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*FAST KINETICS OF UNPAIRED ELECTRONS IN PHOTOSYNTHETIC SYSTEMS**

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Since the initial observation of an electron spin resonance (ESR) signal in chloroplasts, which increases in intensity on illumination,[1] this technique has added significantly to the data on the photosynthetic process.

The ESR signals under discussion are characterized by g values in the range of 2.002–2.007 and by widths, measured between points of maximum slope, in the range of 1–20 gauss. Such signals may be produced by organic free radicals or by unpaired electrons trapped in an extensive lattice, as in a semiconductor. They are not of the type produced by paramagnetic ions of the transition elements, or by molecules in triplet states.[2] For purposes of the following discussion, we attribute the observed ESR signals to "unpaired electrons," giving this term the meaning of implying either organic free radicals (relatively small molecules containing an unpaired electron) or electrons in semiconducting lattice situations, but excluding transition metal ions and triplet molecules.

ESR signals with g values of 2.002–2.005 have been observed in a number of photosynthetic systems.[3–10] In the anaerobic photosynthetic bacterium *R. rubrum*, no signal is observed in the dark, but an intense signal at $g = 2.002$ is observed on illumination. In green plants which produce O_2 photosynthetically, two signals have been observed. One of these (signal II, ref. 5) is observed in the dark; it is centered at $g = 2.005$ and exhibits distinctive hyperfine lines. A second signal (signal I, ref. 5) is absent in the dark and is generated on illumination; this signal has a g value of 2.002 and does not exhibit hyperfine structure. ESR studies of photosynthetic systems have not thus far yielded unequivocal evidence of unpaired electrons associated with semiconductors or with triplet excited states.

The main question under examination in the present investigation is whether the observed unpaired electrons are associated with a component representing the primary light-excited state, or with some later stage in the photosynthetic process. For this purpose, we have studied fast kinetic changes in the intensity of the ESR signal at $g = 2.002$ exhibited by intact cells of *R. rubrum* on illumination. Until

now, measurements of the changes with time in the ESR signals associated with photosynthetic processes have been limited by the time constants of the ESR spectrometers' noise-integrating systems, which are usually several seconds. Although most ESR spectrometers have an inherent time constant which is considerably less than 0.1 sec, the signal resulting from a light stimulus is usually so weak as to require the additional time to average the noise fluctuations to a sufficiently low value so that the signal may be observed satisfactorily.

With the advent of new computers which permit the summation of repeated signals, and, therefore, a considerable increase in signal-to-noise ratio, this difficulty can be circumvented. In the present paper we report kinetic measurements of the changes in ESR signal intensity on illumination of photosynthetic systems, in which a computer of average transients ("CAT," Technical Measurements Corporation)

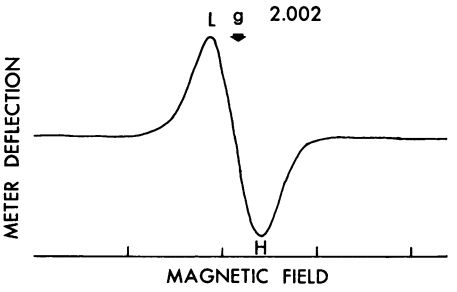

FIG. 1.—Derivative presentation of an ESR signal from an illuminated suspension of *R. rubrum*. The bacteria were grown using the medium and procedures described by Ormerod *et al.*,[11] and were harvested after approximately 42 hr. Here the signal is recorded in the conventional way with a 0.1 second time constant. The "CAT" is not used. Modulation amplitude, 10 gauss. The low field peak is designated by L; the high field peak by H. Each interval on the horizontal axis represents 20 gauss.

has been employed to record changes that occur in times of the order of 1–100 msec. These initial data provide new information regarding the mechanisms responsible for the generation and decay of the free radicals associated with photosynthesis.

Materials and Methods.—Measurements have been made on (*a*) whole cells of *R. rubrum* cultured anaerobically, (*b*) whole cells of Euglena, cultured aerobically, and (*c*) whole cells of Chlorella, aerated with 5% CO_2 in air. The ESR spectrometer used in these studies is basically the same as used in previous work.[1] Magnetic field modulation at a frequency of 1 Mcps is used with phase-sensitive detection, which gives a signal representing the derivative of the absorption in the usual manner. However, the usual noise-integrating and recording system is not used. The signal is integrated in the phase-sensitive detector for a fraction of a millisecond only, mainly to remove frequencies related to the modulation frequency; then it is fed directly into the computer.

