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## Biomedical EPR

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Additional information is available at the end of the chapter

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### Abstract

Free radicals may participate in biological processes. An enzymatic dehydrogenation involved free radical intermediates. The oxidations of organic molecules, although they are bivalent, proceed in two successive steps, the intermediate state being a free radical. In an attempt to correlate the action of such a variety of carcinogenic agents as sodium hydroxide, ultraviolet and ionizing radiations and thousands of organic compounds, a free radical intermediate always suggests itself. Electron paramagnetic resonance (EPR) has brought sufficient sensitivity and discrimination to observe free radical intermediates directly in many of these reactions. EPR is aided by an increased sensitivity in many cases and has made a much greater contribution by distinguishing among paramagnetic ions, odd molecules and free radicals.

**Keywords:** electron paramagnetic resonance (EPR), free radical, paramagnetic ion, oxidation, organic molecule, enzymatic dehydrogenation, carcinogenic agent

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### 1. Introduction

Biomedical application of EPR has gained momentum in recent times. All atoms and molecules cannot be studied by EPR technique. Atoms and molecules with electronic magnetic moment and angular momentum can be studied with this method. Four classes of compounds with biological properties stand out in EPR studies:

- a. odd molecules and free radicals,
  - b. biradicals,
  - c. triplet electronic states and
  - d. transition element ions.
-

In these, single molecules and free radicals can occur biologically in the following situations: univalent redox reactions, enzymatic oxidizations (dehydrogenations), radiation damage and photosynthesis. The biomedical application of electron paramagnetic resonance (EPR) has been grouped together under five headings:

- a. The study of free radicals in living tissue.
- b. The relation between free radicals and carcinogenic activity.
- c. The study of oxidation-reduction systems and enzyme interaction.
- d. Photosynthesis and optical absorption studies.
- e. X-irradiation of biological material.

## 2. The EPR study of free radicals in living tissue

The first study of free radical concentration in living tissues by EPR was conducted by Commoner et al. [1]. The presence of water in biological materials poses difficulties in EPR studies. The interaction of the large dipole moment with the microwave electric field in the liquid phase causes a large quenching and a Q factor decrease. This can be corrected using smaller samples (e.g. sample tubes of 1-mm diameter instead of the normal 5-mm diameter). Working with a high radiofrequency instead of microwaves region can be useful to obtain high sensitivity for aqueous studies. The loss due to the dipole interaction falls with a decreasing frequency. At lower frequencies, a larger volume should be used to increase sensitivity. Such a situation is suitable for free radical concentration studies.

If the experiments are carried out in the microwave area, the loss from water can be removed by freezing the sample and by making the measurements at liquid nitrogen temperatures, or by freezing and drying the material before it is placed in the cavity. Radicals can be adversely affected in both methods. In this case, studies with smaller sample tubes should be preferred at room temperature. Commoner et al. [1] prepared their first samples by free-drying by taking them from many different tissues. Thus, the powder samples were run on an X-band EPR spectrometer. The first results are given in **Table 1**. In metabolically active tissues such as green leaves, liver and kidney, it was noticed that there was a higher content of free radicals. In addition, the free radical concentration was found to be associated with protein components. It has also been shown by fractionation experiments that protein denaturation destroys the radical concentration.

According to the results obtained from studies done with ungerminated and germinated seeds and with leaves exposed to varying amounts of illumination, increasing the free radical concentration is associated with a high metabolic activity. For this reason, although no measurable free radical concentration was found in samples prepared from digitalis seeds before germination, a free radical concentration of  $10^{-7}$  moles/g was obtained from the seeds obtained by the emergence of the primary root.

Material	Radical concentration ( $10^{-8}$ mole/g of dry weight)
<i>Nicotiana tabacum</i> , leaf	65
<i>Nicotiana tabacum</i> , roots	10
Coleus, leaf	180
Barley, leaf	25
Digitalis, germinating seeds	10
Carrot, root	8
Beet, root	6
Rabbit, blood	25
Rabbit, muscle	20
Rabbit, brain	25
Rabbit, liver	60
Rabbit, lung	30
Rabbit, heart	35
Rabbit, kidney	55
Frog, eggs	200
Drosophila, entire	4

