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Free Radicals in Photosynthetic Systems*

MELVIN CALVIN

Department of Chemistry, University of California, Berkeley 4, California

ONE bit of evidence introduced in the preceding paper concerned the possibility that the quantum-conversion act of photosynthesis might be a production of unpaired electrons which were trapped successively 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 simply was stated that there was evidence for unpaired electrons. The nature of this evidence now may be explained. These curves previously shown are known as electron spin-resonance absorption spectra. This article describes electron spin resonance, how it may be used for the detection of unpaired electrons in biological systems, how it has been used, and how it further might be used to identify the nature of the unpaired electrons that might and do occur in biological systems.

Electron spin resonance is another spectroscopic method. Zavoisky[1] was the first to make use of it to detect unpaired electrons in physical systems. It is based on the principle that an electron has a spin giving it a magnetic moment such that, when placed in a magnetic field, the electron orientates itself with respect to the 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 and against the field.

Figure 1 shows the diagrammatic representation of this situation. On the left is the case in which the electrons are in between the pole faces of a magnet, but the electric current is not flowing yet and the electron magnetic moments are arranged randomly. When the field is turned on (as on the right), some of the electrons orientate themselves with the external field, and some against it. These two orientations do not have the same energy. The energies are designated as E_1 and E_2 in the figure, and the difference between them is equal to the product of the magnetic moment μ_0, the gyromagnetic ratio g_0, and the value of the external magnetic field H_0. This difference in energy can be expressed in terms of the frequency $h\nu$, and for an electron in a field of about 3000 gauss, the wavelength corresponding to this transition is about 3 cm.

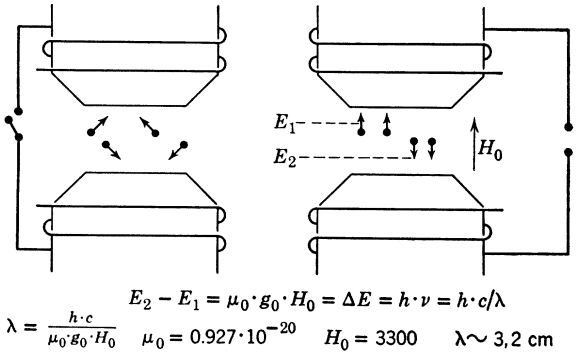

FIG. 1. Energy states of free electrons in an external magnetic field.

One may observe unpaired electrons by shining them with electromagnetic energy of a wavelength corresponding to the transition, and by watching for the absorption of this characteristic frequency, ν, when the magnetic field, H_0, is varied. One then determines the magnetic field at which absorption occurs.

Figure 2 indicates the manner in which the experiment is done, showing the 3-cm generator and the external magnetic field. In the presence of the magnetic field, some of the electrons are oriented parallel with the field, some antiparallel. There will be more in the lower (parallel) state than in the upper (antiparallel), so there results a net energy absorption as transitions between states occur.

Most of the apparatus in use today does not record direct absorption, recording instead the derivative of this absorption. The two graphs in the preceding paper (Figs. 22 and 23) are these derivatives of absorption rather than the absorption itself.

This experiment thus provides a method for the specific detection of unpaired electrons (since paired electrons mutually each quench the magnetic moment of the other). It provides a highly sensitive device, more sensitive in general than that provided by any mass susceptibility measurement, because the latter always must 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 now is being used on biological materials to detect the presence of free radicals and to determine whether or not free radicals

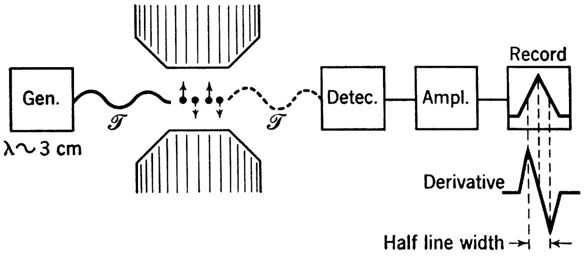

FIG. 2. Absorption of 3-cm waves resulting from transition of free electrons between energy states in an external magnetic field.

