Title

Free Radicals in Photosynthetic Systems

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ABSTRACT

The method of detecting unpaired electrons in liquid and solid systems by electron spin resonance is discussed. The significance of the hyperfine structure in electron spin resonance is discussed and the possible use of these structural features of the electron spin resonance spectrum to elucidate the nature of the photoproduced unpaired electrons in photosynthesizing systems is introduced.

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One bit of evidence introduced in the previous paper (Chapter) concerned the possibility that the quantum conversion act of photosynthesis might be a production of unpaired electrons which were successively trapped and then handed down to other acceptors (chemical acceptors) to do their job by ultimately reducing carbon dioxide.

That particular evidence was not explained. It was simply stated that there was evidence of unpaired electrons. The nature of this evidence may now be explained. Those curves previously shown are known as electron spin resonance absorption spectra. What I would like to do now is describe what electron spin resonance is, how we may hope to use it for the detection of unpaired electrons in biological systems, how it has been used, and how we may hope to use if further to identify the nature of the unpaired electrons that might and do occur in biological systems.

Electron spin resonance is another spectroscopic method. Zavoisky was the first to use it to detect unpaired electrons in physical systems. It is based on the principle that an electron has a spin, and that this spin gives it a magnetic moment such that when placed in a magnetic field it will orientate itself with respect to that magnetic field in certain specific directions. In the case of the electron, the spin is said to be one-half of a unit, and this leads to only two possible orientations of the electron in an external field--with the field and against it.

Figure 1 shows the diagramatic representation of this situation. On the left we have the case in which the electrons are in between the pole faces of a magnet, but the electric current is not yet flowing and the electron magnetic moments are randomly arranged. When we turn on the field (as on the right), some of the electrons will orientate themselves with the external field, and some against it. Now, these two orientations do not have the same energy. These energies are represented here by E_1 and E_2, and the difference between these two energies is equal to the product of the magnetic moment (μ_e), the gyromagnetic ratio (g_o), and the value of the external magnetic field (H_o). This difference in energy can be expressed in terms of the frequency $h\nu$, and for an

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electron in a field of about 3,000 Gauss, the wave length corresponding to the transition is three centimeters.

The way in which these unpaired electrons may be observed is simply to shine electro-magnetic energy on them with a wavelength corresponding to the transition, and watch for absorption of this characteristic frequency ν and varying the magnetic field H_o. One then determines the magnetic field at which absorption occurs. This is the reason it is called electron spin resonance absorption--the field at which resonance absorption with the 3.2 centimeter wave occurs.

Figure 2 indicates the manner in which the experiment is done. Here is the three-centimeter generator, and the external field. In the presence of the magnetic field some of these electrons will be oriented parallel with the field, some antiparallel. There will be more in the lower (parallel) state than there are in the upper (antiparallel), so there will be a net energy absorption as transitions between states occur.

Most of the apparatus in use today does not record direct absorption, although some of them do. Instead they record the derivative of this abosrption and the two graphs in the preceding section (Chapter) were these derivatives of absorption rather than the absorption directly.

This experiment then provides a method for detection unpaired electrons specifically, (since paired electrons mutually quench each other's magnetic moment). It provides a highly sensitive device, more sensitive in general than any mass susceptibility measurement can provide, because the latter must always correct for the diamagnetic material in which the electron is buried. In recent years this device has been used more and more in chemistry, and is now being used on biological materials to detect the presence of free radicals and to determine whether or not free radicals and unpaired electrons are participants in biochemical transformations.

The method can not only tell how many electrons there are, but also may determine something about the environment in which they are situated by the nature of this absorption record. It will be recalled that the energy of the transition is dependent upon the field that the electron itself sees, that is to say the external field due to the magnet, plus any magnetic field which the molecule itself may cause.

Now, the molecule itself is made up of nuclei and other electrons. Most of the other electrons, of course, are paired off, but the nuclei may have magnetic moments and some of them do have magnetic moments producing magnetic fields with which the unpaired electrons may interact.

If the odd electron not only sees the external magnetic field, but also a magnetic field of the molecule itself, one may observe the spectrum of Fig. 3. In this case the molecule is one which has a nitrogen atom in it, and the nitrogen atom itself has a nuclear spin of one unit. This means that the nitrogen nucleus can take up one of three orientations in the external field--parallel, antiparallel or normal to it. So that now the electron near the nitrogen atom may actually be subjected to three different magnetic fields. In one configuration, the magnetic field of the nucleus is added to the external field. In another, it is subtracted from the external field, and in the third case there is no effect upon the external field. So instead of a single peak we should get three, and Fig. 3 shows the three peaks due to the interaction with the spin of the nitrogen nucleus, one in the middle, and one on each side due to [illegible] nitrogen nucleus.

Nitrogen is, of course, not the only nucleus that has a magnetic moment. One of the commonest ones in organic substances is hydrogen. Hydrogen has a spin of a half, and it also can influence the spin spectrum of an odd electron in the case of molecules constructed so that the electron can interact with these protons.

Figure 4 shows a case of tetrachlorohydroquinone (lower right). The resonance field will not be modified by any of the atoms in the molecule since their nuclei are all of spin zero (no magnetic moment) and so you see only one line. If, however, one removes one of these chlorines and replaces it by a proton, and if the electron can interact with that proton at all, the proton, having two possible orientations in the magnetic field will split the resonance into two lines.

Now, with two protons, how many different arrangements can one get to change the external magnetic field? Remember that each one now can arrange itself with or against the magnetic field. One can have both with the field, one can have both against the field, or one can have one with and one against it. So there are three different ways in which these two protons can be arranged with respect to the external magnetic field. The third situation above is twice as probable as either of the other two, because one can have the left-hand proton up and the right down, or vice versa. Therefore, the middle peak should be twice as high as either of the two outside ones as indeed is the case. In both cases they will produce no net modification of the external field. With three protons there are four possible arrangements, and so one gets four peaks--with four protons there are five possible arrangements, and as seen in the figure, the amplitude ratios are the ordinary binomial coefficients.