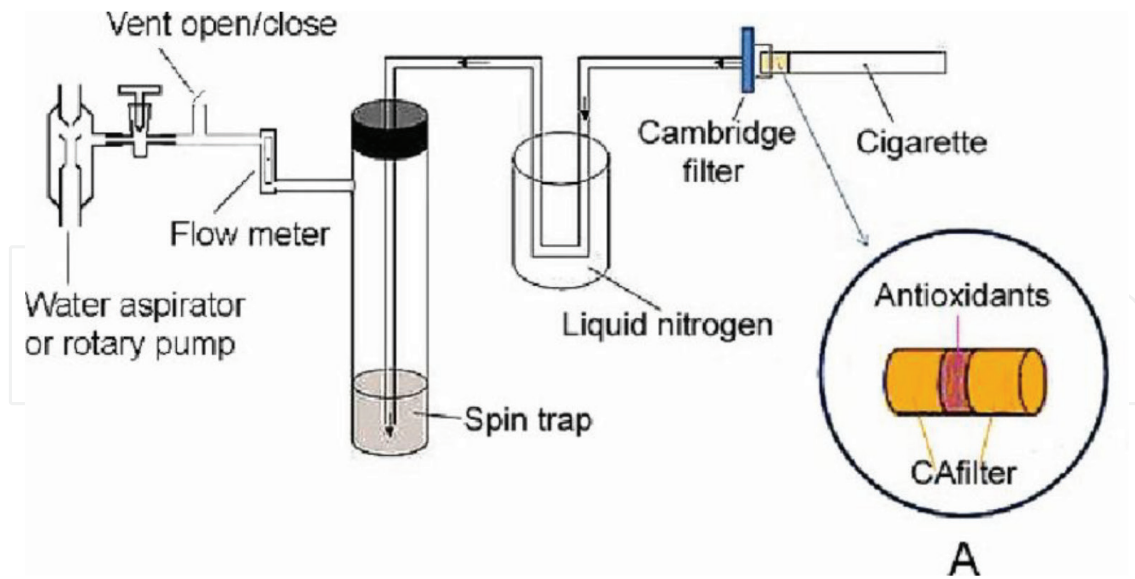
**Table 1.** Radical concentration in different tissues.

These radical concentrations are associated with proteins, reach concentrations varying according to the level of metabolic activity and have the magnitudes consistent with the total electron-transporting content of the tissues [2].

Commoner et al. [1] identified the presence of a high free radical concentration in melanin, a pigmentation found in various biological tissues, Melanin formation in animals by UV or ionizing radiation [3]. This is evidence that free radical production takes place with irradiation of living tissue.

### 3. EPR study of free radicals and carcinogenic activity

Some researchers have acknowledged that free radicals participate in carcinogenic activity [4, 5]. Lipkin et al. [5] suggested that the carcinogenic activities of some large ring structures, together with mild reductive agents, could create negative ion-free radicals. Non-carcinogenic hydrocarbons, such as naphthalene, have also been shown to require very strong reducing agents before such radicals can be formed. Electron paramagnetic resonance is a powerful technique to test the accuracy of these findings.



**Figure 1.** An apparatus for trapping cigarette smoke at low temperatures.

Several experiments have recently been carried out on the radical concentration in cigarette smoke [6]. The arrangement in which the cigarettes are fixed to a filter pump [7] is shown in **Figure 1**. Existing active radicals remain unchanged in the frozen state in the apparatus. After the freezing process is done, when the tube and the contents are processed in the EPR spectrometer for the radical concentration measurement process, it is possible to differentiate between short-lived active radicals and stabilizing radicals.

The concentration of radicals obtained in the frozen smoke condensate before warming was of the order of  $10^{15}$  free electrons per gram. It should be noted that this condensate contains large amounts of solid carbon dioxide and ice. On warming, the condensate separated into an aqueous and an organic or a tarry phase. No radical concentration at all could be detected in the aqueous phase, and that in the organic phase was reduced by a factor of approximately six. The radicals that could still be detected in the latter were found to be highly stabilized, and no diminution of their concentration was found after several days. It would therefore appear from these experiments that the tar constituents of cigarette smoke contain about  $6 \times 10^{15}$  free electron/gram. Some of these radicals, being relatively short-lived, disappear when the condensate is warmed to  $60^\circ\text{C}$  for some minutes. The remainder, however, appears to be very stable and long-lived. It is probable that these stable radicals are very similar to those formed by pyrolysis of other organic matter.

These experiments therefore show that there is a relatively high concentration of both active and stabilized free radicals in cigarette smoke when it is first formed, and it is possible that either or both of these might act as carcinogenic agents. It is important to note in this connection that most bioassays in tobacco carcinogenesis have been carried out using relatively old condensates, and it is now evident that these will be deficient in the unstable free radicals initially present in the smoke.

The subject of carcinogenesis still needs to be resolved and understood. When the effects of various carcinogenic agents such as sodium hydroxide, ultraviolet and ionizing radiation and

thousands of organic compounds are examined, a free radical intermediate always plays a role. Although there is no direct evidence to date, smog studies and recent work by Lyons et al. [6] have found that soot and tobacco smoke contain highly active radicals and signaled possible links with lung cancer.

The concentration of stable free radicals in atmospheric soot is about 100 times larger than in cigarette smoke, but as with the polycyclic hydrocarbons [8], adsorption on comparatively larger particles and further stabilization as a result are likely to render them inaccessible to the cells. It is evident that a large amount of systematic work will have to be performed before the correlation between free radical concentration and carcinogenic activity is established, but these initial experiments show that electron resonance should be of considerable help in these studies.