White light from a zirconium arc is directed through an aperture in the spectrometer cavity onto the sample cell by a lens system which includes a shutter and a photocell for monitoring the beam incident on the sample. The shutter can be operated automatically in a repeated on-off cycle of variable duration. The computer is arranged in one of two ways: (*a*) with the ESR spectrometer held at a fixed magnetic field corresponding to the position of the maximum deflection of the ESR derivative signal, the amplitude of the deflection is recorded by the computer memory bank as a function of time over a period in which the light is turned on, or off, or both. Repetitive on-off cycles are then carried out, with the computer adding each successive time-sweep, thereby recording an over-all average for the total number of sweeps carried out. (*b*) The ESR spectrometer is driven through a 100–200 gauss span of the magnetic field centered around the expected ESR signal during a fixed time (in these experiments, 1 sec). The computer is arranged to record the meter deflection as a function of magnetic field, triggered by the shutter, or externally. In this manner, the computer records the ESR signal (absorption derivative as a function of magnetic field) during a fixed interval after the onset of illumination. Repetitive cycles of this type are carried out, and the summed values are recorded by the computer.

For the second type of experiment the ESR spectrometer is equipped with a special magnetic field regulator and scanner. In this system, the magnetic field is sensed by a sample of a stable free radical sample (*ca.* 100 mg of diphenylpicrylhydrazyl) which is in a microwave circuit powered

by a fraction of the power used for the ESR measurement. This microwave circuit is designed to produce an error signal, which enters into the magnet power supply in such a way as to maintain the magnetic field at the resonant value for the free radical. A small coil is placed around the hydrazyl sample to produce an additional field proportional to the current flowing through it. This current is provided by a sweep circuit and is used to produce the X-input of an X-Y recorder. As this current changes, the feedback action maintains the field constant at the hydrazyl sample, but in doing so it changes the field in the main part of the magnet gap by an amount precisely proportional to the current. Thus, the effects of magnet hysteresis, temperature changes, and stray magnetic fields are avoided, and rapid linear changes in magnetic field can be obtained over the range required to observe the ESR signal.

By the foregoing means, it is possible to record average curves from several hundred repeated signals, which show either the change in the intensity of the ESR signal as a function of time after the onset or cessation of illumination, or the actual shape of the light-induced ESR signal at a predetermined brief interval after illumination.

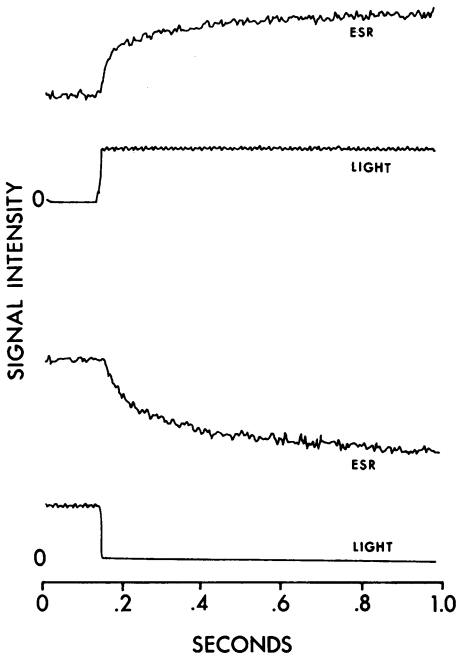

FIG. 2.—Change in intensity of ESR absorption resulting from subjecting a suspension of *R. rubrum* to 400 light-dark cycles (1 sec on—1 sec off) as seen by the "CAT." Here the magnetic field is held constant at the value corresponding to the high field maximum of the derivative of the ESR absorption. The ESR signal is fed into the "CAT," bypassing the relatively long time constant noise-integrating circuit used to record Fig. 1. Half of the "CAT" channels record the ESR signal, while the other half simultaneously record the output of a photocell which is sampling the light incident on the suspension of *R. rubrum*. The upper curves illustrate the increase in the ESR signal when the light is turned on; the lower curves illustrate the decay when light is turned off. Modulation amplitude, 6 gauss; temperature, 22°C. The culture conditions are as indicated in legend to Fig. 1.