Therefore, one may not only determine the number of odd electrons that are present in a system, but also ascertain something about the kind of an environment they are in. Figure 5 shows the compound, dibenzene chromium cation, which has one unpaired electron, and the question which arose in my mind was the location of this unpaired electron. We found that this unpaired electron of the chromium can actually see the 10 protons of the two benzene rings of this compound associated with the chromium atom. Ten protons have 11 possible arrangements, and so one should have 11 lines. The outside ones are very weak, because the probability of having all of the 10 protons oriented in the same direction is very small compared with the mixed configurations. So one can see how in transition elements we can determine something about the environment of the transition elements by this method as well.

Now, the last example before going to the biological material is a nice one. It's a perinapthalene radical--a beautiful symmetrical radical (Fig. 6). During preparation of perinapthalene it became accidentially oxidized, and when put in the machine, this is what we saw. It looked very formidable, but it didn't turn out too badly. The odd electron sees two different kinds of protons. If it is examined carefully, one will see that there are seven groups of lines--each group a quadruplet. This means that there are six electrons of one kind and three of another. The electron interacts more strongly with the one group of six protons than it does with the other group of three.

Now, how much of this can be used for the investigation of biological free radicals? Well, the answer is that it is only just beginning to be used. Beinert has been working with a fatty acyl CoA which he found produced a transient, colored intermediate when mixed with its substrate. This, he proposed, was a

free radical intermediate, and when it was examined at Stanford and Commoner Associates, an electron spin resonance signal was seen which could be distinguished from the resonance due to the Cu^{+2} in the enzyme (Fig. 7). Commoner and co-workers at St. Louis have presented evidence of free radical intermediates in the reactions of alcohol dehydrogenase and cytochrome oxidase when those enzymes are brought into contact with their substrates.

How does one distinguish this kind of a signal of a free radical from the one shown in the previous Chapter? First of all, radicals of this kind do not fade, of course, at low temperatures. They are frozen in. That is exactly what happens when one cools the system of chloroplasts, which was described previously. The fact of the matter is that one can find structured systems in which we can induce the signal at low temperature, but in which it will also fade at low temperature at a very high rate.

The occurrence of a signal due to an oxidation mechanism alone is illustrated by Fig. 8 in which the behavior of a methanolic extract of Rhodospirillum is shown when exposed alternately to oxygen and nitrogen. When a similar methanolic extract of Chlorella is illuminated in an oxygen and then in a nitrogen atmosphere, the increased signal of the former (Fig. 9) is considered as the sum of the contribution of an oxidative, a photooxidative, and an odd electron produced and trapped in a free radical. The relative contributions of these processes is apparent from the figure. Figure 10 then shows how cooling, even in oxygen, reduces the signal amplitudes. Here one sees that cooling to -145°C has practically eliminated the dark spectrum.

The spin resonance signal for Rhodospirilum is shown in Fig. 11 after five minutes of illumination, and then examined at the indicated temperatures. At 25°C it is the second from the smallest signal, at -55°C it is bigger, and at -15° it is bigger still. When the temperature is further reduced to -160°C, the intensity falls to the smallest shown. The time behavior is shown in Figure 12. The spectrometer is set on the peak of the signal, and one may now observe the rate of rise and the rate of decay of each of these signals at each temperature as a function of time. The room temperature signal is the second smallest shown, and it rises as fast as the instrument can follow. This signal then immediately decays after illumination ceases, to within the time constant of the machine. There is, however, no rapid decay at -15°, and at -55° there is a very rapid rise, a further slow rise, then followed by a small component of rapid decay, and a much longer slow decay. At -160° all we have left is very rapid rise and very rapid fall. Thus, I believe we have eliminated the possibility that we are producing free radicals directly by illumination. This behavior must be due to either untrapped carriers or trapped carriers in well shielded traps.

How can one account for this whole sequence of things? Referring back to Fig. 13 (introduced in the previous chapter) one sees that the first act is the absorption of light to produce an exciton; the exciton is then converted into conductive carriers, the carriers into chemical radicals, and the chemical radicals lead to stable chemicals. All that is required to account for the data of Fig. 12 is the presumption that each one of these successive acts has a higher temperature coefficient. The first step has no temperature coefficient, the second may have a very slight one, the third a higher one, and so on. At room temperature the energy was transported over into the chemical radicals, but these were decaying into stable chemicals very rapidly by the usual enzymatic process. The possibility that we are looking at a triplet state is eliminated by the nature of the signal. A triplet signal would be a double band very characteristic

of two unpaired electrons in the same molecule. A similar sequence of temperature effects is seen in the luminescence intensity (Fig. 14) and is similarly accountable presuming the integrated intensity to be a measure of the back reaction.

Another representation of the same scheme is shown in Fig. 15. The chlorophyll ground state is represented by a band instead of by a single line, because of the interaction of the chlorphyll molecules in these arrays previously shown in electron micrographs, and the excited singlet state would actually be a broader band. The triplet state is shown as a rather narrow band overlapping with the excited sequence. We haven't put the triplet directly in line here because we never observe triplet light emission.

This gives a working hypothesis to account for at least some of the problems, outlined at the beginning, that one must account for--the requirements to be satisfied, in order to convert this 35 kilocalories formula into useful chemical potential.

Figure Captions

Fig. 1. Energy States of Free Electrons in an External Magnetic Field.

Fig. 2. Absorption of 3 cm Waves Due to Transition of Free Electrons Between Energy States in an External Magnetic Field.

Fig. 3. ESR Spectra of Nitrosyl Disulfonate.

Fig. 4. ESR Spectra of Chloroquinones.

Fig. 5. Paramagnetic Resonance Absorption Spectrum of Bis-Dibenzene Chromium Cation.

Fig. 6. Paramagnetic Resonance Absorption Spectrum of Perinaphthenyl Radical.

Fig. 7. ESR Spectra from Coenzyme A Dehydrogenase plus Substrate.

Fig. 8. Rhodospirillum Methanol Extract Dark Signal at Room Temperature.

Fig. 9. Light and Dark Signals from Chlorella-methanol Extracts in Two Atmospheres at Room Temperature.

Fig. 10. Light and Dark Signals from Chlorella-methanol Extract at Two Temperatures.