#### 4. EPR study of oxidation: reduction and enzyme systems

It has already been seen that free radicals have been postulated as necessary intermediates in biological oxidation-reduction systems [9] and that the preliminary measurements of Commoner et al. [1] appear to support this hypothesis. Free radical processes have also been postulated as taking part in most enzyme reactions [10], and kinetic studies often show that chain processes are present. A large number of electron resonance studies of enzyme reactions are now in progress to investigate the details of these reaction mechanisms. At the time of writing, no detailed results on actual enzyme systems have been published, but some preliminary basic work on simpler oxidation-reduction systems has been reported.

One series of organic compounds closely related to biologically important molecules are the phthalocyanines. These are large planar molecules consisting of a conjugated ring system very similar to that of the porphyrin ring and containing either two protons or a divalent metal atom at the center. If these are oxidized by such agents as ceric sulfate, a two-stage oxidation process takes place via a transient intermediate stage which often gives the solution a markedly different color for a few seconds. There has been considerable speculation [11] as to whether this intermediate oxidation stage involved a change of valency of the central metal atom or the liberation of an unpaired electron in the ring system. Electron resonance studies are able to give a very direct answer to such a problem as this, since different valence states of the central metal atom will have characteristic  $g$ -values well displaced from the free-spin value, whereas a mobile electron in the ring system will have a narrow 'free radical' resonance line very close to  $g = 2.0$ .

It was found experimentally [12] that all the phthalocyanines studied had intermediate oxidation states that gave a narrow resonance line with a  $g$ -value very close to that of a free spin. This was therefore conclusive proof that the oxidation process involved mobile electrons in the conjugated ring system. In some cases [12], it was possible to follow the oxidation process through its different stages and watch the growth and decay of the intermediate on the oscilloscope of the resonance spectrometer.

Another oxidation system of considerable biological importance is the oxidation of ferrihemoglobin to its metastable state. This system has been studied in some detail [13, 14] by electron



resonance. The systematic analysis of these results is a good illustration of the fact that it is important to consider all possible interactions when investigating biological systems. As in the case of the phthalocyanines, the oxidation intermediate will either involve an unpaired electron in the conjugated ring system or a change in the valency state of the central iron atom. Chemical investigations [15] give inconclusive evidence as to which of these mechanisms is actually present.

In the initial electron resonance studies [13], it was found that a strong narrow free radical line with a  $g$ -value of 2.0023 was obtained; methemoglobin or metmyoglobin was oxidized by hydrogen peroxide or periodate. By analogy with the results obtained from the phthalocyanines, it was therefore assumed that the oxidation involved electron removal from the  $\pi$ -orbitals of the porphyrin ring. Furthermore detailed and systematic investigations of this system [14] confirmed that there is a close connection between the free radical and the peroxide compound, as shown by four separate sets of results. First, the formation of the free radical is specific for the methemoglobin peroxide reaction, since it does not occur in model systems such as serum albumin-Fe-H<sub>2</sub>O<sub>2</sub> or metmyoglobin cyanide-Fe-H<sub>2</sub>O<sub>2</sub>. Secondly, the removal of excess peroxide by catalase does not affect the free radical. Thirdly, the amount of free radical is proportional to the initial concentration of metmyoglobin, under comparable conditions. Fourthly, the free radical is destroyed by substances reducing the peroxide compound (ferrocyanide, nitrite, iodide, *p*-cresol).

In spite of the above conclusions, however, there are further results which show that the free radical cannot be identified with the peroxide compound itself. The reasons for this can be summarized as follows. First, the calculated concentration of free radical in most favorable case was only 9% of the peroxide compound. Secondly, its concentration varied widely under conditions in which the amount of peroxide compound remained approximately constant. Thus, an increase of pH from 6 to 10 decreased the free radical concentration by 80%, while the peroxide compound was formed equally well at these pH values. Thirdly, the preaddition of stoichiometric quantities of ferrocyanide to the metmyoglobin before the formation of peroxide compound almost eliminated the free radical. Such treatment has been found [15] to remove the first oxidizing equivalent of hydrogen peroxide lost during the formation of the peroxide compound.

These experiments therefore suggest that the free radical is not the peroxide compound itself, but a product of oxidation by the first oxidizing equivalent, which is likely to be a hydroxyl radical [15]. The free radical signal is thus probably due to oxidation of part of the globin molecule, and although it takes place at the same time as the peroxide compound is formed, it is not associated directly with this oxidation mechanism. It is nevertheless of considerable interest as it is an example of a reversible univalent oxidation state in a biological system which does not involve valence changes of a transition metal. The fact that the formation of the actual peroxide compound involved a change in the binding of the iron atom was demonstrated conclusively by observing the metmyoglobin resonance at  $g = 6.0$  [16] as oxidation occurred. This resonance was found to decrease as the peroxide compound was formed, indicating a change from ionic to covalent binding [17] as the oxidation took place.