Results.—(a) *Rhodospirillum rubrum:* Figure 1 shows a typical ESR signal from a packed suspension of *R. rubrum* cells illuminated by white light. The signal is an apparently simple absorption centered at $g = 2.002$ with a width (between maxima in the absorption derivative) of about 10 gauss; it exhibits no hyperfine structure. Figure 2 illustrates the changes in signal intensity observed when *R. rubrum* cells are subjected to 1-sec light-dark cycles. The rise time (time for the signal to increase to one half its final recorded value) for the increase in signal intensity after illumination is about 30 msec; the time constant for decay in the dark is about 34 msec. These represent the most rapid light-dependent changes in ESR signal intensity observed thus far in photosynthetic systems.

The kinetic changes in signal intensity during a 125-msec period following illumination are shown in Figure 3. It will be noted that the time required for the shutter to open is about 6 msec; the shutter passes one-half maximum light intensity after about 3 msec. In the first 3-msec period after the shutter begins to open there is no evidence of any increase in the ESR signal intensity; thereafter, the intensity begins to increase with its characteristic kinetics. This lag cannot be due to the response time of the ESR spectrometer, for a direct measurement made under the conditions of this experi-

ment shows that the response time is 0.5 ± 0.2 msec (i.e., the time for the spectrometer to exhibit one half its full response to an imposed signal). Neither can this lag be ascribed to a light intensity threshold effect, since it is also observed when the intensity is one half that used in the experiment reported in Figure 3.

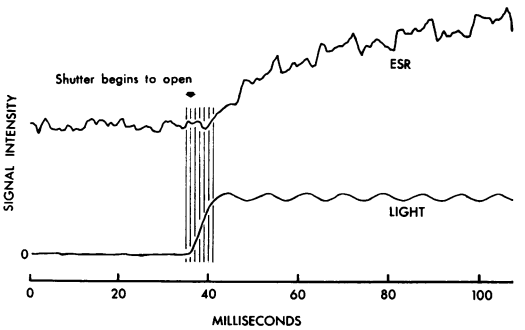

FIG. 3.—Light-induced rise in signal intensity under conditions of greater time resolution. Here *R. rubrum* were subjected to 1,626 cycles of 1 sec light—1 second dark, of which the "CAT" monitored a 125 msec portion. Otherwise experimental conditions as in Fig. 2, except temperature *ca.* 17°C. The ripple in the photocell output results from the use of alternating current in the lamp.

The presence of a corresponding delay in the decrease of the ESR signal during a dark period following illumination is shown in Figure 4, which is a record of the ESR signal intensity during a 62.5-msec period after the shutter has interrupted the light beam. The instrument settings and the preparation of the *R. rubrum* are comparable to those in Figure 3. It can be seen that a delay lasting at least 2–3 msec in the response of the ESR signal to cessation of illumination is present.

The foregoing experiments describe changes in ESR signal intensity at a single magnetic field which is chosen to correspond with one of the two positions of maximum deflection in the absorption derivative shown in Figure 1. However, since the latter is determined some minutes after the onset of light, it is not necessarily descriptive of the ESR signal present during the first second after onset of light. As indicated in *Materials and Methods*, by linking the "CAT" computer to a spectrometer equipped with a magnetic field regulator, it is possible to determine the total ESR absorption derivative at a brief interval after onset of light. Figure 5 illustrates such an experiment, in which the entire span of magnetic field in which the signal occurs is scanned in a period of about 300 msec following onset of light. The signal is quite similar to that observed under steady illumination with respect to g value, but is somewhat wider (an effect which may be due to instrumental conditions). Thus, an ESR signal similar to that exhibited by *R. rubrum* under constant illumination is already present within 300 msec after illumination. It is possible that experiments conducted at shorter intervals after onset of light and with a narrow modulation (i.e., less than 6 gauss, which is used to determine the signals shown in Figs. 1 and 5) will reveal the presence of a different type of signal. Such experiments will require an improved instrument sensitivity.