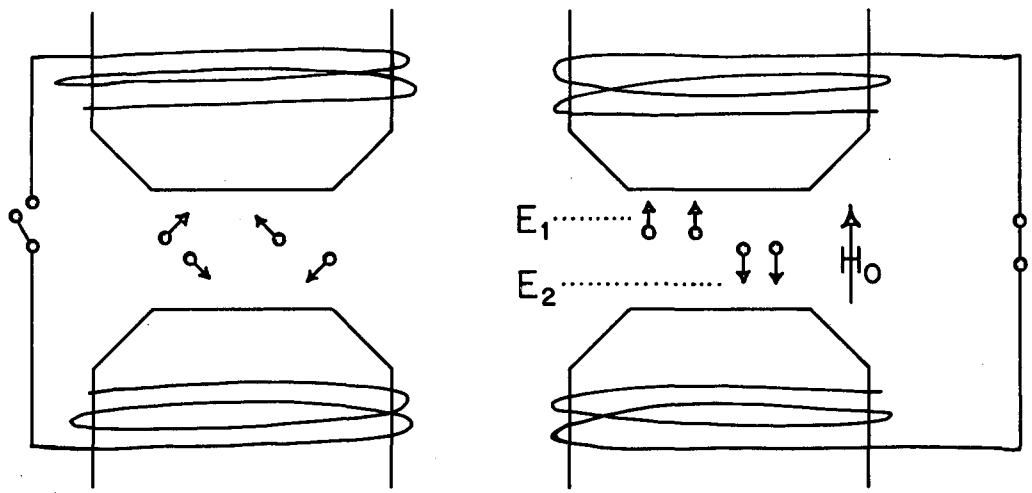
Fig. 11. ESR Signals from Rhodospirillum rubrum 5 minutes Continuous Illumination.

Fig. 12. Rise and Decay of ESR signals from Rhodospirillum rubrum.

Fig. 13. Hypothetical Scheme for Light Energy Utilization on Chloroplasts.

Fig. 14. Integrated Intensity of Spinach Chloroplast Luminescence from 0.0015-5.0 Seconds after Excitation as a Function of Temperature.

Fig. 15. Proposed Scheme for Various Photochemical Processes in Photosynthesis.



$$E_2 - E_1 = \mu_0 \cdot g_0 \cdot H_0 = \Delta E = h \cdot \nu = h \cdot c / \lambda$$

$$\lambda = \frac{h \cdot c}{\mu_0 \cdot g_0 \cdot H_0} \qquad \mu_0 = 0.927 \cdot 10^{-20} \qquad H_0 = 3{,}300 \qquad \lambda \sim 3{,}2 \text{ cm}$$

MU-15462

Fig. 1.

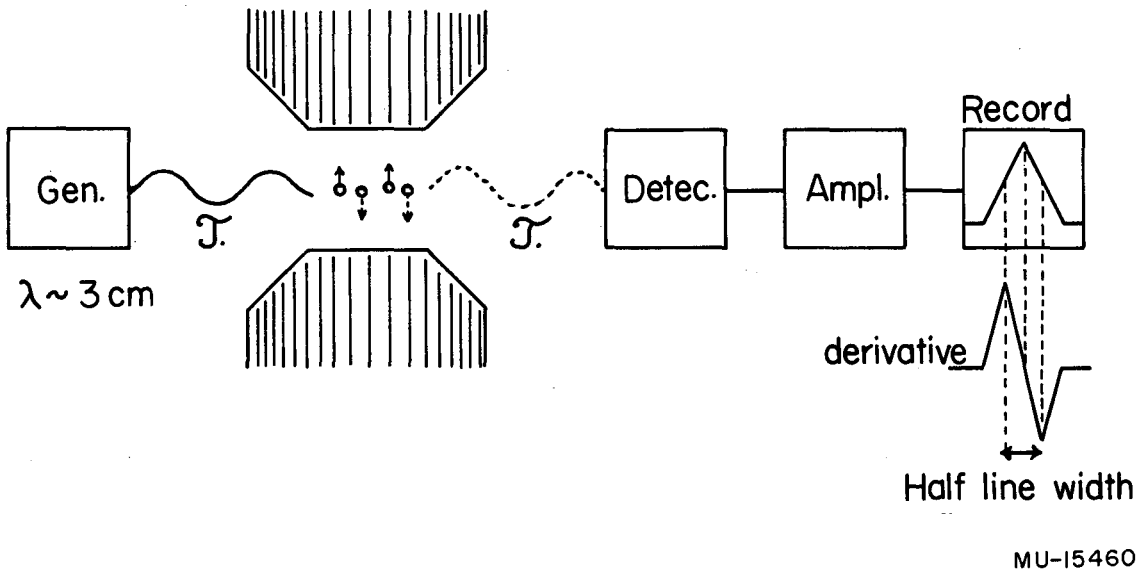

Gen.
λ~3 cm
Detec.
Ampl.
Record
derivative
Half line width
MU-15460

Fig. 2.