It may be noted in this connection that a detailed study of the different derivatives of hemoglobin by electron resonance not only allows the actual orbitals involved in the binding to be determined [17] but also gives detailed structural information on the porphyrin and histidine planes [16–20].

This work on the oxidation states of hemoglobin and myoglobin indicates the necessity for careful systematic studies if the electron resonance spectra of these biological systems are to be correctly interpreted. This remark will apply with an even greater force to the catalase and peroxidase [14] and other complex enzyme systems that are being currently investigated.

It may be noted that a kinetic study of these enzyme reactions not only allows the variation of free radical concentration to be followed but also enables correlations to be made between this and the concentration of any paramagnetic atoms that are present. The exact role of different metallic ions in enzyme reactions has been a matter of speculation for some time, and in some cases, they are assumed to play an essential step in the oxidation-reduction mechanism, and in others, they appear to enter only as impurities. If the concentration of the metallic ions is monitored [21] at the same time as that of the free radicals, these effects can be clearly distinguished.

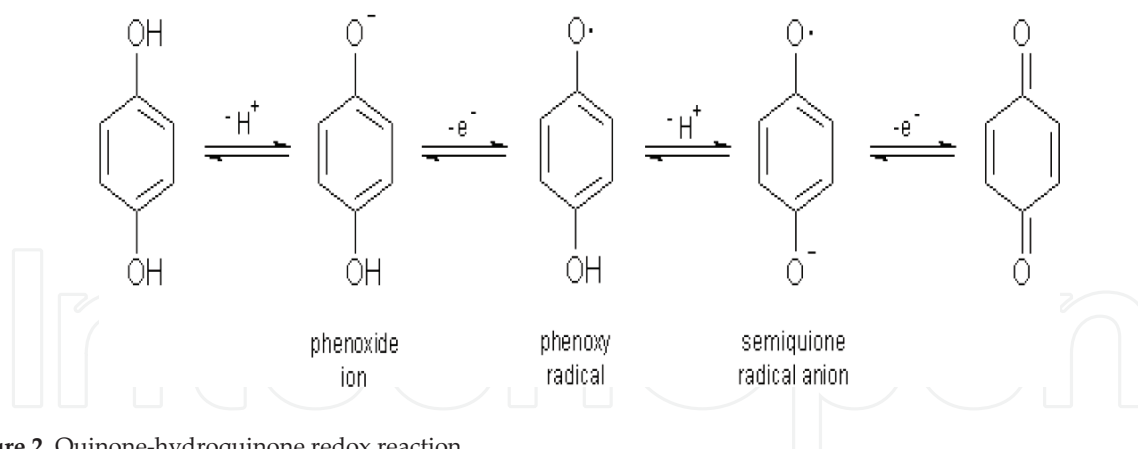
The idea that free radicals can participate in biological processes has recently been explored. It has been suggested by Haber and Willstatter [22] that free radical intermediates form a large part of the enzymatic dehydrogenations. In the single electron transfer hypothesis of biological oxidation, most of the reactants in metabolic redox systems are double molecules. In this case, all the electrons in these molecules are found in pairs. Thus, it is considered that all of the electron transfers must be in pairs. In the two-electron transfer, certain molecules must be present which proceed by a one-electron step. An example of this is the process of delivering an electron under oxidation or reduction to metallo-organic substances of cytochrome. It has been suggested by Michaelis that all the oxidation of organic molecules proceeds in two successive stages and that free radical is an intermediate state [23]. Michaelis proved this experimentally.

Spectrophotometric kinetic studies have shown that various biologically active substances such as free radicals are present as intermediates between fully reduced and fully oxidized states. Prior to the discovery of the electron paramagnetic resonance technique, static susceptibility measurements have been a direct method of detecting more than  $10^{-6}$  moles of paramagnetic molecule solution. Brill [24] has recently successfully carried out an experiment that can detect a  $10^{-7}$  molar solution of paramagnetic molecules. However, Pauling and Coryell [25] gave many values for the magnetic susceptibility of hemoglobin. The hypothesis of Michaelis was not confirmed with complete success due to this low sensitivity. Thus, EPR has greatly contributed to the distinction between paramagnetic ions, odd molecules and free radicals through increased sensitivity.

There are many EPR studies related to non-enzymatic reduction of quinone and quinoid compounds [26]. Two consecutive one-electron transfers occur in these compounds. Furthermore, the lifetime of paramagnetic semi-quinone radicals depends on the isomeric form which is compatible with the differences in oxidation potentials and pH of the solution. While the free radical generated by the dehydrogenation of the hydroxyl group is stabilized in the alkaline solution, the free radical generated by the dehydrogenation of the amino group is stabilized in the acid solution. The compounds which give free radical concentrations are as follows:

p-benzoquinone, o-benzoquinone, catechol, pyrogallol, gallic acid, guaiacol, arsinol, resorcinol, 4-ethyl resorcinol, 4-propyl resorcinol, 4-butyl resorcinol, 4-amyl resorcinol, 4-hexyl resorcinol, 2-nitro resorcinol, gentisic acid, homogentisic acid, polyporic acid, tocopherol, 1,5





**Figure 2.** Quinone-hydroquinone redox reaction.

naphthalenediol, 1,4 naphthoquinone, 1,2 naphthoquinone, juglone, menedione, vitamin K<sub>1</sub>, vitamin E, phthiocol, anthraquinone, anthraquinone  $\beta$ -sulphonic acid, alizarin, carminic acid, phenanthrene quinone, phenolphthalein, ascorbic acid, nicotinic acid, p-phenylene diamine, p-aminophenol, benzidine, diacetyl, benzil, ninhydrin, riboflavin, FMN, indigo, adrenaline.

