trapped, at least for part of the signal in \textit{R. rubrum}.

It will be helpful now to relate our arguments to the redox scheme
for bacterial photosynthesis shown in Figure I-3 (page I- ). Although only a few compounds are indicated in the figure, each dashed arrow represents an electron transport chain containing many different molecules. In the dark, all these compounds will be in some mixture of oxidized and reduced states. When the light is turned on, electrons are transferred in the direction of the arrows: bacterial chlorophyll and all compounds to the right of it become more oxidized; all compounds to the left of the solid arrow become more reduced. Loach\textsuperscript{12,37} has found that the action of light in producing the EPR signal in \textit{R. rubrum} can be mimicked by mild oxidation. Thus the light-induced EPR signal is produced by oxidation and the molecule responsible for it should be on the redox chain terminating in bacterial chlorophyll.

Figure V-1 shows the absorption spectrum and light-minus-dark difference spectrum of \textit{R. rubrum} chromatophores. In freshly prepared chromatophores, the decay kinetics of the various light-induced OD changes were indistinguishable.\textsuperscript{33} After the preparation aged for a day or so, however, Kuntz found that the decay kinetics of the various OD changes were no longer identical.\textsuperscript{33} It also appears that the relatively high intensity of the detecting beam used by Kuntz contributed to the kinetic separation.\textsuperscript{38,39} In any event, Ruby and Kuntz\textsuperscript{49} found that, in a sample showing the separated kinetics, the decay kinetics of the light-induced EPR signal did not match the decay kinetics of the 865 nm OD change, but instead matched the decay kinetics of the 433 nm OD change. The OD change at 865 nm is identified with the one-electron photo-induced oxidation of the special bacterial chlorophyll in the photochemical reaction center.\textsuperscript{14,37} On this basis I conclude that the unpaired
Fig. V-1. Absorption spectrum and light-minus-dark difference spectrum of *R. rubrum* chromatophores as measured by Kuntz. Note that the vertical scale below 650 μm in the difference spectrum is expanded twofold. The sample used for the difference spectrum had an O.D. of 2.2 at 880 μm.
electron is not on special bacterial chlorophyll, but instead is associated with the molecule responsible for the 433 nm OD change (or another molecule with the same decay kinetics). Unfortunately, the compound responsible for the 433 nm change has not been identified. 34,53,60

Clayton 14 has found that all the light-induced OD changes in \( R. \) spheroides, a photosynthetic bacteria quite similar to \( R. \) rubrum, can also be produced by chemical oxidation. The light-minus-dark difference spectrum of \( R. \) rubrum, including the peak at 433 nm, is almost identical with that of \( R. \) spheroides. If we assume that Clayton's results can be applied to \( R. \) rubrum, then the increase in absorption at 433 nm is the result of an oxidation. This is a crucial deduction, since it will help us decide which molecules can be responsible for the OD change.

Accepting for the present my calculation that the extinction coefficient of our molecule at 433 nm is of the order of \( 10^4 \), let us consider the possible candidates. First of all, chlorophyll is eliminated because the decay kinetics at 433 nm can differ from those at 865 nm. 35,44 Although some cytochromes absorb in the region of 433 nm, they all exhibit a blue shift on oxidation. Typical cytochrome spectra are shown in Figure V-2 29 and V-3. 43 The 433 nm peak in \( R. \) rubrum, if anything, is part of a red shift, and thus cytochromes are eliminated. Quinone extinction coefficients are much lower than \( 10^4 \) in this region, 19 so they also are ruled out. We are left with the flavins, which have a sufficiently high extinction coefficient in this region and also show an increase in absorption on oxidation. 41 The absorption spectra of oxidized and reduced FMN, a flavin common in photosynthetic systems, is shown in Figure V-4. 8
Fig. V-2. Absorption spectra of oxidized and of reduced cytochrome c from horse heart; this preparation contained 0.45 per cent iron. [From D. Keilin and E. C. Slater, Brit. Med. Bull., 9, 89 (1953).]
Fig. V-3. Difference spectrum for oxidized-minus-reduced cytochrome c.\textsuperscript{43}
Fig. V-4. Spectra of oxidized and reduced flavin mononucleotide (FMN).
I would now like to make a few suggestions for further experiments in this field. If we wish to determine the molecular species responsible for the EPR signals observed in photosynthetic systems, it seems to me that the most fruitful approach to take at this point is the correlation of the EPR signal and OD changes. A comparison of the EPR and OD kinetics in a green system might well be fruitful. A more extensive study of the 433 nm change in \textit{R. rubrum} seems warranted. In particular, Vrendenberg and Duysens report that while all the cytochrome changes in \textit{R. rubrum} are frozen out at low temperature, the 433 nm change is still observed at 100 °K. It would be interesting to compare the kinetics of the 433 nm peak and the EPR signal at liquid nitrogen temperature to see if they are still identical. Another interesting question which could be investigated is the effect of cell age on the EPR signal. It is usually implicitly assumed that one is dealing with a homogeneous sample, but this is probably not the case with micro-organisms. The doubling time of healthy, growing \textit{R. rubrum} cells is about six hours, and a given sample should contain cells of all ages. There is no a priori reason to suppose that cells of different ages would have, for example, exactly the same kinetic response to light or the same response to changes in temperature. It may be that some of our observations are artifacts resulting from a non-homogeneous sample. This possibility could be tested by repeating the experiments with synchronously growing cultures.
APPENDIX A