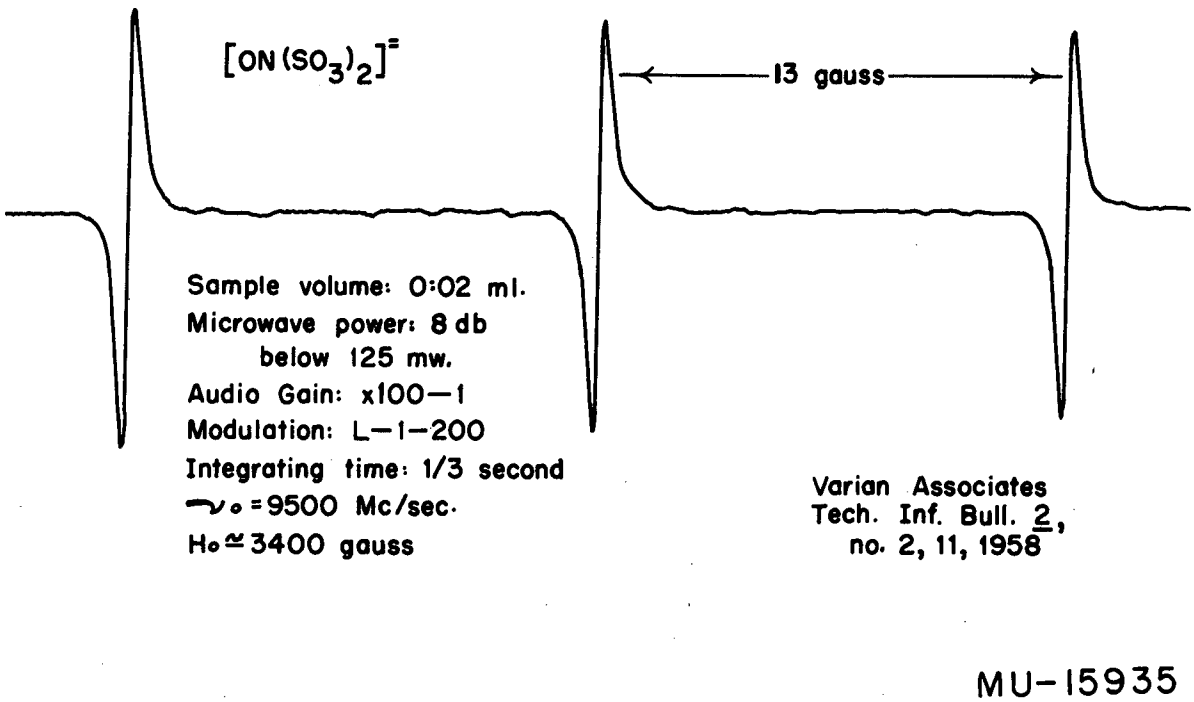

[ON(SO3)2]=
13 gauss
Sample volume: 0:02 ml.
Microwave power: 8 db
below 125 mw.
Audio Gain: x100—1
Modulation: L—1—200
Integrating time: 1/3 second
ν0 = 9500 Mc/sec.
H0 ≃ 3400 gauss
Varian Associates
Tech. Inf. Bull. 2,
no. 2, 11, 1958
MU-15935

Fig. 3.

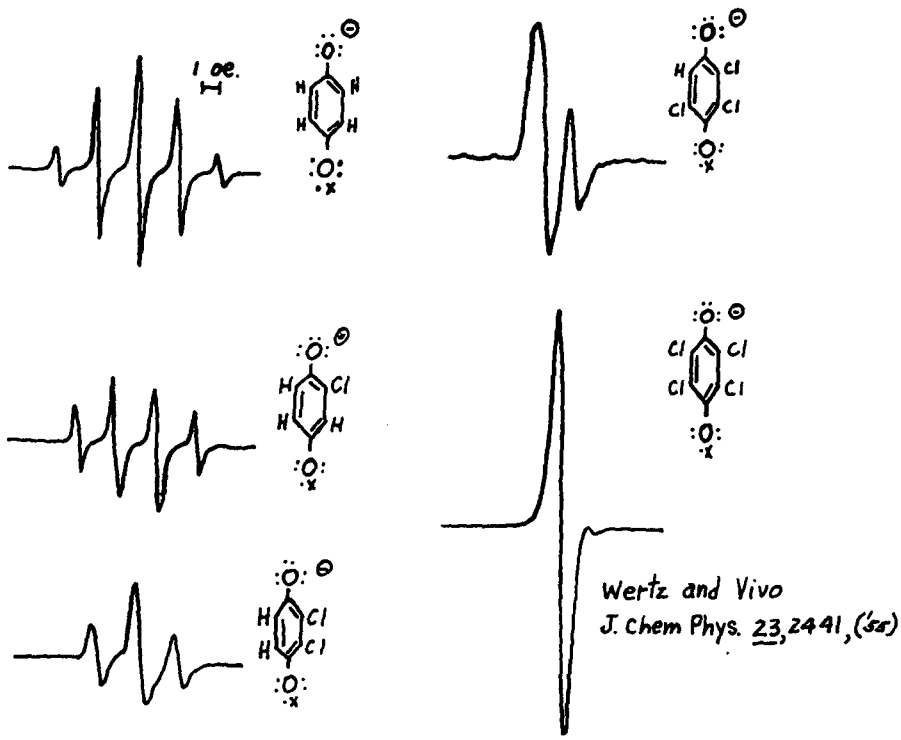


Fig. 4.