If the lifetime of the free radical intermediate such as the parabenzoquinone redox reaction shown in **Figure 2** is short, the probability of a polymeric reaction is reduced.

Beinert [27], Commoner et al. [28] and Ehrenberg and Ludwig [29] conducted a number of studies on EPR studies of free radicals resulting from enzymatic oxidation-reduction reactions. Beinert has shown that the EPR signals observed for flavin enzyme and fatty acyl (CoA) dehydrogenase are associated with the re-oxidation of the enzyme-substrate complex by molecular oxygen [27]. However, more studies should be done to determine whether they will be confronted with other double flavin enzymes. Beinert could be shown by comparison with optical kinetics that the radicals observed in a single flavin enzyme and cytochrome reductase are intermediates in the enzyme substrate reaction itself.

In enzyme chemistry, the detection of paramagnetic ions in enzymatic reactions is important. In this subject, EPR plays an important role. Bray et al. [30] investigated the role of molybdenum in xanthine oxidase reactions, despite the oxidation state; Malmstrom et al. [31] investigated the role of Mn<sup>2+</sup> in enolase reactions; Malmstrom et al. [32] investigated the role of Cu<sup>2+</sup> in laccase; Beinert and Sands [33] investigated the role of Cu<sup>2+</sup> in cytochrome oxidase and the role of Fe<sup>2+</sup> in cytochrome-reductase [34]. In addition, after all the iron reduction (28 per cent), Beinert and Sands [33] showed that no free radical signal reduced by DPNH was produced. Because DPN shows a reaction sequence in which the electron flow sequence progresses in the form of substrate→flavin→iron. The answer to whether or not the iron is in contact with other electron receivers can only give EPR.

## 5. EPR studies on photosynthesis and optical absorption

The free radicals play an important role in photosynthesis, and it had been proposed previously that the excitation of chlorophyll during photosynthesis involved mono- [9] or bi-radical [35]

formation. In the initial experiments [1], the leaves were lyophilized before insertion into the cavity, and hence kinetic studies on the specimens were not possible. In a more recent series of experiments, however, Commoner, Heise and Townsend [36] were able to study aqueous suspensions of chloroplasts, in situ in the cavity, under different conditions of illumination. This was achieved by the use of very small specimen tubes in conjunction with a high-sensitivity 100-kc/s field modulation spectrometer.

The chloroplasts, which are known to be responsible for the essential steps of photosynthesis [35], were prepared from leaves of *Nicotiana tabacum* by gentle hand maceration in an ice bath in a buffer solution of pH 8. After filtration and centrifugation, the chloroplasts were resuspended in 55% sucrose and stored in the cold. The spectrometer employed a reflection-type cavity and had a 2-mm diameter hole in the shorting end through which the light from a car headlamp was focused. In this way, the free radical concentration in the chloroplast suspension could be studied under different conditions of illumination, and the rate of growth and decay was measured accurately.

It was found that there was only a very small radical concentration in the absence of any illumination, but that this increased by about sixfold as soon as the 50 c.p. headlamp was switched on. Rapid tracing of the signal enabled an accurate plot of the growth of radical concentration to be obtained. It was found that the concentration rose exponentially to a steady value after the onset of illumination with an exponential time constant of 12 s. The decay of radical concentration when the lamp was switched off was also of an exponential form, but with a somewhat longer constant of 45 s. These results show conclusively that a steady-state radical concentration was being measured and not a system of trapped or stabilized radicals.

The variation of radical concentration with intensity and the wavelength of the incident illumination were also studied [36]. The concentration was found to rise with an increasing intensity but reached a saturation value at high levels, and this is also true of the photosynthetic activity of both chloroplasts and whole cells. It was also shown that the free radical concentration was produced by the same wavelength range 4000–7000 Å, which is responsible for photosynthesis. These experiments therefore provide conclusive evidence that one-electron intermediates with unstable molecular configurations are produced during photosynthetic reactions.