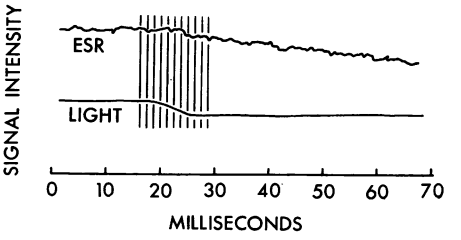

FIG. 4.—Decay of light-induced signal seen under conditions of high time resolution. A preparation of *R. rubrum* was subjected to 2,000 cycles of 1.5 sec light, 0.5 sec dark, of which the "CAT" monitored a 62.5-msec portion. Otherwise experimental conditions were as indicated in the legend to Fig. 3.

(b) *Euglena:* Figure 6 shows the change in signal intensity as a function of time for an 8-sec period following illumination of a sample of packed cells of *Euglena gracilis*. The curve is the computer-recorded average of repeated cycles of 8-sec alternating periods of light and dark, with only the period after illumination being recorded. The time constant for the appearance of the signal (time required for the development of one half the maximum intensity) is about 400 msec. Comparable results have been obtained with *Chlorella pyrenoidosa*. In both cases, the time constants for decay of the signal in the dark are comparable to the light-onset time constants.

Interpretation of Results.—The data of Figure 3 provide evidence which is directly relevant to the possible occurrence of unpaired electrons as an immediate consequence of the primary act of light absorption. If this were the case, unpaired electrons would be associated with the excited state generated by light absorption, and an ESR signal should begin to develop within the time required for the excitation process, which is of the order of 10^{-9} sec. However, as shown in Figure 3, there is a lag of 2–3 msec between the onset of light and the onset of the ESR signal. Hence, some process or processes with a mean lifetime of 2–3 msec must intervene between the primary act of light absorption and the generation of unpaired electrons. That a 2–3 msec delay is also observed before the onset of decay in the dark is additional evidence for the existence of an intermediate process characterized by this lifetime. We conclude, therefore, that the ESR signal observed in *R. rubrum* is not due to unpaired electrons associated with the excited state generated by the primary act of light absorption, i.e., by the initial step in the photosynthetic process.

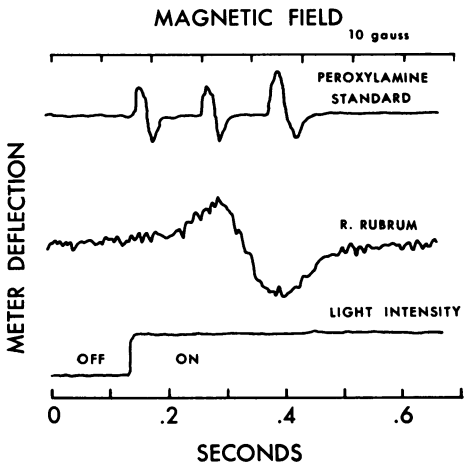

FIG. 5.—Rapid scan of ESR signal from suspension of *R. rubrum* shortly after beginning of illumination. Some distortion of the spectrum can be expected due to increase of the signal during the scan. Additional experimental conditions are: modulation amplitude, 6 gauss; 2,180 repeated light-dark cycles of 1 sec on—1 second off; temperature *ca.* 18°C. Culture conditions are as indicated in legend to Fig. 1. The upper trace, which was taken with a solution of peroxylamine disulfonate free radical, serves to establish the magnetic field scale for the scan.

The observed signal may represent unpaired electrons in some relatively stable lattice trap, the 2–3 msec lag reflecting the mean time required for the electrons to reach the trap. Alternatively, they may be due to the chemical redox processes activated in photosynthesis.

Thus, at least two processes, the primary act of light absorption (i.e., the elevation of the absorber to an excited state) and an energy or electron transfer process, precede the formation of unpaired electrons which give rise to the ESR signal exhibited by *R. rubrum*. Figures 2 and 3 show that the unpaired electrons, once formed, are subject to a decay process with a time constant of about 30 msec. The intensity of the ESR signal represents the resultant concentration of unpaired electrons established by the two preceding processes and by the decay process, which

may itself be a complex of parallel or successive events. On illumination, the initial processes generate an increasing concentration of unpaired electrons which, in view of the above relationship, accelerates the rate of decay. The resultant is a kinetic curve for the appearance of the ESR signal following onset of illumination which exhibits, approximately, the time constant and monotonic character of the decay process.