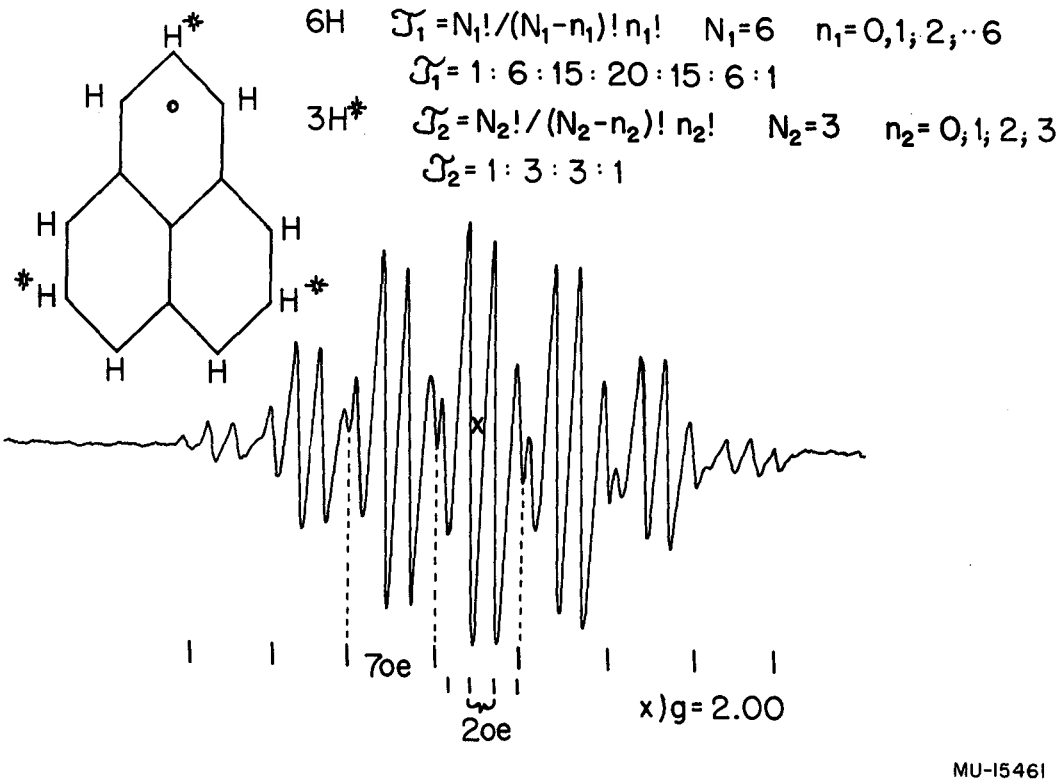

6H
$\mathcal{I}_1 = N_1!/(N_1-n_1)!\,n_1!$ $N_1=6$ $n_1=0,1,2,\cdots 6$
$\mathcal{I}_1 = 1:6:15:20:15:6:1$
3H*
$\mathcal{I}_2 = N_2!/(N_2-n_2)!\,n_2!$ $N_2=3$ $n_2=0,1,2,3$
$\mathcal{I}_2 = 1:3:3:1$
H*
H
H
H
H
*H
H*
H
H
7oe
2oe
x)g = 2.00
MU-15461

Fig. 5.

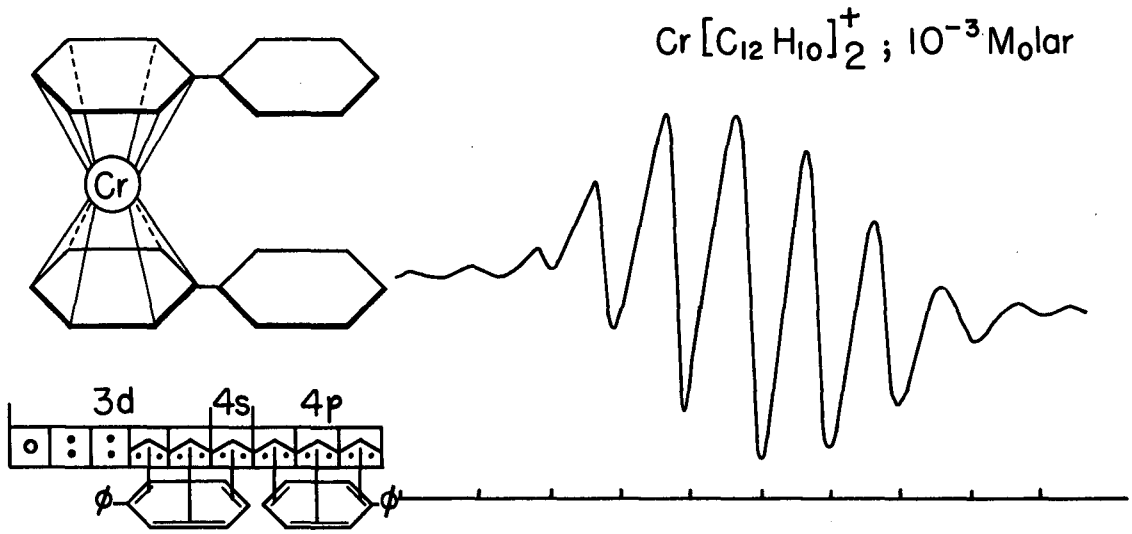
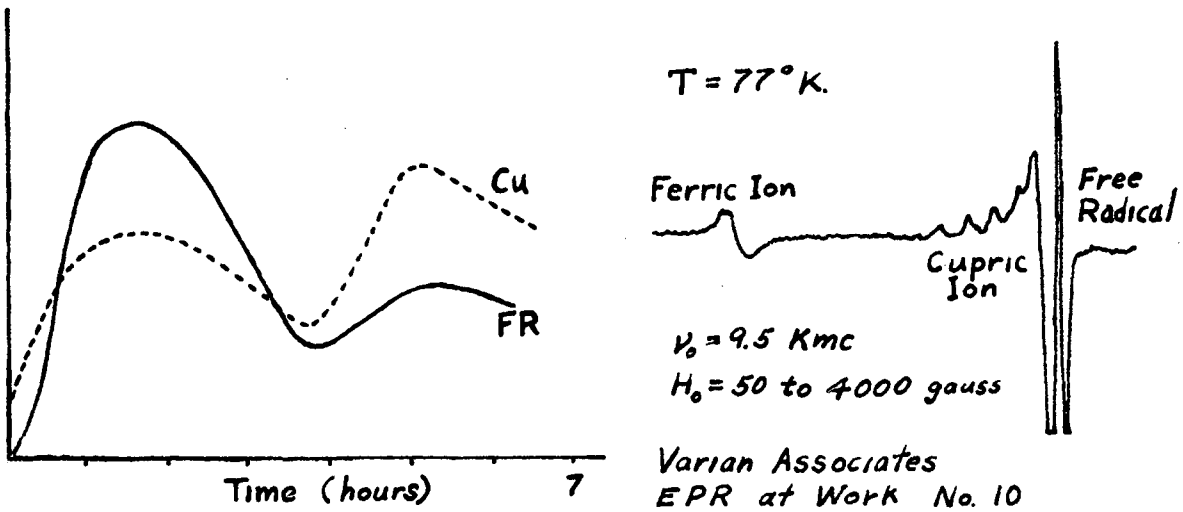
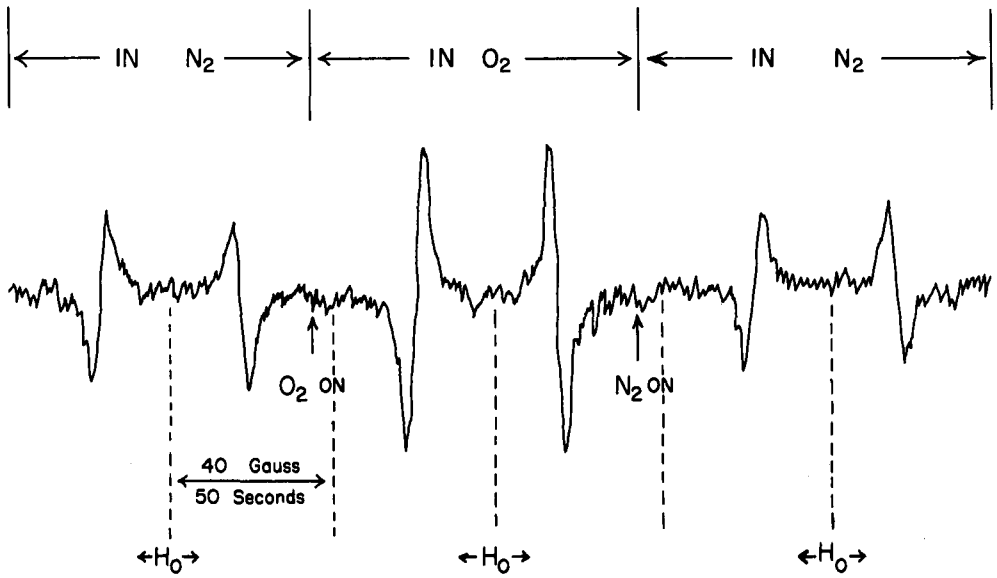