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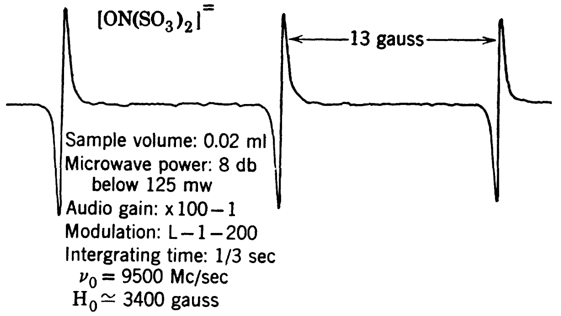

FIG. 3. ESR spectra of nitrosyl disulfonate [from Varian Associates Technical Information Bulletin, No. 2, 11 (1958)].

and unpaired electrons are participants in biochemical transformations.

The measurements obtained by this method not only can reveal how many electrons there are, but also, by the nature of the absorption record, may reveal something of the environment in which the electrons are situated. One recalls that the energy of the transition is dependent upon the field that the electron sees—that is, the external field due to the magnet, plus any magnetic field which the molecule may cause. 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. Some have magnetic moments that produce magnetic fields with which the unpaired electrons may interact. If the odd electron sees not only the external magnetic field, but also that of the molecule, a spectrum similar to that shown in Fig. 3 from Varian Associates[2] may be observed. In this case, the molecule is one which has in it a nitrogen atom, which 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. The electron near the nitrogen atom actually may 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. In the third case, there is no effect on the external field. So instead of one there should be three peaks. Figure 3 shows the three peaks attributable to the interaction with the spin of the nitrogen nucleus: one in the middle, and one on each side resulting from the three possible orientations of the nitrogen nucleus.

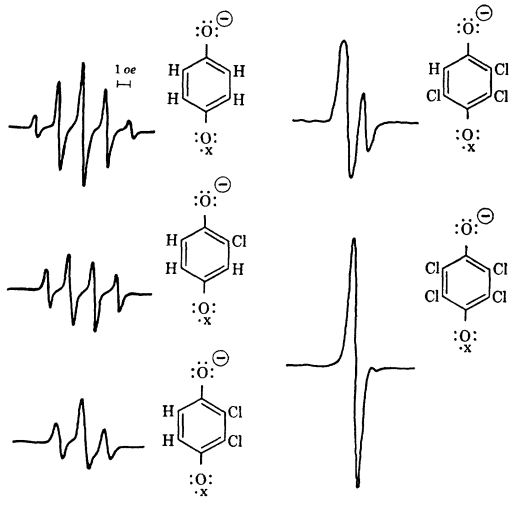

FIG. 4. ESR spectra of chloroquinones [from J. E. Wertz and J. Vivo, J. Chem. Phys. 23, 2441 (1955)].

Nitrogen is not the only nucleus having a magnetic moment. One of the most common 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 where molecules are constructed such that the electron interacts with these protons.

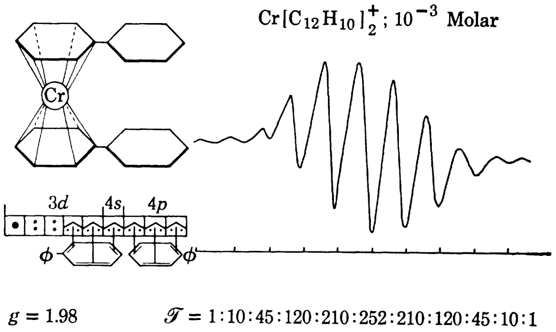

FIG. 5. Paramagnetic-resonance absorption spectrum of bisdibenzene chromium cation.