Further measurements on illuminated chloroplasts were then made by Sogo et al. [37]. In particular, they studied the variation of radical rise and decay time with the temperature of the specimen. Their results are summarized in **Table 2**, and the most striking feature of these results is that the signal growth time is approximately the same when the chloroplasts are frozen at  $-140^{\circ}\text{C}$  as when they are at room temperature. This fact would appear to rule out the ordinary enzymatic oxidation-reduction reactions as the free radical intermediates in photosynthesis. The longer decay time at  $-140^{\circ}\text{C}$  also suggests that excitation to the triplet state is not responsible for the observed signal, and it is unlikely that this would be observed in any case. It would therefore seem that the intermediate associated with photosynthesis is some form of electron produced by a dissociated bond or trapped in a quasi-crystalline lattice. It is noticeable [37] in this connection that the lines at room temperature show evidence

Substance	Light intensity (quanta/sec.)	Temperature (°C)	Signal growth time	Signal decay time
Dried leaves	$10^{15}$	25	Minutes	Hours
Dried whole chloroplasts	$10^{15}$	25	Minutes	Hours
		60	Seconds	Seconds
Wet whole chloroplast	$10^{15}$	25	Seconds	1 minute
		-140	Seconds	Hours
Wet small chloroplast fragments	$10^{15}$	25	Seconds	Minutes
Wet large chloroplast fragments	$10^{15}$	24	30 s	30 s
	$10^{16}$	25	6 s	30 s
	$10^{16}$	-140	10 s	Hours

**Table 2.** Growth and decay time of radical concentration in photosynthesis material.

of exchange narrowing, but are wider at the lower temperatures, indicating a reduced mobility of the unpaired electrons.

Tollin and Calvin [38] have also performed detailed studies of the luminescence of similar chloroplast samples and have correlated these results with the electron resonance measurements. As a result of this, it would appear that the hypothesis of electron trapping and the production of holes is the most plausible theory of photosynthetic reaction. This semi-conductor theory of chloroplast action has been proposed by several investigators [39, 40] and is also supported by glow-curve and resistance measurements [40]. In this theory, the luminescence that occurs immediately after illumination ceases will be largely due to radiative recombination of nearly free electrons and holes. Following this, the thermal excitation of electrons and holes from the shallowest traps will produce further emission, and the decay constant will be a function of the trap depth and the temperature. The close correlation of the luminescence and electron resonance is explained in this way, and also the much longer decay times obtained at low temperatures, when the thermal excitation energy is much smaller while the trap depth remains more or less the same.

These initial electron resonance studies of photosynthesis are only of a preliminary nature, however, and the interpretation of the results must still be considered as somewhat speculative. They do illustrate very well, however, the kind of information that can be obtained, and the technique should prove to be a very powerful complement to all the normal luminescence and phosphorescence studies.

Photosynthesis, the biochemical process in which photoenergy transforms into chemical energy, involves steps of oxidation reduction. Thus, EPR is one of the important methods that clarify the photosynthesis process. Calvin and Sogo [41] and Commoner et al. [28] observed light-sensitive EPR signals on the chlorophyll system. Other important work in this regard was carried out by Sogo et al. [37] and Tollin and Calvin [38].

## 6. EPR study of X-irradiation of biological material

A study of the breakdown processes that occur in living tissue as a result of X- or  $\gamma$ -ray irradiation is one of the most important problems in modern medical physics. Electron resonance is one of the most direct and sensitive methods of investigation in this particular field, and the initial results have already shown that a large variety of different spectra can be obtained from different specimens. The energy of the incident quanta is not only great enough to break a very large number of different bonds but the secondary radical and non-radical species can also produce further change in the cell structure. Thus, most biological specimens contain a large percentage of water, and it is known that the OH $\cdot$  radicals formed in this by primary photolysis will combine and produce relatively high concentrations of hydrogen peroxide. This is very toxic for a large number of cellular reactions and may easily produce breakdown processes, the products of which can then be further attacked by primary or secondary radicals. Deductions of the mode of breakdown from the observed electron resonance spectra should therefore be made very tentatively until all possible mechanisms have been considered.

Electron resonance studies of this kind can be divided into two broad categories:

- a. quantitative studies of free radical production as a function of radiation dosage and
- b. qualitative analysis of the types of radical structure formed in the breakdown process.

In quantitative studies, it is possible to study either the dynamic concentrations which are formed in situ in the resonator, or the larger concentrations obtained in a solid or a viscous medium by a suitable trapping technique. In order to obtain dynamic concentrations of sufficient intensity, it is necessary to employ very powerful radiation sources, and this often requires pulse techniques. The use of pulsed irradiation can also give useful additional information. Thus, it is possible, in principle, to trigger the electron resonance spectrometer at a specified time delay after the irradiation pulse and hence obtain a series of measurements on the free radical decay between successive pulses. A high sensitivity detection with long-time constants is not feasible under such conditions, however, and this is another reason why high radiation dosages must be employed. Several laboratories are working on systems suitable for these quantitative dynamic studies, but to date, most information has come from the qualitative analysis of the different radical structures present in the breakdown process.