Cr [C12 H10]2+ ; 10-3 Molar
Cr
3d
4s
4p
g = 1.98
T = 1:10: 45: 120 :210:252:210:120:45:10:1
MU-15463

Fig. 6.



MU-15933

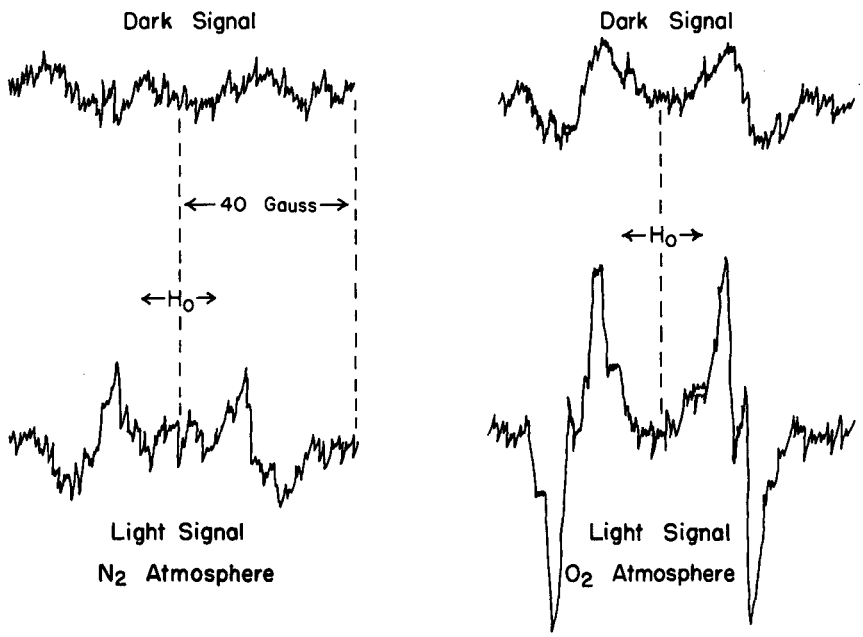
Fig. 7.



Rhodospirillum Methanol extract dark signal at room temperature

MU-15650

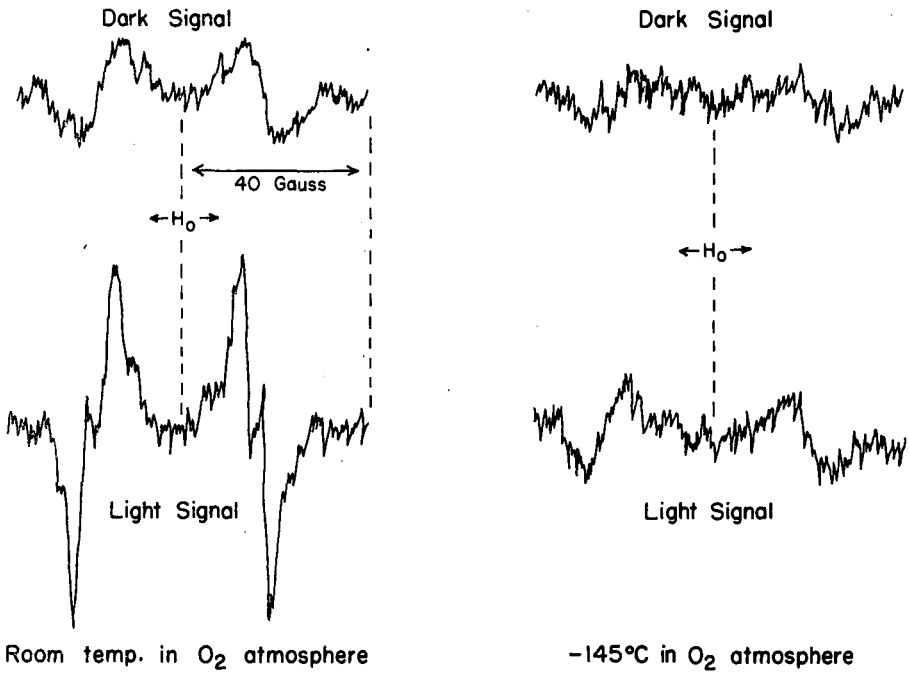
Fig. 8.