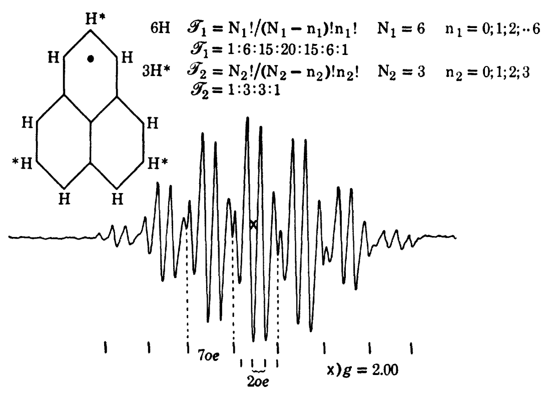

FIG. 6. Paramagnetic-resonance absorption spectrum of perinaphthenyl radical.

Figure 4, from work by Wertz and Vivo,[3] shows the case for tetrachlorohydroquinone (lower right). The resonance field is not modified by any of the atoms in the molecule since their nuclei are all of spin zero (no magnetic moment), and thus only one line obtains. If, however, one removes one of the chlorines and replaces it by a proton, and if the electron can interact with that proton, the proton, having two possible orientations in the magnetic field, splits the resonance into two lines.

How many different arrangements can one obtain to change the external magnetic field if two protons are present? Both of the magnetic moments may be had with or against the field, or they can be had one with and one against the field. There are, therefore, three different ways in which these two protons can be arranged with respect to the external magnetic field. The third situation is twice as probable as either one of the other two. Therefore, the middle peak should be twice as high as either of the two outside ones. Indeed, this is the case. In both cases, they produce no net modification of the external field. If three protons are present, there are four possible arrangements, and one gets four peaks; with four protons, there are five possible arrangements, and the amplitude ratios are the ordinary binomial coefficients, as seen in the figure.

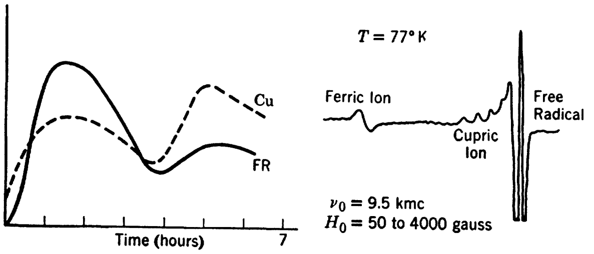

FIG. 7. ESR spectra from coenzyme-*A* dehydrogenase plus substrate [from H. Beinert, EPR Talk No. 10, Varian Associates (1958)].

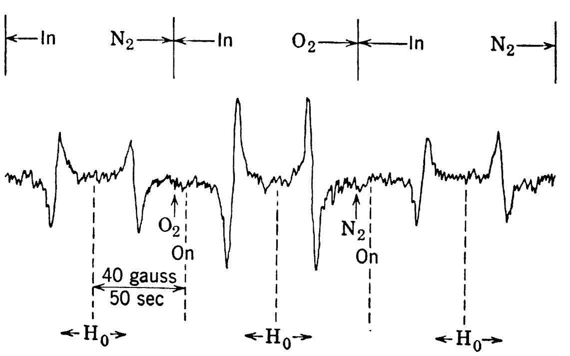

FIG. 8. Dark signal from *Rhodospirillum* methanol extract at room temperature

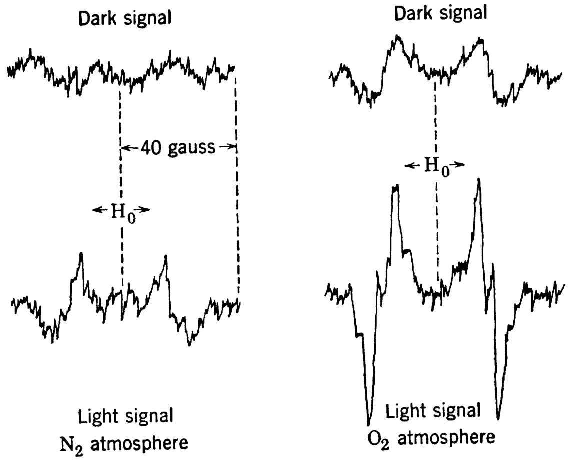

FIG. 9. Light and dark signals from *Chlorella* methanol extracts in two atmospheres at room temperature.

