12 July 1957, Volume 126, Number 3263 SCIENCE

Biological Activity of Free Radicals

Barry Commoner, John J. Heise, Barbara B. Lippincott, Richard E. Norberg, Janet V. Passonneau, Jonathan Townsend

Electron transport, the physical process that governs the chemistry of biological oxidations is, as yet, poorly understood. Most of the known reactants and products in metabolic oxidation-reduction systems are ordinary molecules in which all electrons necessarily occur in pairs. It might be expected, then, that electron transfers among these molecules would also occur in pairs. However, in several important instances, the chain of electron transport also includes metalloorganic substances, such as cytochrome, in which oxidation-reduction involves only a single electron. To account for the necessary linkage between such one-electron steps and apparent two-electron steps, Michaelis proposed the hypothesis "that all oxidations of organic molecules, although they are bivalent, proceed in two successive univalent steps, the intermediate state being a free radical" (*1*). Unlike ordinary molecules, a free radical contains an unpaired electron.

A second unresolved issue is the physical relationship between electron donor and electron acceptor. In ordinary solution, electron transfer occurs during collision between the donor and acceptor molecules. In biological systems, however, the reacting molecules are bound to the large protein component of the enzyme. It is then possible that the donor and acceptor are not in direct contact and that the necessary electron transfer occurs by a type of conduction through the substance of the intervening protein. This notion was first proposed in Szent-Gyorgyi's continuum theory (*2*) and, despite subsequent discussion and some experiment, it is as yet neither proved nor disproved. Such a system is analogous to a semiconductor, as first pointed out by Szent-Gyorgyi (*3*), in which electron transfer is a univalent process. During this process, a semiconductor would contain unpaired electrons.

These considerations imply that free radicals are intermediates in metabolic oxidation-reductions. Equally indirect evidence suggests that carcinogenesis by chemical agents may result from their metabolic conversion, within the cell, into unusually stable free radicals (*4*). This view is supported by the fact that physical carcinogenic agents—that is, ionizing radiation and ultraviolet light—induce the formation of free radicals *in vitro* (*5*). Similar theoretical considerations have led to suggestions that free radicals and components of the semiconductor type participate in photosynthesis (*3, 6*).

Thus, there have been inferences, but until now no firm proof, that free radicals and similar components that contain unpaired electrons are involved in a number of fundamental biological processes and that free radical mechanisms may be a ubiquitous attribute of the chemistry of living substance. Experimental evidence might be expected to elucidate these processes and the general mechanism of biological electron transport.

Electron Spin Resonance

A direct experimental approach to these questions was made possible by the discovery by Zavoisky in 1945 (*7*) of the electron-spin-resonance (ESR) absorption and by the subsequent development of ESR spectrometers capable of revealing the presence of unpaired electrons due to the absorption of incident microwave energy under the influence of an appropriate external magnetic field. The spectrometer records the absorption of incident microwave energy of a fixed frequency as the strength of the magnetic field is varied. Electron spin resonance arises from the interaction of the inherent magnetic moment of an unpaired electron with the external magnetic field. The absorption of microwave energy of a given frequency (ν), which represents the ESR signal, occurs at a particular value of the external magnetic field (H), the relationship between the two factors being determined by the equation

$$\nu = \text{a constant} \times g \times H$$

In this equation, variations in the term g reflect the interactions of the electron's magnetic moment with the electron's environment. For organic free radicals, g varies about the free-electron value of 2.0023, in a narrow range of about 2.0010 to 2.0070 (*8*). A given free radical exhibits a characteristic value of g within this range.

A free radical may be characterized as well by the width of the ESR absorption, which is generally expressed as the line width (in gauss) at one-half the maximum absorption. Finally, certain free radicals may be characterized by the detailed structure of their electron spin resonance. This structure arises from the fact that certain atomic nuclei possess a magnetic moment which may interact with the magnetic moment of a neighboring unpaired electron. The otherwise simple absorption of the electron then becomes resolved into multiple lines or hyperfine structure. For a given free radical, such hyperfine splitting is characteristic, and sometimes unique, with respect to relative peak heights and spacing along the magnetic field. The characteristics of the ESR line may also serve to distinguish among the three generic types of components that contain unpaired electrons—that is, free radicals, semiconductors, and paramagnetic ions of the transition elements.

This technique was first applied to biological materials by Commoner, Townsend, and Pake (*9*). Although the spectrometer then available precluded the use of wet (and therefore biologi-

Dr. Commoner, Mr. Heise, Mrs. Lippincott, and Dr. Passonneau are on the staff of the Henry Shaw School of Botany, Washington University, St. Louis, Mo. Dr. Norberg and Dr. Townsend are on the staff of the department of physics, Washington University.

cally active) samples, it was shown that electron spin resonance occurs in a wide variety of lyophilized tissues and that the magnitude of the signal is related to the tissue's relative metabolic rate. Since direct proof that free radicals are metabolic intermediates requires evidence that they occur during the course of biochemical processes, a new highly sensitive spectrometer for samples containing about 0.1 milliliter of liquid water has now been constructed (for a brief description, see *10*). With this apparatus, it has become possible to study various active biochemical and biological systems. The present article (*11*) summarizes the results of this work, which show that free radicals and probably components of the semiconductor type are active constituents of certain biological oxidation-reduction systems and of living cells.