Further kinetic studies, both in photosynthetic bacteria and in green algae, are in progress. If signal-to-noise ratios permit, a study of the details of the kinetic curves may provide data on the sequence of reactions involving unpaired electrons, the rate constants for these reactions, and the influence of temperature and other relevant parameters on them.

Summary.—(1) A computer of average transients ("CAT") has been adapted for use with an electron spin resonance spectrometer in order to determine high-speed kinetic changes in the intensity of ESR signals. Applied to photosynthetic systems, the equipment is capable of describing such kinetic changes on a millisecond time scale with an adequate signal/noise ratio.

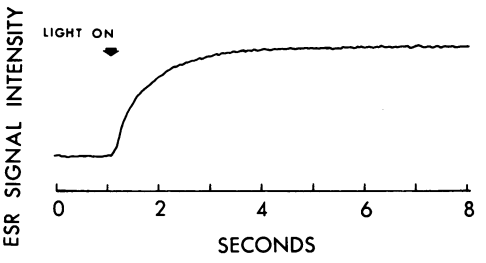

FIG. 6.—Change in signal intensity resulting from subjecting a suspension of *Euglena gracilis* to 678 light-dark cycles (8 sec on—8 sec off). The organism was inoculated (*ca.* 5%) into "Euglena medium,"[12] grown in constant light (*ca.* 500 ft-c), and harvested 6 days later. Temperature *ca.* 7°C.

(2) On illumination with white light, intact cells of *R. rubrum* exhibit a monotonic increase in ESR signal intensity which reaches half its asymptotic value in about 30 msec. Similar kinetics are exhibited by the decay of the ESR signal when illumination ceases. In the case of intact cells of Euglena, the corresponding time constants are of the order of several hundred msec.

(3) Following either onset or cessation of illumination, *R. rubrum* exhibits a 2–3 msec lag before the resultant change in ESR signal intensity begins. This result shows that the unpaired electrons associated with the ESR signal are not due to a light-excited state of the primary absorber in photosynthesis. In *R. rubrum* photosynthesis one or more electron or energy transfer processes must intervene between the primary light absorption event and the generation of unpaired electrons.

(4) The data suggest that the kinetic changes in ESR signal intensity exhibited by *R. rubrum* on onset or cessation of illumination are chiefly governed by the rates of one or more processes which result in the decay of the unpaired electrons generated by light.

We wish to thank Dr. Howard Gest for kindly supplying the culture and for much helpful advice and information concerning *R. rubrum*.

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† Trainee supported by U.S. Public Health Service training grant 2G-714.

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*THE DESTABILIZING EFFECT OF RIBONUCLEASE ON THE HELICAL DNA STRUCTURE**

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There has been considerable recent interest in the possible effects of proteins upon the biological properties of DNA; the role of histones in regulation of RNA synthesis has been discussed by a number of workers,[1,2] while Monod and his colleagues[3] have suggested that in inducible enzyme synthesis the regulatory substance acting upon DNA is a protein. All of these studies have been concerned with the ways in which protein may regulate the stability of the helical DNA structure, making the bases more or less accessible for their potential roles in DNA and RNA synthesis.

The experimental investigations of the effect of histones upon DNA stability demonstrate that they further stabilize the already stable two-stranded helical structure.[1,2] In this paper we report an interaction between DNA and bovine pancreatic ribonuclease which leads to a marked destabilization of the helical DNA structure. It seems likely that this property of RNAase may have more general implications with regard to the mode of action of nucleases, polymerases, and other enzymes which act upon DNA. It is also conceivable that this kind of destabilization may be a prototype of protein-DNA interactions which have a regulatory role in biological systems.

Materials and Methods.—Optical studies of thermal denaturation were carried out in a Zeiss PMQ II spectrophotometer, as described previously.[4] Optical rotation measurements were made in a Rudolph Model 80 polarimeter, with photoelectric detector and oscillating polarizer. Temperature was determined to $\pm 0.050°$ by averaging the readings obtained in two dummy tubes placed in the thermostat fluid line on either side of the sample tube. The Spinco Model E ultracentrifuge was used for analytical studies; measurements were made at 59,780 rpm. The Spinco Model L was used for sucrose gradient measurements, employing the SW-39 rotor and the gradient generating technique described by Martin and Ames.[5] Conductance was measured using a Wheatstone bridge circuit in which two adjacent arms were conductivity cells, one containing