Not only may one determine, therefore, the number of odd electrons present in a system, but also one may ascertain something about the kind of an environment in which they are. Figure 5 shows the compound, dibenzene-chromium cation, which has one unpaired electron. The question of interest is the location of this unpaired electron. It was found, in our laboratory, that this unpaired electron of the chromium actually can see the ten protons of the two benzene rings of this compound associated with the chromium atom. Ten protons have eleven possible arrangements, and there should be eleven peaks in the curve. Those on the outside are very weak, because the probability of having all ten protons oriented in the same direction is very small as compared with the mixed configurations. One can determine, therefore, something about the environment of the transition elements by this method.

As a final chemical example, consider a perinaphthalene radical—a beautifully symmetrical radical (Fig. 6). During the preparation of the perinaphthalene, it accidentally became oxidized, and a very formidable absorption spectrum was obtained. The odd electron sees two different kinds of protons. On careful examination, one sees that there are seven groups of lines—each group a quadruplet. This means that there are six protons of one kind and three of another. The electron interacts more strongly with the six-proton group than it does with the three-proton group.

How much of this can be used for the investigation of biological free radicals? Beinert[4] 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 University and at Varian Associates, an electron spin-

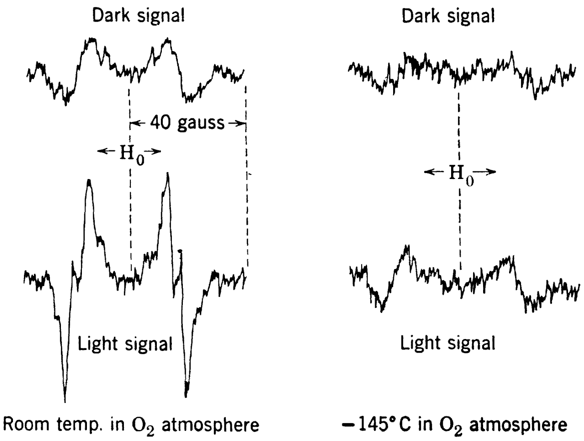

FIG. 10. Light and dark signals from *Chlorella* methanol extract at two temperatures.

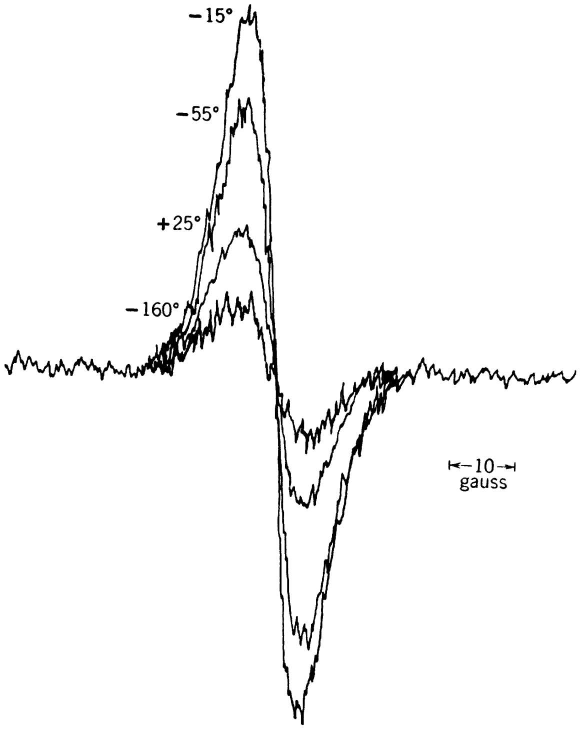

FIG. 11. ESR signals from *Rhodospirillum rubrum*, 5 min continuous illumination.