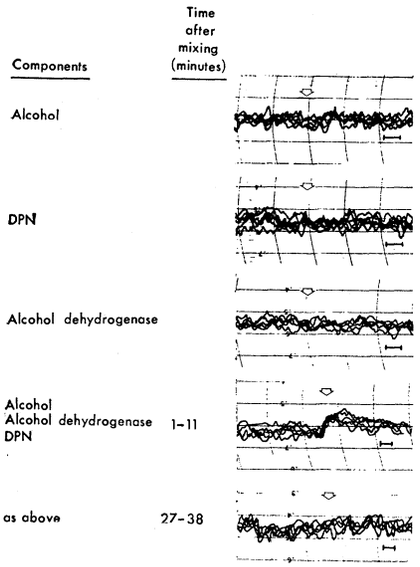

Fig. 1. Electron spin resonance (ESR) from the separate components of the alcohol dehydrogenase system, and from the complete system: 0.06 ml of 50-percent alcohol, 30 mg of oxidized diphosphopyridine nucleotide (DPN^+), 50 mg of alcohol dehydrogenase (Sigma), and 0.12 ml of Tris buffer at pH 8.0. The magnetic field increases toward the right along the abscissa. The bars at the right of each record represent a 10-gauss increment. The arrows mark the position of the electron spin resonance of a standard free radical, peroxylamine disulfonate ion, for which $g = 2.005$. The ordinate represents the rate of change, with respect to field strength of microwave energy (9000 Mcy/sec) absorbed by the sample, as the strength of the magnetic field is varied. The records shown represent superimposed tracings of sequential runs. The electron spin resonance shown by the complete system, which is indicated by the deflection from the base line, occurs at a magnetic field which corresponds to a g value of about 2.006. Ambient temperature: about 35°C.

Simple Enzyme Systems

The simplest biochemical process in which a free radical intermediate might occur is an enzyme-mediated, coupled oxidation-reduction of two substances—for example, substrate-dehydrogenase-coenzyme systems. In this reaction, two electrons are transferred from the dehydrogenase substrate to the coenzyme. If the process takes place in two successive single-electron steps, free radical intermediates capable of exhibiting electron spin resonance should occur. Since such free radicals are frequently rather unstable substances, one must anticipate that the steady-state concentrations may be very small.

To attain a maximal concentration, the components are placed in the ESR spectrometer cell in the dry form, and about 0.1 milliliter of the appropriate buffer is then mixed with these materials. The cell is inserted into the resonance cavity as quickly as possible, and rapid sweeps are made of the region of the external magnetic field in which electron spin resonance due to free radicals is expected to occur.

Results obtained with the alcohol dehydrogenase system are presented in Figs. 1 and 2. No electron spin resonance is obtained from the dehydrogenase, alcohol, or oxidized diphosphopyridine nucleotide (DPN^+) when these are examined separately. However, within 1 minute after all three components are mixed, a discernible ESR signal appears which persists for about 10 minutes and then gradually disappears. After 27 minutes, the signal is no longer detectable.

Alcohol dehydrogenase also catalyzes the reverse process in which reduced diphosphopyridine nucleotide (DPNH) is oxidized and acetaldehyde is reduced. Results obtained with this reaction are shown in Fig. 2. The separate components yield no ESR signal. The complete system exhibits an electron spin resonance during the first 15 minutes after the reaction starts, and the signal rapidly disappears thereafter.

The observed signals occur at a magnetic field which represents a value for g of about 2.006; this is within the range typical of free radicals. The electron spin resonances described in Figs. 1 and 2 represent (on the basis of comparison with a standard amount of a known free radical) roughly 10^{-11} mole of unpaired electrons. This may be compared with the amount of diphosphopyridine nucleotide (DPN) present, which is 10^{-5} mole, or 10^6 times the number of unpaired electrons.

Thus, the signal observed in the alcohol dehydrogenase system exhibits a g value which is consistent with an electron spin resonance arising from an organic free radical. The electron spin resonance depends on the activity of the complete system, appears as a transient phenomenon during the course of either the "forward" or "reverse" process, and reflects an exceedingly low steady-state concentration of unpaired electrons. In all these respects, the observed electron spin resonance conforms with the expectation, based on Michaelis' hypothesis, that free radical intermediates are formed during the activity of the alcohol dehydrogenase system. Similar results have been obtained with two other dehydrogenase systems: (i) lactic acid dehydrogenase, lactic acid, and diphosphopyridine nucleotide; and (ii) glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and triphosphopyridine nucleotide (TPN).

Comparable experiments have been carried out with a more complex enzyme system: reduced diphosphopyridine nucleotide, cytochrome reductase, cytochrome *c*, cytochrome oxidase, and O_2, in which electrons are transferred from component to component in the order given. The terminal electron donor, reduced diphosphopyridine nucleotide, is supplied in excess, so that the over-all activity of the system is regulated by the concentration of the ultimate electron acceptor and the turnover rates of the intervening cyclic oxidation-reduction steps.

The results are shown in Figs. 3*A* and 3*B*. When reduced diphosphopyridine nucleotide and cytochrome reductase are mixed, a transient electron spin resonance appears, which disappears within 10 minutes. When the system contains reduced diphosphopyridine nucleotide, cytochrome reductase, and cytochrome

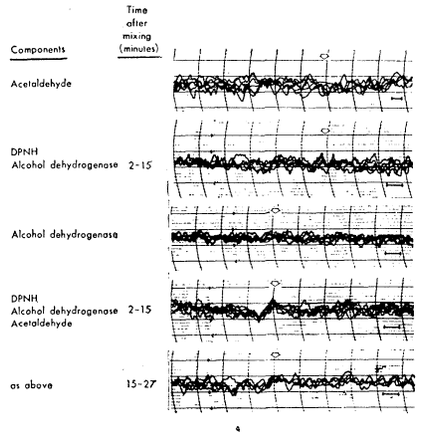

Fig. 2. Electron spin resonance from the alcohol dehydrogenase system, with reduced diphosphopyridine nucleotide (DPNH) and acetaldehyde as substrates. Details are the same as those described in Fig. 1, except that the system contained 30 mg of reduced diphosphopyridine nucleotide and 0.1 ml of acetaldehyde.

