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ELECTRON SPIN RESONANCE IN THE STUDY OF RADIATION DAMAGE*

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Abstract—It has been demonstrated by a Duke University microwave group that the electron spin resonance of the resulting unpaired electron can give specific information about the radiation damage in proteins, nucleic acids, and many other biologically significant chemicals. The structures of their electron resonances show that free radicals of various types are formed from the different amino acids and simpler peptides by ionizing radiations. However, in numerous proteins only two structural patterns are obtained, either separately or in combination. One of these is like the common pattern obtained for cysteine, cystine, and glutathione and is believed to arise from an unpaired electron (electron hole) on the protein sulfur. The other pattern (obtained alone in proteins which have no sulfur) is a doublet characteristic of the interaction of the electron spin with the spin of a single proton. The latter appears to arise from an electron on a carbonyl oxygen interacting with a proton of the hydrogen bridge, or possibly on a —CH— of the peptide chain which has lost an R side group. There is no evidence that the ionizing radiation breaks the polypeptide backbone structure of the proteins. The results seem to require that an electron hole or vacancy created at a given location in the protein molecule can migrate to other locations where it has lower energy.

I. INTRODUCTION

YESTERDAY evening when coming over from the airport I discovered that I was in the car with a biologist. After making this discovery, about half way over, I asked my new acquaintance what it is that the biologists expect of the physicists, what help—if any—we physicists can be to them. He told me that we could give them better instruments. What they need as biologists, he said, are newer and better instruments to see into things. He made no mention of information or theory. I didn't ask him whether we were to bring the instruments or just send them by mail. Nevertheless, I think that a physical instrument which brings information out of biological things should be accepted as a ticket of admission to a discussion of information theory in biology, especially one held under the auspices of physicists!

The instrument which I offer as an admission ticket was not invented by me. Electron magnetic resonance was discovered in 1945 by a Russian scientist, ZAVOISKY (1). Nor can I claim to be the first to apply electron resonance to the study of radiation damage. That, I believe, was first accomplished by HUTCHISON (2), who in 1949 detected *F*-center resonance in neutron-irradiated alkali halides. Our group at Duke University, we are proud to say, was among the first to show the applicability of electron magnetic resonance in the study of biological substances, and the first, we think, to detect such resonances in irradiated proteins. COMBRISSON and UEBERSFELD (3), independently of our

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work, found resonances in certain amino acids. Their results did not agree with ours, except with those for glycine.

Our group has now obtained electron spin resonances of scores of biological substances which have been subjected to ionizing radiation. These include amino acids (4), peptides (5), fatty acids (6), nucleic acids (7), various proteins (4, 8), enzymes (8), hormones (9), and vitamins (9). Some of these results we think we understand, at least partially; others we do not pretend to understand. This does not discourage us, however. Some twenty to thirty years were required for obtaining reasonably definitive interpretations of x-ray diffraction patterns of a few of the simpler proteins. Nevertheless, it must have been apparent from the first that these patterns contained a wealth of information which would eventually be decoded by the persistent scientist. In electron spin resonance we now have a direct method for studying radiation damage which is comparable to the x-ray diffraction method for the study of structures. It is, in fact, a specific for such studies, for it 'sees' not the normal biological matter but the radicals, or broken pieces of molecules torn apart by ionizing radiations.

Descriptions of microwave spectrometers for observation of electron magnetic resonances are available (10, 11). Such spectrometers can now be obtained commercially. Descriptions of theoretical methods and applications to chemical and biochemical problems are given in recent publications (11, 12, 13, 14, 15, 16).

In the observation of electron magnetic resonance the sample to be investigated is placed in a microwave cavity at a point where the magnetic component of the microwave radiation is strongest. The cavity containing the sample is so placed in a d.c. magnetic field that the lines of the d.c. field lie perpendicular to the magnetic component of the microwave radiation. When the d.c. field is adjusted to the proper strength for resonance, microwave radiation will be absorbed. The value of the field for resonance is:

$$H = \frac{h}{g}\frac{\nu}{\beta} \tag{1}$$

Numerically,

$$H\,(\text{gauss}) = 0.7143\nu\,(\text{Mc/sec})/g \tag{2}$$

where g is the spectroscopic splitting factor for the paramagnetic species. It is found that for practically all organic free radicals, including those produced in solids by ionizing radiation, the g value is very close, within a fraction of a per cent, to the g factor for the free electron spin, 2.0023. This comes about because possible orbital moments are largely averaged out by the motion of the unpaired electron, or by the spreading out over a number of atoms (delocalization) of its molecular orbital. The persistent observation of a g factor near that of the free electron spin has led to the designation of this resonance as electron spin resonance.