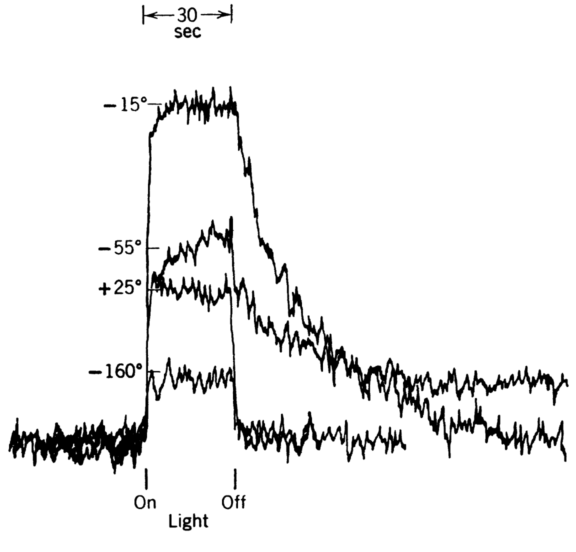

FIG. 12. Rise and decay of ESR signals from *Rhodospirillum rubrum*.

resonance signal was seen which could be distinguished from the resonance owing to the Cu^{2+} in the enzyme (Fig. 7). Commoner[5] and co-workers presented evidence of free-radical intermediates in the reactions of alcohol dehydrogenase and of cytochrome oxidase when these enzymes are brought into contact with their substrates.

How is this kind of a signal of a free radical distinguished from that shown in the preceding paper (Fig. 22)? Firstly, radicals of this kind do not fade, at low temperatures. They are frozen in. That is exactly what happens upon cooling the system of chloroplasts as described earlier. The fact is that structured systems can be found wherein the signal can be induced at a low temperature, but wherein it also fades at a low temperature at a very high rate.

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

Figure 11 shows the spin-resonance signal for *Rhodospirillum* after 5 min of illumination at the indicated temperatures. At 25°C, it is the next smallest signal;

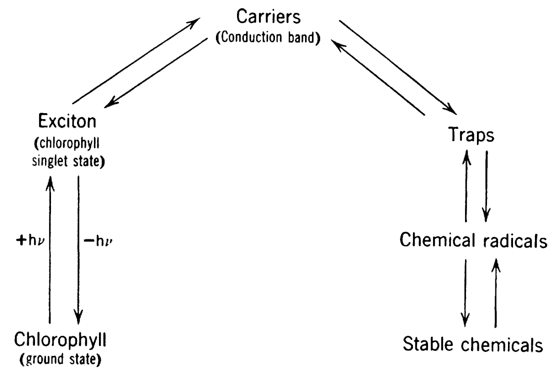

FIG. 13. Hypothetical scheme for light-energy utilization on chloroplasts.

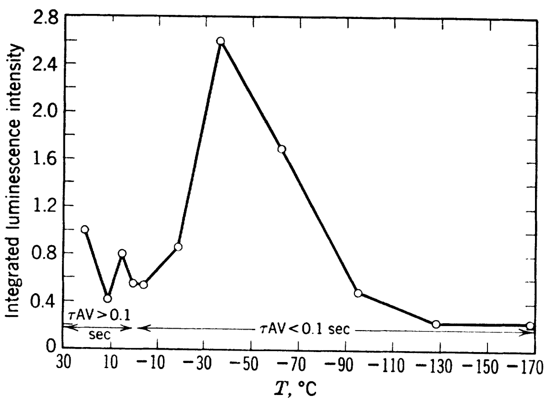

FIG. 14. Integrated intensity of spinach-chloroplast luminescence from 0.0015 to 5.0 sec after excitation, as a function of temperature.