c, a larger electron spin resonance appears immediately after mixing. The signal slowly decreases in amplitude, although it is still evident 1½ hours later. If cytochrome oxidase is then added, an immediate enhancement of the ESR signal occurs, which persists relatively undiminished for at least 1½ hours. Control runs, carried out with each of the components separately (See Fig. 3*B*) show no electron spin resonance with the possible exception of a very weak signal in the case of reduced diphosphopyridine nucleotide. It is probable that this electron spin resonance reflects the slow, spontaneous reoxidation of reduced diphosphopyridine nucleotide by oxygen. An even weaker signal of doubtful significance may be present in the spectrometer run made with the reductase alone.

The activity of this system is wholly dependent on the reductase, since electron transfer between reduced diphosphopyridine nucleotide and cytochrome *c* does not occur. It is significant, therefore, that when atabrine, a competitive inhibitor of cytochrome reductase (*12*), is added to an active system, the electron spin resonance abruptly disappears (see Fig. 3*B*).

The electron spin resonances of the cytochrome reductase system exhibit somewhat variable values of *g*. For the electron spin resonances first observed after reduced diphosphopyridine nucleotide, reductase, and cytochrome *c* are mixed, *g* is about 2.006. In longer runs, *g* appears to drift toward higher values. The shift suggests that variable concentrations of two or more types of free radical occur, or that the changing composition of the system influences the character of a single free radical.

The results conform to the expectation that free radical intermediates occur during the activity of the cytochrome reductase system. The small, transient electron spin resonance observed when reduced diphosphopyridine nucleotide and cytochrome reductase are mixed is evidence that short-lived free radical intermediates are formed during the limited transfer of electrons from reduced diphosphopyridine nucleotide to the flavin moiety of cytochrome reductase. When cytochrome *c* is also present, the electron spin resonance is more intense and more sustained in time. This is to be expected from the fact that cytochrome *c* serves to reoxidize the flavin, thereby augmenting the rate and duration of the cyclic oxidation-reduction. As the cytochrome *c*, which is the terminal electron acceptor in this system, becomes reduced, the transfer rate and the amplitude of electron spin resonance diminish. The enhanced electron spin resonance owing to the subsequent addition of cytochrome oxidase is similarly consistent with the oxidase's biochemical activity—that is, the regeneration of oxidized cytochrome *c* and the resulting enhancement of the rate of the over-all process. Finally, the effect of atabrine is evidence that the electron spin resonance exhibited by the system is a specific function of the enzymatic activity of cytochrome reductase. Thus, the results of experiments with electron spin resonance conform with expectations based on the biochemical activities of the cytochcrome reductase system.

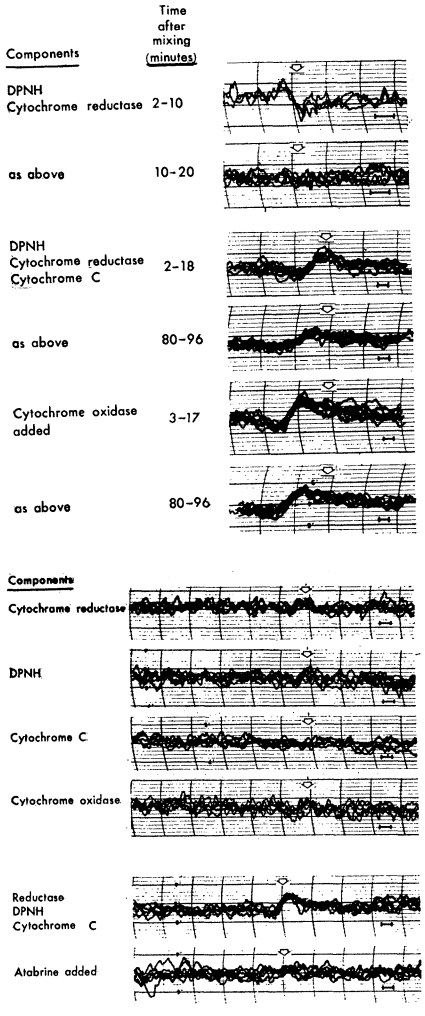

Fig. 3*A* (Top). Electron spin resonance from the cytochrome reductase system: 8 mg of reduced diphosphopyridine nucleotide, 30 mg of cytochrome reductase (prepared from pig heart by the method of Edelhoch *et al.*, *22*), 30 mg of cytochrome *c*, 30 mg of cytochrome oxidase (prepared from beef heart by the method of Waino *et al.*, *23*), and 0.1 ml of glycyl glycine buffer, *p*H 8.7. The tracings have been normalized with respect to the ESR position. Other procedures were the same as those described in the legend for Fig. 1. Fig. 3*B* (Bottom). Results obtained from control runs of the separate components of the cytochrome reductase system (four upper runs), and from the addition of 30 mg of atabrine to the complete system 20 minutes after the reaction began. Other procedures were the same as those described in the legend for Fig. 1.

The available data do not yet permit identification of the specific free radicals responsible for the observed electron spin resonance. The occurrence of an electron spin resonance in the alcohol dehydrogenase system suggests that diphosphopyridine nucleotide forms a free radical intermediate. There is spectrophotometric evidence that flavin-containing enzymes form rather stable free radical intermediates (*13*), which are apparently identical to the free radical, verdoflavin, described by Michaelis in nonenzymatic oxidation-reductions (*14*). We find that verdoflavin yields a typical electron spin resonance, with $g = 2.005$.