The identification of different radical species from the hyperfine pattern of their electron resonance spectra has been considered at some length. It was seen there that it is often possible to pick out the presence of specific groups in the presence of other free radicals, and this method of analysis can now be extended to the more complex biological compounds. The results of Gordy et al. [40] on X-irradiated cystine and various fibrous proteins may be taken as a specific example of this. The electron resonance spectra of cystine, hair, nail and feather are obtained [42], and they are seen to be very similar. This similarity is even more striking when it is found that the spectra are not symmetrically placed around  $g = 2$  and have splittings that vary with the strength of the external field. It would therefore appear that in each case, the free radicals must have an unpaired electron strongly localized on a sulfur or an oxygen atom

with an associated anisotropic  $g$ -factor. It is noticeable in this connection that the spectra are very similar to those obtained from frozen solutions of sulfur in oleum [42].

Gordy et al. [43] in fact explain these spectra as due to an unpaired electron localized on the S-S bond of the cystine. Thus, if an electron is ejected by the irradiation from some point in the molecule, the vacancy probably moves to the S atoms to form an additional lower energy three-electron bond.

There is thus now a hole shared by the two sulfur atoms through exchange of their electrons, and the loss of an electron from the sulfur lone pair orbitals tends to strengthen rather than weaken the bond in this case.

From the marked similarity of the spectra of the irradiated hair, feather and toe-nail, it would appear that the radicals in these proteins must also be associated with their cystine content. The above hypothesis also explains why only the cystine spectrum is observed although the cystine group is a small fraction of the total amino acid content of the protein. If the three-electron S—S bond represents a lower energy state, the cystine is likely to donate an unpaired electron to any other ionized group formed by the irradiation. In this way, the two sulfur atoms of the cystine molecule act as an electron reservoir and supply electrons to fill vacancies at other points in the protein molecule, and such a mechanism should very much reduce the damage produced by the irradiation. It is therefore evident that in these particular cases, the electron resonance spectra have not only shown that the cystine type grouping is present in these proteins, but have also indicated that the breakdown process is transferred to the S—S bond, and molecular fracture is therefore not so likely to take place in these compounds.

These results indicate the type of reasoning that is used when deducing radiation effects from resonance spectra of complex molecules. Another example is given by the spectra observed from irradiated glycylglycine, silk, cattle hide and fish scale [43]. A symmetrical doublet with a frequency-independent splitting of 12 gauss is obtained in all of these cases. This is attributed to the direct dipole-dipole interaction between the hydrogen bonding proton and the unpaired electron localized on an oxygen atom of the adjacent polypeptide chain.

This method of analysis by 'correlation of similar spectra' will have to be employed in most of the biological studies in the immediate future. The conclusions deduced in this way must be considered as tentative and often as just one possible explanation among several others. As further results are obtained, however, a greater background of information will be built up on the most likely type of hyperfine interaction present in any particular system.

Gordy et al. [44, 45] have started a series of measurements on the electron resonance spectra observed from various X-irradiated proteins, to try and build up some systematic data for this purpose. With this in mind, the simpler peptide and polypeptides were studied first [44], so that their spectra could then be compared with those obtained from the more complex proteins. These investigations included X-irradiated glycylglycylglycine, DL-alanyl-DL-alanine, acetyl-DL-alanine, glycyl-DL-valine, acetyl-DL-leucine, and DL-alanylglycylglycine. The electron

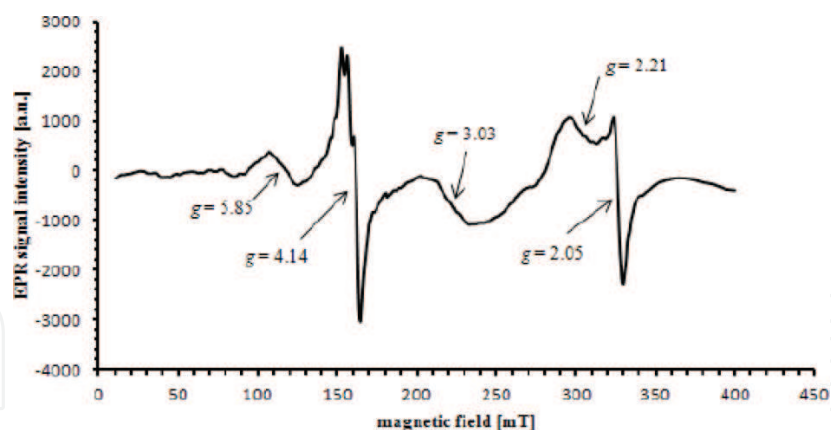


resonance spectra of the more complex proteins such as histone, insulin, hemoglobin and albumin were then investigated [45] and compared with those of the simpler proteins. It was found that two types of pattern were obtained in each case. One of these is very similar to that obtained from the irradiated cystine, and the other is a doublet similar to that obtained from the irradiated glycylglycine. It would therefore appear that the electron-donating power of the S-S bonds and the hydrogen bonding across the polypeptide chains may be general features in most protein structures. The work is also being extended to irradiated hormones and vitamins [44] such as progesterone parathyroid, vitamin A, vitamin K, ascorbic acid and also to irradiated nucleic acids [45] such as DNA, RNA, adenosine, cytidine and inosine. These measurements are all of a preliminary nature at the moment, and much careful and systematic work will have to be done before any definite conclusions can be established. The field is a very wide one, however, and when sufficient systematic measurements have been made, some detailed information on the different mechanisms associated with irradiation damage should emerge.