Light and dark signals from Chlorella-methanol extract in two atmospheres at room temperature

MU-15648

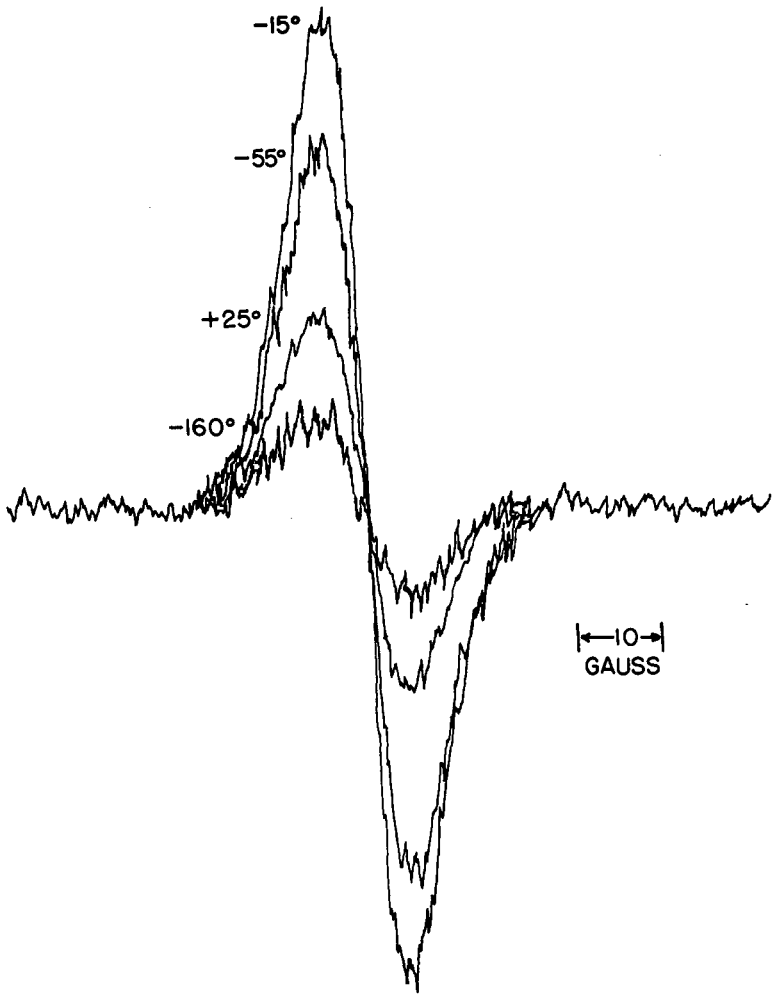
Fig. 9.



Light and dark signals from Chlorella-methanol extract at two temps.

MU-15649

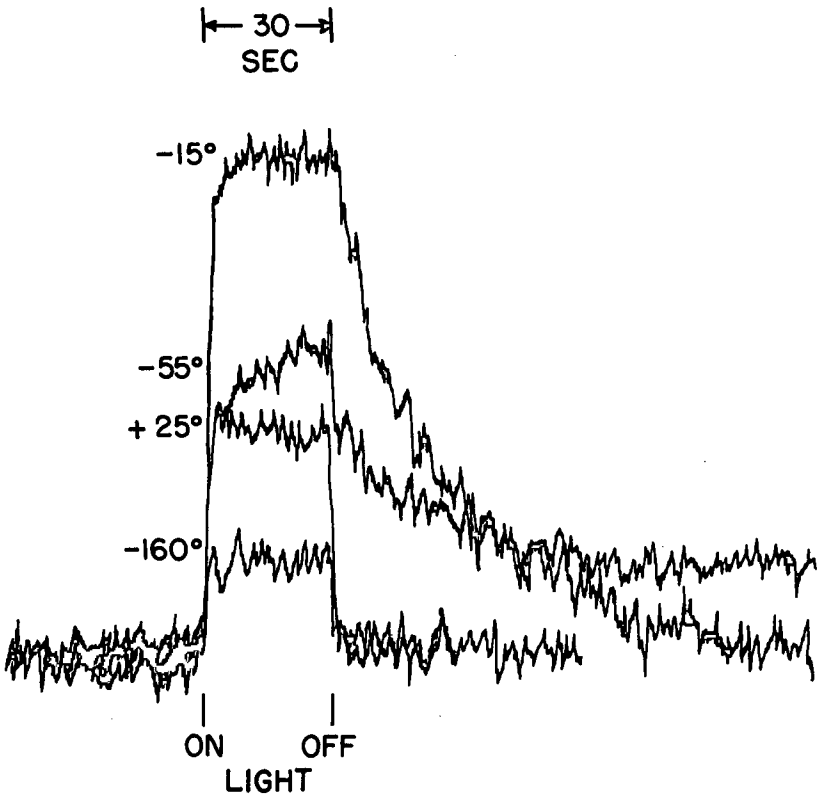
Fig. 10.



ESR SIGNALS FROM *RHODOSPIRILLUM RUBRUM*
5 MINUTES CONTINUOUS ILLUMINATION

MU-15137

Fig. 11.



RISE AND DECAY OF ESR SIGNALS
FROM *RHODOSPIRILLUM RUBRUM*

MU-15138

Fig. 12.