It is probable, therefore, that the signals observed in the cytochrome reductase system represent, at least in part, electron spin resonance arising from free radical intermediates in the oxidation-reduction of both diphosphopyridine nucleotide and the enzyme's flavin prosthetic group. It cannot yet be determined whether the observed electron spin resonance may also include contributions due to reduced cytochrome *c* and cytochrome oxidase, which might produce an electron spin resonance with a rather high value of *g*.

With respect to the several systems studied, the foregoing results appear to confirm Michaelis' hypothesis that free radical intermediates occur in enzymatic oxidation-reduction processes.

Chloroplast System

An electron spin resonance, which is augmented by illumination, was discovered in isolated tobacco chloroplasts by Commoner, Heise, and Townsend (*10*). This observation has been recently confirmed (*15*). From the extensive studies of a number of investigators (*16*), it is known that such preparations contain (i) the chlorophyll-lipoprotein complex which absorbs incident photons; (ii) enzyme systems which together with the chlorophyll complex catalyze the photolysis of water, with the release of O_2 when a suitable electron acceptor is present; and (iii) various electron-transport systems, especially those involving the cytochromes, ascorbic acid, flavin mononucleotide, and triphosphopyridine nucleotide.

The electron transport systems may be expected to develop free radical intermediates of the type already discussed, and the chlorophyll complex, when activated by light, has on theoretical grounds been supposed to form an excited triplet state (which is a biradical,

and therefore presumably capable of yielding an electron spin resonance) or components of the semiconductor type (*6, 15*).

In the initial studies of Commoner, Heise, and Townsend, it was shown that the chloroplast preparations respond to illumination by generating an electron spin resonance which, on the basis of the evidence then available, was ascribed to either the light-excited chlorophyll complex or the electron-transport enzyme systems associated with photosynthesis, or both. In what follows, it is shown that both types of constituents occur and that experiments with electron spin resonance are capable of elucidating, in part, the electron transport processes related to photosynthesis.

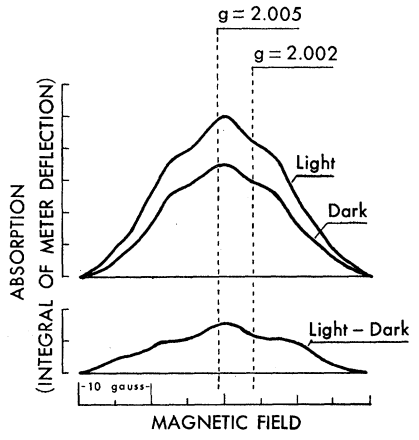

Fig. 4. Electron spin resonance from unwashed spinach chloroplasts in 0.5*M* sucrose. The absorption curves were obtained from the absorption derivatives yielded by the spectrometer (see Fig. 5, for an example) by means of an automatic analog computer. Illumination was with light from a 500-watt tungsten projection lamp filtered through 5 cm of 0.5-percent $CuSO_4$ solution. Magnetic field increases to the right. Ambient temperature: about 35°C.

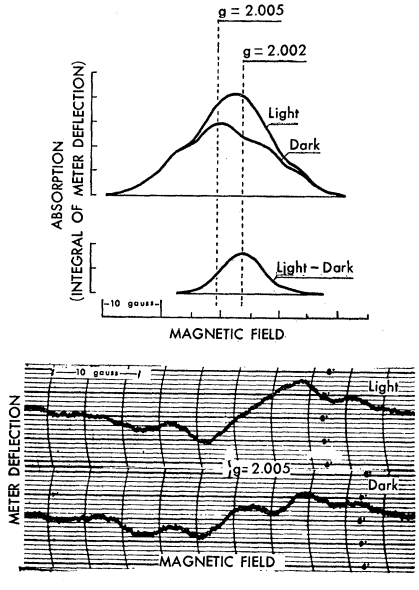

Fig. 5. Electron spin resonance from spinach chloroplasts washed twice with 0.5*M* sucrose solution. The lower curves represent the actual spectrometer records, and the upper curves represent the integral curves derived from them. Other experimental details as described in Fig. 4.

When chloroplast preparations are examined in the dark, an electron spin resonance is observed which exhibits characteristics that are consistent from preparation to preparation (see Table 1 and Figs. 4, 5, and 6). These can best be seen from the ESR absorption, which is obtained by machine integration (on an automatic analog computer) of the absorption derivative yielded by the ESR spectrometer. (The relationship between the absorption derivative and the absorption is illustrated in Fig. 5).

Electron-spin-resonance absorptions obtained from various chloroplast preparations are shown in Figs. 4, 5, and 6. In each case, the absorption exhibited in the dark is a fairly broad line (width at one-half maximum height, about 19 gauss) with a maximum at approximately $g = 2.005$. These absorptions appear to be resolved into a symmetrical group of five hyperfine lines of unequal amplitude extending over a span of about 25 gauss. Such compact, symmetrical, and unequal hyperfine groupings near the free electron g value are characteristic of organic free radicals rather than paramagnetic ions or semiconductors.

In general, the peaks observed conform to the amplitude ratio 3/2/1 (for the central, first peripheral, and second peripheral peaks, respectively). There is a limited number of atomic nuclei with magnetic moments, that occur in considerable quantity in biological materials: hydrogen, nitrogen, and phosphorus. Consideration of the types of hyperfine splitting observed in known free radicals containing these atoms (*17*) indicates that, for instances in which only five lines occur, the ratio of 3/2/1 is uniquely associated with free radicals in which the unpaired electron is in the neighborhood of two chemically equivalent nitrogen atoms. The splitting observed in electron spin resonance in the dark (6 to 7 gauss) is in the range of splittings which have been found in a number of free radicals containing two equivalent nitrogen atoms in a conjugated structure (*18*).