Growth medium used for *Nostoc muscorum*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration in Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO$_3$</td>
<td>20 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>4.0</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>2.0</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>1.0</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>0.5</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>0.092</td>
</tr>
<tr>
<td>MnCl$_2$</td>
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</tr>
<tr>
<td>ZnSO$_4$</td>
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</tr>
<tr>
<td>CuSO$_4$</td>
<td>0.00063</td>
</tr>
<tr>
<td>CoCl$_2$</td>
<td>0.00034</td>
</tr>
<tr>
<td>MoO$_3$</td>
<td>0.00021</td>
</tr>
<tr>
<td>FeSO$_4$</td>
<td>0.072</td>
</tr>
<tr>
<td>Versenol 120</td>
<td>0.06 ml</td>
</tr>
</tbody>
</table>
Decomposition of the Rise and Decay Kinetics

The kinetic curves obtained in these experiments were plotted on a semi-log scale and decomposed into exponentials. Usually two exponentials were sufficient to fit the data. The time constants obtained by this method are listed in Tables III and IV.

The following notes refer to these tables:

a The sample in these runs was dried films of R. rubrum chromatophores. In all other runs the sample was dried films of whole cell R. rubrum.

b This column gives the nominal time constant of the instrument. This is the value indicated on the lock-in amplifier. A 12 db/octave roll-off was used, and with this setting the actual instrumental time constant was measured to be approximately 1.5 times the indicated value. The minimum overall instrument time constant was about 0.01 seconds.

c These columns give the measured exponential time constants. In a few cases the measured time constants are comparable to the instrumental time constant. In order to correct for this, a correction factor of 1.5 times the corresponding RC value was subtracted from all measured time constants before they were used to calculate the effective time constants. The relative contributions of the exponentials necessary to reproduce the experimental curves are indicated by the percentages in parentheses.
### Table III