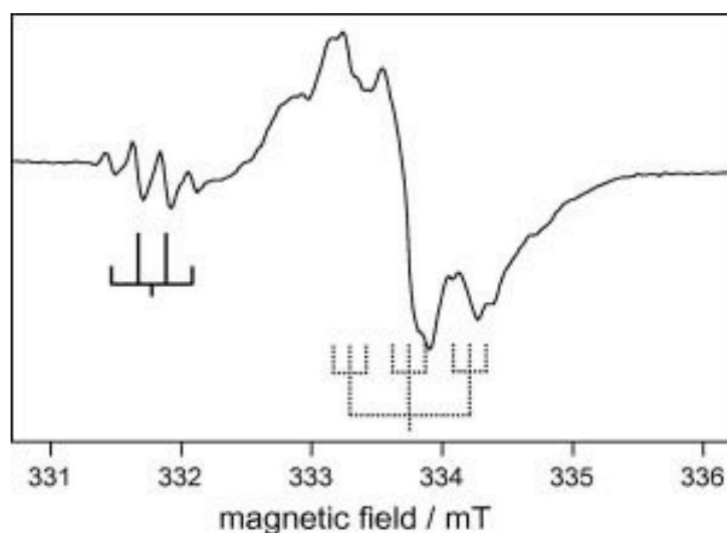
Free radical reactions in a biological system are controlled by both pH and antioxidants. The effect of antioxidants on radiation damage in the biological system is a known fact. Contrary to this effect, it is formed by sensitizers, which are good oxidants. The best examples are O<sub>2</sub> and quinones. In addition, various organic compounds can form active free radicals after reduction. An experiment showing free radical production as a secondary process in radiation damage can be summarized as follows: water and a mixture of 3% alcohol-riboflavin were reduced to 10<sup>-5</sup> M riboflavin by ethyl alcohol radicals, resulting from OH and H radicals generated by 650 kV X-rays. This experiment was performed by Gordy et al. [43]. Information on the level of antioxidant can be obtained by titrating against a standard free radical solution and using EPR. Quantitative determinations of reduced free radical and therefore antioxidant concentrations are described by Blois et al. [46]. At this point, it is necessary to make more detailed studies on the clinical applications of EPR. EPR spectroscopy allows the observation of free radical intermediates *in vivo*. Commoner et al. [1] performed the first experiments with lyophilized samples. EPR studies were carried out for samples such as yeast, blood, rabbit organs, germinated digitalis seeds and barley leaves [47]. In the EPR spectrum of whole human blood, partial conversion of hemoglobin to methemoglobin was observed, and physical damage to both molecules was observed by lyophilization. In addition, a free radical signal associated with the breakdown of porphyrin ring structures of hemoglobin was recorded.

Electron paramagnetic resonance (EPR) spectroscopy is the most reliable technique for relationship between reactive oxygen species and disease (or aging), the measurement of biological free radicals and redox states. It has been used *in vitro* to measure oxygen radicals such as hydroxyl radical and superoxide anion radical in combination with the spin-trapping technique [48]. The measurement of EPR is nondestructive and is unaffected by the turbidity of the sample, so people are interested in using EPR for the *in vivo* measurement of biological radicals. However, there are difficulties with this. First, steady concentrations of biological radicals are too low to detect directly with EPR spectroscopy during their very short half-life.





**Figure 3.** EPR spectrum of whole blood of healthy person contains the signals from  $\text{Cu}^{2+}$  in ceruloplasmin ( $g = 2.05$ ), high spin  $\text{Fe}^{3+}$  in transferrin ( $g = 4.14$ ), high spin  $\text{Fe}^{3+}$  in methemoglobin ( $g = 5.85$ ), low-spin ferriheme complex ( $g = 2.21$ ) and cytochrome ( $g = 3.03$ ). Spectrum recorded at 170 K.



**Figure 4.** EPR spectrum of the  $\gamma$ -irradiated single crystal of 2-thiothymine.

Second, water in the body of the animal causes dielectric loss of the electromagnetic waves used for EPR measurement [49].

EPR spectra of some biological samples are shown in **Figure 3** [50] and **Figure 4** [51].

## 7. Conclusion

Free radicals play a role in biological oxidation-reduction reactions, radiation damage, photosynthesis and carcinogenesis. Electron paramagnetic resonance is a very good technique to directly observe free radical intermediates in most of these reactions. In particular, there are many environmental factors that affect human life. It is inevitable that this technique is guiding in discussing the effects of these factors and taking various measures accordingly.

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