In a few preparations, we have observed what may be an additional pair of weak hyperfine lines on the periphery of the main electron spin resonance. This may indicate that the five lines of the electron spin resonance in the dark are but the most intense components of a more complex structure. Resolution of this question may lead to specific information concerning the chemical nature of the substance responsible for the electron spin resonance observed in unilluminated chloroplasts.

Figures 4, 5, and 6 also show the results obtained when the chloroplast preparations are illuminated. In all cases, the electron spin resonance is increased in amplitude over the value obtained in the dark. The relative concentrations of unpaired electrons in various preparations can be estimated from the areas of the respective integrated ESR absorptions. Figures 4, 5, and 6 show that, as the chloroplast preparation is more extensively washed or dialyzed, the electron spin resonance in the dark decreases in amplitude, while maintaining its characteristic hyperfine structure. In contrast, the reasonance in the light

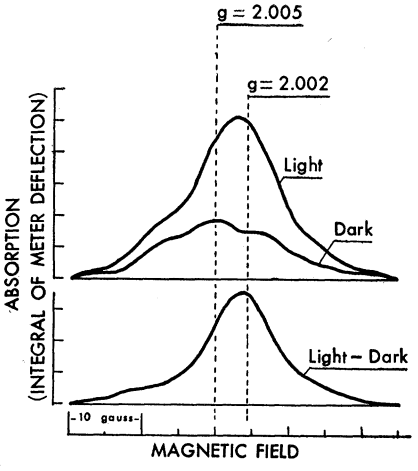

Fig. 6. Electron spin resonance from spinach chloroplasts washed and dialyzed against 3 lit of 0.5*M* sucrose solution at 4°C over a 24-hour period. The curves represent integrals obtained from spectrometer records of the type shown in the lower part of Fig. 5. Other experimental details as described in Fig. 4.

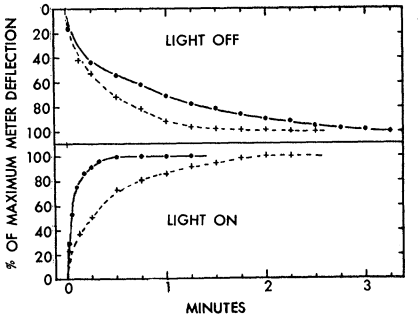

Fig. 7. Electron-spin-resonance meter deflections (after onset and cessation of light) for a spinach chloroplast preparation in 0.5*M* sucrose solution (broken lines), and the same preparation after dialysis against distilled water (solid lines). The points were read off spectrometer records of the type shown in the upper part of Fig. 8.

Table 1. Characteristics of electron spin resonances of spinach chloroplast preparations and living *Chlorella* cells.

Type of preparation	Width at one-half maximum of ESR absorption line (gauss)		Hyperfine splitting (gauss)	
	Dark	Light minus dark	Dark	Light minus dark
Chloroplasts, unwashed and undialyzed, in 0.5*M* sucrose	19	21	6	6 to 7
Chloroplasts, washed but undialyzed, in 0.5*M* sucrose	19	8	7	None
Chloroplasts, washed and dialyzed, in 0.5*M* sucrose	19	11	6 to 7	None
Chlorella, living cells	19	19	About 7	About 7

shows a relatively constant amplitude.

The shapes of the electron spin resonances of various illuminated preparations are complex and different from one another. The electron spin resonance exhibited during illumination is the sum of the electron spin resonance in the dark and the additional resonance induced by light; the latter can be extracted from the data by subtracting the resonance in the dark from that in light. These difference curves are shown in Figs. 4, 5, and 6.

When a chloroplast suspension which has been exposed to minimal washing is illuminated, the light-induced electron spin resonance is identical in g value, width, and shape with the original electron spin resonance in the dark (Fig. 4). Hence, in this case, the unpaired electrons generated by light appear as an additional amount of the free radical previously present in the unilluminated chloroplasts.

However, as the preparation is progressively washed and dialyzed, and the magnitude of the electron spin resonance in the dark is decreased, illumination induces the appearance of a new electron spin resonance. This new absorption differs from the electron spin resonance in the dark in three respects. (i) The absorption peak occurs at a magnetic field which is the same, within the few parts in 10^4 accuracy presently available, as that of a free electron ($g = 2.002$); in contrast, the g value of electron spin resonance in the dark is 2.005. (ii) The resonance line lacks resolvable hyperfine splitting, whereas such splitting does occur in the dark resonance. (iii) The line width is about 8 to 10 gauss, as against about 20 gauss for dark resonance. These characteristics are evident in the difference curves obtained from washed and dialyzed chloroplasts (Figs. 5 and 6). The slight absorption on the periphery of these difference curves suggests that the dark resonance may also contribute slightly to the effect of light.

The light-induced electron spin resonance at $g = 2.002$ appears to be associated with the chlorophyll-lipoprotein complex. Takashima (*19*) has reported the isolation of crystal-like rosettes of chlorophyll-lipoprotein by extraction of chloroplasts in α-picoline, followed by extensive dialysis and final precipitation by addition of dioxane (*20*). When the dark green rosettes prepared from spinach chloroplasts in this way are examined in the ESR spectrometer, they exhibit a weak signal in the dark which is significantly augmented on illumination. The absorption occurs at about $g = 2.002$ and exhibits a half-width of about 12 gauss. In these respects, it resembles the light-induced electron spin resonance of dialyzed spinach chloroplast preparations.