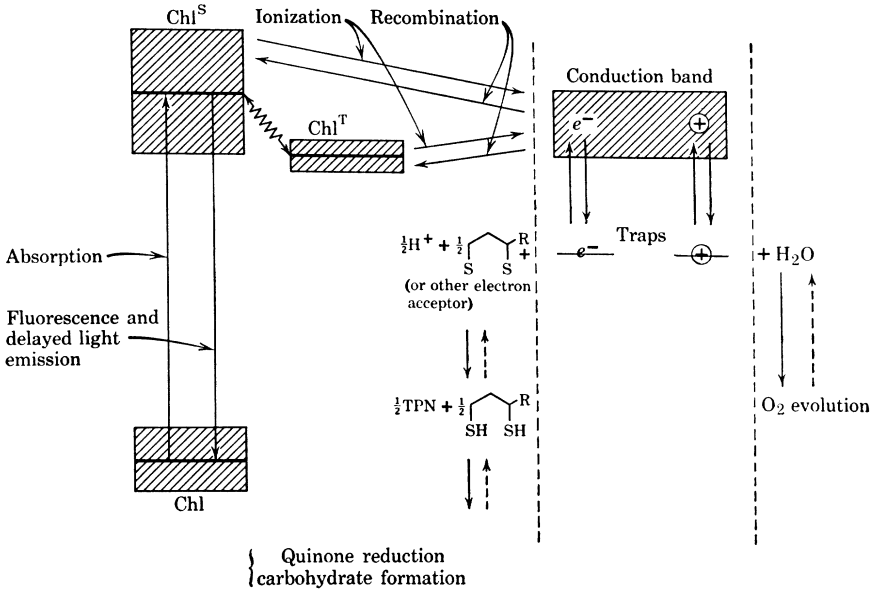

FIG. 15. Proposed scheme for various photochemical processes in photosynthesis.

at −55°C, it is larger; and at −150°C, it is larger yet. When the temperature is further reduced to −160°C, the intensity falls to the smallest shown. The time behavior is shown in Fig. 12. The spectrometer is set on the peak of the signal, and one may observe the rates of rise and of decay of each of these signals at each temperature as a function of time. The room-temperature signal is the next smallest shown, and it rises as rapidly as the instrument can respond. This signal decays immediately after illumination ceases, to within the time constant of the apparatus. There is, however, no rapid decay at −15°C; at −55°C, there is a very rapid rise, a further slow rise, followed by a small component of rapid decay, and a much longer slow decay. At −160°C, all that remains is very rapid rise and very rapid fall. Thus, I believe the possibility has been eliminated that these free radicals are being produced directly by illumination. This behavior must be the result either of untrapped carriers or of trapped carriers in well-shielded traps.

How can this whole sequence of events be accounted for? Figure 13 (discussed in the previous article) indicates the first act, the absorption of light to produce an exciton. The exciton then is converted into conductive carriers, these into chemical radicals, and the chemical radicals lead to stable chemicals. To account for the data shown in Fig. 12, it is necessary to presume 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 temperatures, the energy is transported into the chemical radicals, but these decay very rapidly into stable chemicals by the usual enzymatic process. The possibility that a triplet state exists 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 curve (Fig. 14) and similarly is 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—as a result of the interaction of the chlorophyll molecules in these arrays previously shown in electron micrographs—and the chlorophyll excited singlet state actually would be a broader band. The triplet state is shown as a rather narrow band overlapping the excited sequence. The triplet band has not been put directly in line because triplet light emission is not observed.

The foregoing scheme gives a working hypothesis to account for some of the problems, outlined at the onset —that the initial 35 kcal of energy be converted into useful chemical potential.

BIBLIOGRAPHY

[1] E. Zavoisky, J. Phys. U.S.S.R. **9**, 211 (1945).
[2] Varian Associates Technical Information Bulletin, No. 2, 11 (1958).
[3] J. E. Wertz and J. Vivo, J. Chem. Phys. **23**, 2441 (1955).
[4] H. Beinert, EPR Talk No. 10, Varian Associates (1958).
[5] B. Commoner, J. J. Heise, B. B. Lippincott, R. E. Norberg, J. V. Passonneau, and J. Townsend, Science **120**, 57 (1957).