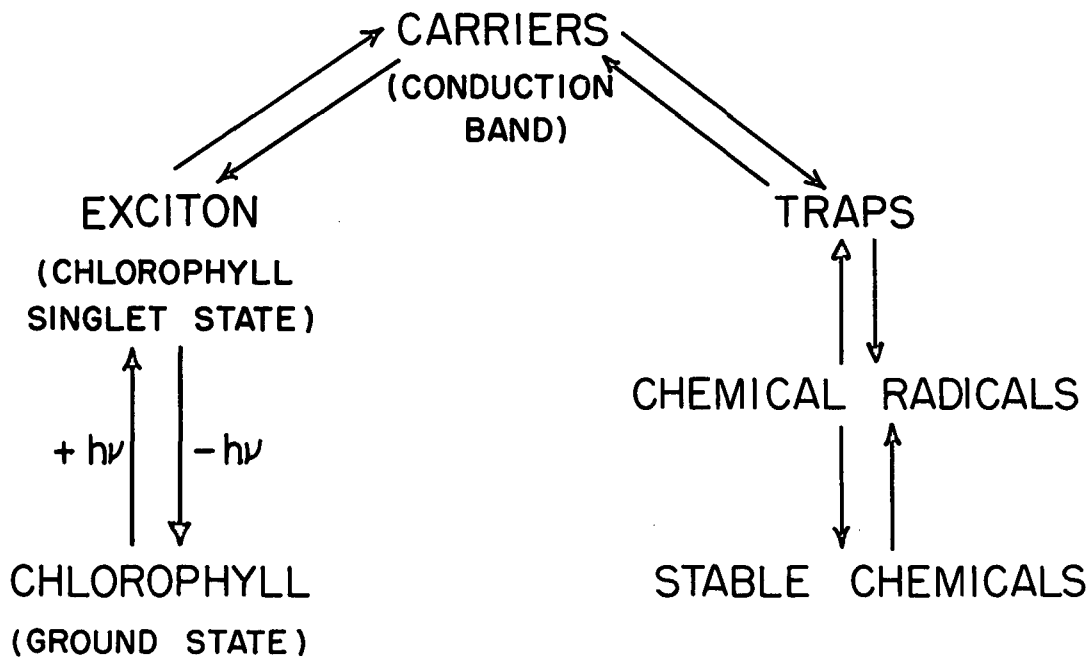
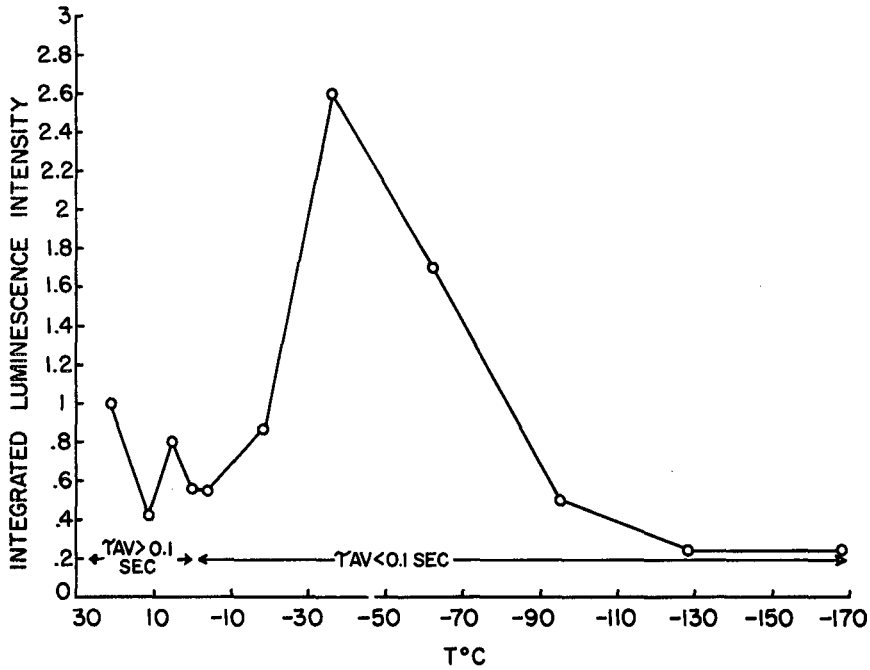

CARRIERS
(CONDUCTION BAND)
EXCITON
(CHLOROPHYLL SINGLET STATE)
TRAPS
CHEMICAL RADICALS
+ hν
− hν
CHLOROPHYLL
(GROUND STATE)
STABLE CHEMICALS
MU-15135

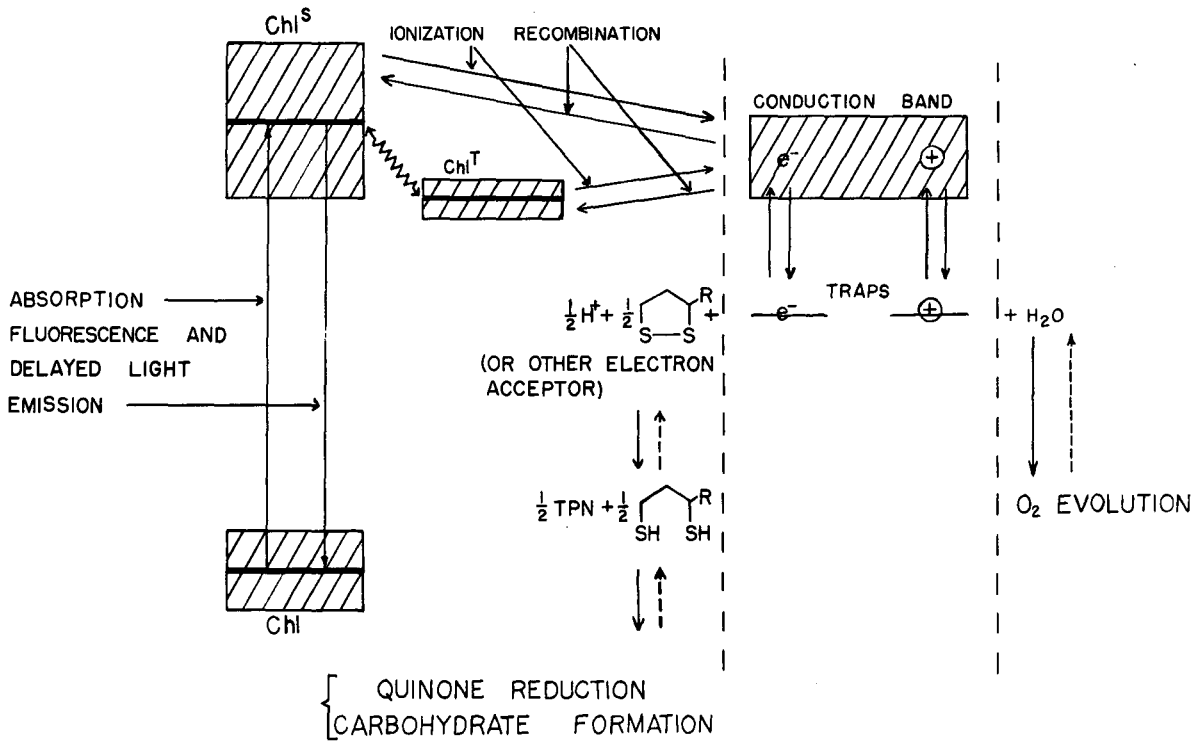
Fig. 13.



INTEGRATED INTENSITY OF SPINACH CHLOROPLAST LUMINESCENCE FROM 0.0015-5.0 SECONDS AFTER EXCITATION AS A FUNCTION OF TEMPERATURE

MU-15136

Fig. 14.



PROPOSED SCHEME FOR VARIOUS PHOTOCHEMICAL PROCESSES IN PHOTOSYNTHESIS

MU-13256-C

Fig. 15.