This observation and the persistent association of the resonance at $g = 2.002$ with dialyzed and water-disrupted chloroplasts indicate that this electron spin resonance arises from light-induced, unpaired electrons in the chlorophyll-lipoprotein complex. That the line is relatively narrow and the g value close to that of the free electron suggest that the light-excited complex may be somewhat analogous to a semiconductor. Recent investigations by Arnold and Sherwood on the conductivity of dry chloroplast films lead to the same conclusion (*21*).

If the ESR spectrometer is set at the magnetic field at which the maximum absorption of microwave energy occurs, the meter deflections induced by turning the light on and off can be recorded against time, in the manner illustrated by the curves shown in Figs. 7 and 8. Such records provide a kinetic analysis of the responses of the several types of chloroplast preparations.

Dialysis has a marked effect on the rates of response of chloroplast preparations to onset and cessation of light (see Fig. 7). A preparation which has been washed in sucrose solution, but not dialyzed, achieves one-half of its maximum response to light at about 15 seconds after onset. The half-time for decay in the dark is nearly the same, 14 seconds. In contrast, after the same preparation has been dialyzed against water, one-half of the maximum light-induced deflection is obtained at 2.2 seconds after onset, while the decay curve exhibits a half-time of 25.5 seconds.

Thus, dialysis enhances the rate at which the light-induced electron spin resonance appears and reduces the rate of decay in the dark. As noted previously, dialysis also reduces the amplitude of electron spin resonance in the dark and induces the appearance, in the light, of the electron spin resonance associated with the chlorophyll complex. Taken together, these facts suggest that chloroplasts contain a diffusible component which transfers unpaired electrons from the light-activated chlorophyll complex to the free radical which gives rise to the electron spin resonance in the dark. Conceivably, this component and the free radical responsible for the dark resonance are identical. In unwashed and

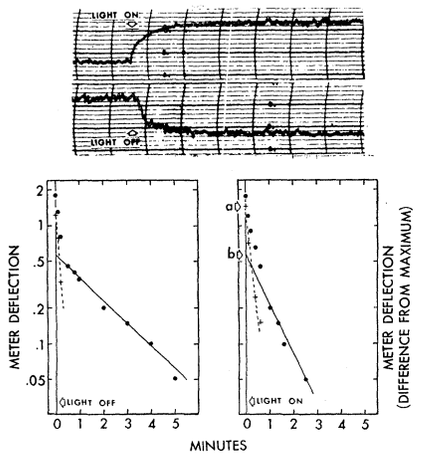

Fig. 8. Variation with time, after onset and cessation of light, in the ESR meter deflection obtained from a chloroplast preparation briefly dialyzed against 0.5*M* sucrose. The two upper curves show the change in the magnitude of the ESR signal (that is, the meter deflection at the magnetic field at which this deflection is at its first maximum) with time after the onset and cessation of illumination. The lower figures represent semilogarithmic plots of the meter deflections against time. Solid circles represent points taken from the actual records (above). The solid line represents the slow response, with intercept *b* being the contribution of this response to the total deflection. The broken line represents the fast response which is determined by the difference between the total response (solid circles) and the extrapolated line (solid) representing the slow response. The contribution of the fast response to the total meter deflection is represented by the intercept of the broken line at time zero (point *a*).

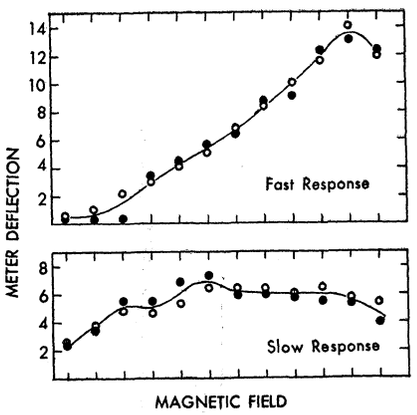

Fig. 9. Contribution of fast and slow responses to the meter deflection at a series of magnetic fields when a spinach chloroplast preparation in 0.5*M* sucrose is illuminated and darkened. Open circles refer to the increase in meter deflection on illumination. Closed circles refer to the decrease in meter deflection when the light is turned off. The points which determine the fast response curve represent intercepts such as *a* of Fig. 7; the points which determine the slow response curve represent intercepts such as *b* of Fig. 7. Each division of the abscissa represents about 0.8 gauss. Other experimental details as described in Fig. 4.

undialyzed chloroplasts, this transfer system is fully active. As a result, unpaired electrons generated by light in the chlorophyll complex are rapidly transferred to the free radical. In this circumstance, illumination augments only the resonance due to this free radical, the steady-state concentration of unpaired electrons in the chlorophyll complex being too small for detection. This condition is illustrated by the results obtained with unwashed chloroplasts shown in Fig. 4.

If the diffusible component is removed by dialysis, the rate of electron transfer from the light-excited chlorophyll complex is reduced. The steady-state concentration of unpaired electrons in the complex is thereby enhanced, and under illumination the electron spin resonance characteristic of the chlorophyll complex ($g = 2.002$) is then detectable as a signal superimposed on the five-component dark resonance centered about $g = 2.005$. This condition is illustrated by the responses of the washed and dialyzed preparations shown in Figs. 5 and 6.

These considerations suggest that, in preparations that have not been totally freed of the diffusible carrier, illumination will generate electron spin resonances due to both the electron-transport free radical and the activated chlorophyll complex. The latter represents an inherently rapid photoactivation process upon which the former is dependent. Hence, of the two signals, the resonance due to the chlorophyll complex ought to appear more rapidly in the light and decay more rapidly in the dark.