**RISE KINETICS**

<table>
<thead>
<tr>
<th>Run</th>
<th>Temperature (°K)</th>
<th>$R_c^b$</th>
<th>Fast$^c$</th>
<th>Slow$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>78-1$^a$</td>
<td>297</td>
<td>0.01</td>
<td>1.81 (100%)</td>
<td></td>
</tr>
<tr>
<td>80-2$^a$</td>
<td>297</td>
<td>0.01</td>
<td>3.41 (100%)</td>
<td></td>
</tr>
<tr>
<td>87-1</td>
<td>297</td>
<td>1.0</td>
<td>3.4 (47%)</td>
<td>9.1 (53%)</td>
</tr>
<tr>
<td>87-1</td>
<td>297</td>
<td>1.0</td>
<td>2.3 (58%)</td>
<td>21.9 (42%)</td>
</tr>
<tr>
<td>161-1</td>
<td>297</td>
<td>0.1</td>
<td>.43 (48%)</td>
<td>3.04 (52%)</td>
</tr>
<tr>
<td>178-6</td>
<td>297</td>
<td>0.1</td>
<td>.59 (77%)</td>
<td>3.5 (23%)</td>
</tr>
<tr>
<td>180-5</td>
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<td>0.1</td>
<td>.35 (72%)</td>
<td>2.0 (28%)</td>
</tr>
<tr>
<td>180-4</td>
<td>266</td>
<td>0.1</td>
<td>.13 (70%)</td>
<td>1.5 (30%)</td>
</tr>
<tr>
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<td>263</td>
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<td>.12 (35%)</td>
<td>.75 (65%)</td>
</tr>
<tr>
<td>180-2</td>
<td>237</td>
<td>0.1</td>
<td>.15 (67%)</td>
<td>.67 (33%)</td>
</tr>
<tr>
<td>180-1</td>
<td>233</td>
<td>0.1</td>
<td>.076 (42%)</td>
<td>.41 (58%)</td>
</tr>
<tr>
<td>79-5$^a$</td>
<td>.79</td>
<td>0.01</td>
<td>.098 (57%)</td>
<td>.48 (43%)</td>
</tr>
<tr>
<td>81-2$^a$</td>
<td>79</td>
<td>0.01</td>
<td>.096 (46%)</td>
<td>.69 (54%)</td>
</tr>
<tr>
<td>179-7</td>
<td>79</td>
<td>0.01</td>
<td>.076 (59%)</td>
<td>.19 (41%)</td>
</tr>
<tr>
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<td></td>
<td>.064 (100%)</td>
</tr>
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<td>186-1</td>
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<td>0.01</td>
<td>.03 (50%)</td>
<td>.31 (50%)</td>
</tr>
<tr>
<td>82-3$^a$</td>
<td>4.2</td>
<td>0.01</td>
<td>.028 (27%)</td>
<td>.31 (73%)</td>
</tr>
<tr>
<td>185-2</td>
<td>1.96</td>
<td>0.01</td>
<td>.124 (100%)</td>
<td></td>
</tr>
<tr>
<td>186-4</td>
<td>1.68</td>
<td>0.01</td>
<td>.047 (37%)</td>
<td>.152 (63%)</td>
</tr>
</tbody>
</table>
### Table IV

**DECAY KINETICS**

<table>
<thead>
<tr>
<th>Run</th>
<th>Temperature (°K)</th>
<th>RC (sec)</th>
<th>Exponential Time Constants (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fast</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slow</td>
</tr>
<tr>
<td>78-2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>297</td>
<td>0.01</td>
<td>8.95 (100%)</td>
</tr>
<tr>
<td>80-4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>297</td>
<td>1.0</td>
<td>19.5 (100%)</td>
</tr>
<tr>
<td>87-1</td>
<td>297</td>
<td>1.0</td>
<td>4.2 (34%) 16 (66%)</td>
</tr>
<tr>
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<td>297</td>
<td>0.1</td>
<td>3.4 (32%) 18.1 (68%)</td>
</tr>
<tr>
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</tr>
<tr>
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<td>273</td>
<td>0.1</td>
<td>.50 (48%) 8.3 (52%)</td>
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<td>.24 (35%) .84 (22%) 4.4 (44%)</td>
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<td>.20 (46%) 2.71 (54%)</td>
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<tr>
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<td>.13 (43%) 1.09 (57%)</td>
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<td>.15 (59%) 1.2 (41%)</td>
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<tr>
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<td>0.01</td>
<td>.05 (69%) 1.04 (31%)</td>
</tr>
<tr>
<td>186-1</td>
<td>80</td>
<td>0.01</td>
<td>.05 (61%) .48 (39%)</td>
</tr>
<tr>
<td>82-4&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>0.01</td>
<td>.072 (48%) .71 (52%)</td>
</tr>
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<td>0.01</td>
<td>.059 (42%) .405 (58%)</td>
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<tr>
<td>186-4</td>
<td>1.68</td>
<td>0.01</td>
<td>.13 (51%) .56 (49%)</td>
</tr>
</tbody>
</table>
APPENDIX C

Abbreviations Used

ADP  adenosine diphosphate
ATP  adenosine triphosphate
BChl  bacterial chlorophyll
Chl  chlorophyll
Cyt  cytochrome
EPR  electron paramagnetic resonance
GHz  gigahertz \(10^9\) cycles per second
nm  nanometers \(10^{-9}\) meters = 1 millimicron
OD  optical density (Absorbance)
TPN  triphosphopyridine nucleotide
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