This hypothesis is subject to experimental verification. Figure 8 shows the time course of the response of a partially dialyzed chloroplast preparation to onset and cessation of light. A plot of the log of the deflection against time shows that the responses are complex exponential functions. At least two processes are involved. The faster process has a half-time of about 5 to 10 seconds for both onset of light and decay. The slower process has a half-time of about 40 seconds for onset of light, and about 100 seconds for decay. The relative contributions of the fast and slow processes to the total ESR meter deflection can be calculated from the semilogarithmic plot by the procedure described in Fig. 8. At the magnetic field at which the maximum meter deflection takes place, the fast process accounts for about two-thirds of the total.

By means of a similar experiment it is possible to characterize the separate electron spin resonances responsible for the fast and slow responses. Measurements such as those shown in Fig. 8 are made at a series of fixed magnetic fields beginning at a value below the center of the resonance and continuing to a field strength just past the first maximum deflection in the derivative curve. From a semilogarithmic plot of these data, the relative contributions of the fast and slow processes are determined at each value of the magnetic field. From these values, one may plot the separate fast and slow meter deflections as a function of the magnetic field. This plot, which is shown in Fig. 9, describes, in part, the electron spin resonances individually responsible for the two processes.

The fast process is apparently associated with an unstructured resonance, while the slower process is the result of a resonance which may exhibit a hyperfine splitting. The maximum deflection of the fast resonance occurs at a magnetic field about 4 gauss above the maximum of the slower resonance. Comparison of these results with the data of Figs. 4, 5, and 6 indicates that the fast response resembles the electron spin resonance associated with the light-activated chlorophyll complex, while the slow response resembles the resonance due to the electron-transport free radical.

From this evidence, it may be concluded (i) that the unstructured electron spin resonance at $g = 2.002$ represents unpaired electrons associated with the chlorophyll lipoprotein complex; (ii) that this resonance is uniquely dependent on light, and on illumination is generated at a rate which probably exceeds the spectrometer's time of response; and (iii) that this reasonance decays in part by means of electron transfer, via a diffusible component, to the organic free radical responsible for five-component electron spin resonance centered about $g = 2.005$. This free radical itself may be diffusible.

These conclusions are in keeping with expectations based on the photochemical and oxidation-reduction processes which occur in chloroplast preparations. The light-excited state of the chlorophyll complex which gives rise to the resonance at $g = 2.002$, may be viewed as the product of the primary photochemical process of photosynthesis. The resonance centered at $g = 2.005$ may be ascribed to a free radical form of one of the constituents of the electron-transport system which mediates the transfer of electrons from the primary photochemical process to the subsequent chemical events of photosynthesis.

Living Cells

A goal of these investigations has been the elucidation of electron-transport systems within intact, functional, living cells. The studies just described have accordingly been extended to encompass, at least in a preliminary way, the activity of living cells of *Chlorella*.

If densely packed suspensions of freshly harvested cells of *Chlorella pyroidenosa* (Emerson strain 3) are exam-

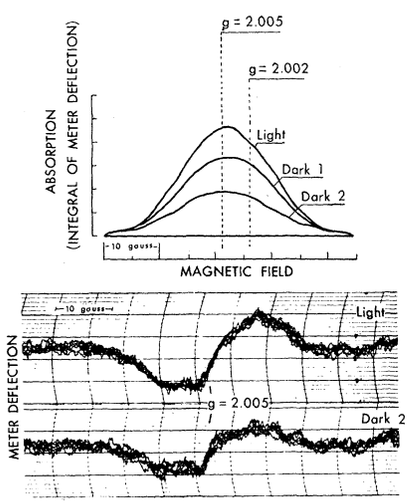

Fig. 10. Electron spin resonance of a suspension of living cells of *Chlorella* in the light and in the dark before illumination (Dark 1) and after illumination (Dark 2). The cells were suspended in a salt solution free of trace elements after being harvested from a nutrient containing urea, trace elements, and basic salts. The lower curves represent successive tracings of the direct spectrometer signals; the upper curves are integrals derived from such tracings. Other experimental details as described in Fig. 4.

ined in the ESR spectrometer, results such as those shown in Fig. 10 are obtained. These cells exhibit an electron spin resonance in the dark, which is enhanced by illumination. In both conditions, the g value is about 2.005 and the line width about 19 gauss. In these respects the electron spin resonance of *Chlorella* cells is similar to the resonance line centered at $g = 2.005$ previously found in spinach chloroplasts. This resemblance is reinforced by the lowermost curve shown in Fig. 10, which represents the electron spin resonance obtained from the *Chlorella* preparation when it is again darkened after a period of illumination under relatively anaerobic conditions. This electron spin resonance exhibits some indication of hyperfine splitting, which, while it is less well resolved than the structure shown by spinach chloroplasts, appears to be based on the same splitting.

These results show that living *Chlorella cells,* like the chloroplast preparations, respond to illumination by generating unpaired electrons. The characteristics of the *Chlorella* electron spin resonance are similar to those of the free radical, which in relatively intact chloroplast preparations gives rise to the five-component resonance line centered at $g = 2.005$. Thus, in both the living cell and in the unwashed chloroplast, light-induced, unpaired electrons appear to be rapidly transferred from the primary photochemical component to a free radical constituent of the electron-transport system, where they give rise to a detectable change in steady-state concentration.

The behavior of *Chlorella* cells in the ESR spectrometer is more complex than that of isolated chloroplasts. The structure of the *Chlorella* electron spin resonance changes with time, especially after the light is turned off, and in the dark shows evidence of a number of closely spaced hyperfine bands not detected in chloroplasts. Although illumination does not appear to change the g value or band width, it does seem to suppress structure, possibly because new free radical species contribute to the signal.

The results obtained with *Chlorella* are consistent with the chloroplast data already described and indicate that the operation of the intact photosynthetic system in the living cell involves univalent electron-transfer processes.

Conclusions

The foregoing results show that in the several instances studied, components that contain unpaired electrons occur as intermediates in biological oxidation-reductions. Although the electron spin resonance signals thus far obtained from isolated oxidation-reduction enzyme systems are too small to permit a detailed characterization, the observed g values are consistent with those exhibited by organic free radicals. The kinetic behavior of the electron spin resonances observed in enzyme systems and their response to variations in system constituents are in keeping with Michaelis' hypothesis that biochemical oxidation-reduction occurs in successive univalent steps which give rise to free radical intermediates. As experimental procedures are improved, and more intense signals are obtained, it should prove possible to determine the specific molecular composition of the free radical intermediates of these and similar enzyme systems.

The detection of the five-component electron spin resonance line centered at $g = 2.005$ in chloroplasts is perhaps more complete evidence for the participation of a specific organic free radical in biochemical oxidation-reduction. The fine structure which it exhibits is of a type observed only among organic free radicals. The enhanced amplitude of this electron spin resonance in illuminated unwashed chloroplast preparations, and apparently in living *Chlorella* cells as well, is evidence that the free radical from which it arises participates in the electron-transport process associated with photosynthesis. This result supports the less detailed data on isolated enzyme systems as evidence of the general validity of Michaelis' free radical hypothesis.

The results of the studies of isolated chloroplasts, and living cells of *Chlorella* provide evidence in support of the proposal that the flow of electrons associated with photosynthesis is a univalent process. The data provide direct support for Szent-Gyorgyi's suggestion that the chloroplast is analogous to a semiconductor and show that the ESR technique is capable of analyzing the interactions among components of this complex metabolic process.

The results obtained with *Chlorella* may be regarded as direct evidence of the participation of a specific free radical in the metabolic activity of a living cell. Electron spin resonances have also been obtained from other living cells, including several species of bacteria and mammalian tissues.

In the aggregate, these results appear to establish the usefulness of the ESR technique as a new means for analyzing the physical mechanisms of biochemical processes.

A final comment is pertinent. Ten years ago, Leonor Michaelis wrote of his free radical hypothesis "The road for the exploration of individual metabolic catalyses will be long. Although it is still far ahead, one is encouraged to believe that the correct road sign has been found" (*1*). The foregoing results are a tribute to his foresight.

References and Notes

1. L. Michaelis, in *Currents in Biochemical Research,* D. E. Green, Ed. (Interscience, New York, 1946).
2. A. Szent-Gyorgyi, *Muscular Contraction* (Academic Press, New York, ed. 2, 1951).
3. ———, *Science* **93**, 609 (1941).
4. C. J. Kensler, S. O. Dexter, C. P. Rhoads, *Cancer Research* **2**, 1 (1942); H. F. Park, *J. Phys. and Colloid Chem.* **54**, 1383 (1950); A. F. Fitzhugh, *Science* **118**, 783 (1953).
5. J. Weiss, *Nature* **157**, 584 (1949); L. H. Gray, *Progr. in Biophys.* **2**, 240 (1950).
6. E. I. Rabinowitch, *Photosynthesis and Related Processes* (Interscience, New York, 1945).
7. E. Zavoisky, *J. Phys. U.S.S.R.* **9**, 211 (1945).
8. J. E. Wertz, *Chem. Revs.* **55**, 829 (1955).
9. B. Commoner, J. Townsend, G. E. Pake, *Nature* **174**, 689 (1954).
10. B. Commoner, J. J. Heise, J. Townsend, *Proc. Natl. Acad. Sci. U.S.* **42**, 710 (1956).
11. This work was supported in part by the Office of Naval Research, and by the U.S. Air Force through the Air Force Office of Scientific Research of the Air Research and Development Command under contract No. AF18(600)1592. We thank S. I. Weissman for his helpful discussions of these results, and we acknowledge with thanks the assistance of A. Cederquist in the construction of the ESR spectrometer, and that of L. Smith in performing the machine integrations of ESR signals. We also thank Robert Emerson for a culture of his strain 3 of *Chlorella.*
12. L. P. Vernon, H. R. Mahler, N. K. Sarkar, *J. Biol. Chem.* **199**, 599 (1952).
13. R. Kuhn and T. Wagner-Jauregg, *Ber. deut. chem. Ges.* **67**, 361 (1934).
14. L. Michaelis, M. P. Schubert, C. V. Smythe, *J. Biol. Chem.* **116**, 587 (1936).
15. M. Calvin and P. B. Sogo, *Science* **125**, 499 (1957).
16. R. Hill and C. P. Wittingham, *Photosynthesis* (Methuen, London, 1955).
17. For this we are indebted to S. I. Weissman.
18. C. A. Hutchison, R. C. Pastor, A. G. Kowalsky, *J. Chem. Phys.* **20**, 534 (1952).
19. S. Takashima, *Nature* **169**, 182 (1952).
20. Y. Chiba, *Arch. Biochem. and Biophys.* **54**, 83 (1955).
21. W. Arnold and H. K. Sherwood, *Proc. Natl. Acad. Sci. U.S.* **43**, 105 (1957).
22. H. Edelhoch, O. Hayaishi, L. J. Tepley, *J. Biol. Chem.* **197**, 97 (1952).
23. W. W. Waino *et al., J. Biol. Chem.* **173**, 145 (1948).