In the vector model, the electron spin vector would precess about the direction of the applied field H. Quantum mechanically there are only two stable orientations for this precessing vector, which represents an average or the 'expectation value' for the electron spin momentum. These correspond to the two observable components, $+\frac{1}{2}$ and $-\frac{1}{2}$, of the electron spin vector along a fixed direction in space. Because of the interaction of the magnetic moment of the spinning

electron with H, the potential energy of the electron is slightly greater for one of the orientations than for the other. The difference in energy for the two orientations is equal to the microwave quantum energy $h\nu$ which will induce the spin vector to flip over from one orientation to the other. The classical Larmor precessional frequency of the electron spin vector about the direction of H is equal to the absorbed microwave frequency. Thus the precessing electron is in tune with, or at resonance with, the microwave radiation.

In normal organic matter about us, the electrons are all—or nearly all—in the lowest orbital levels, with the maximum limit of two electrons in each molecular orbital. According to the Pauli principle, two electrons can share an orbital only if their spins are aligned in an antiparallel manner. If it is assumed that the spin vector of one electron flips over in an imposed field, that of its orbital mate must flip in the opposite direction at the same time, thus preventing any observable absorption or emission of radiation. To produce an observable electron spin resonance in normal organic matter, one must by some means lift electrons out of the completely filled orbitals of the ground level. Strong ionizing quanta, such as those of x-rays, can eject electrons from ground molecular orbitals with sufficient energy to free them entirely from the parent molecule. If a molecule loses a single electron through ionizing irradiation, the ionized molecule—if it holds together—will have a single unpaired electron in one of its orbitals. This electron is now free to flip over in an external field without the opposite flipping of a partner. The singly ionized molecule is thus paramagnetic and can execute electron spin resonance. Furthermore, the electron which is knocked away from one molecule may become attached to a neighboring molecule and thus convert it into a negatively charged radical. Since the latter molecule is presumed to have all its bonding orbitals filled, the new arrival must go into an orbital of higher energy and remain unpaired. Thus resonance of electrons on negatively charged molecules might likewise be detected. If the electron is ejected with sufficient energy it may, of course, ionize several molecules before coming under the control of a particular molecule. The end result is the same, however, except that a single quantum has, in effect, been able to ionize more than one molecule. Two types of charged radicals are thus produced. If the barrier to the return passage of the electron between the molecules is high, as is the case in most organic solids, a sufficiently high concentration of charged radicals can be built up in this way to give a detectable electron spin resonance. The molecules may be small ones such as the amino acids or long-chain macromolecules such as the proteins or nucleic acids. The only requirement is that the separated electrons cannot easily become paired again, i.e. that the radicals produced by ionizing radiation have a lifetime sufficiently long for a detectable quantity to be built up.

II. NATURE OF INFORMATION CONTENT IN ELECTRON SPIN RESONANCE

If the spin of an odd electron of a radical were entirely free from perturbing influence of its environment, its resonance would be a single, sharp, isotropic line with a g factor of 2.0023. Not much information is contained in such a simple signal, although one could measure the lifetime of the radical from

its rate of decay. Also, the very fact that electrons could achieve such freedom within an organic solid might itself be classed as desirable information. Fortunately, however, the electron resonance signals are often rich with information about the environment of the unpaired electrons. Our problem is to decode their messages. There are at least three important sources of information in these resonances. The first is the hyperfine structure arising from interactions of the electron spin moment with magnetic moments of various nuclei around or near the unpaired electron. The second is the small residual spin-orbit interaction which in some instances makes the g factor slightly anisotropic and different from the free spin value of 2.0023. The third is the information which can be obtained from the line widths and shapes. The most important of these sources is the nuclear hyperfine structure.

Most instruments used for detection of electron spin resonance plot the intensity of absorption at a particular frequency as a function of d.c. magnetic field. The appearance of the plot depends upon the instrument as well as upon the actual, intrinsic shape of the resonance. I shall not discuss possible variations in the actual line-shapes, but shall here assume that the resonances have gaussian shape when the intensity of absorption at a constant frequency is plotted as a function of d.c. magnetic field. A high-fidelity receiver and recorder (or cathode ray scope) would reproduce closely the actual shape of the resonance curve, as shown in Fig. 1(a). The high-fidelity systems are not, however, the most sensitive systems. The most sensitive methods of detection employ modulation of the resonance relative to the observation frequency. A narrow-band amplifier is tuned either to the modulation frequency or to a higher harmonic of this frequency. If one uses a frequency modulation which is very small as compared to the width of the resonance and a phase-sensitive amplifier tuned to the modulation frequency, a curve like that in Fig. 1(b) is obtained. This curve represents the first derivative of the actual line-shape. If one uses such a receiver and tunes to the second harmonic of the modulation frequency, a curve like that in Fig. 1(c) is obtained. This curve represents the second derivative of the actual line-shape. Both the first and second derivative curves are commonly employed in display of electron spin resonances. In interpretation of the curves it is desirable to know what method of detection has been employed, especially when there are structural components incompletely resolved. In the illustrations which follow we shall sometimes use first and sometimes second derivative displays.

This brief description of the appearance of the signals and the simplified theory of the structure of the resonance given below will, I hope, make it possible for you, whether you are a biologist, chemist, physicist, or hybrid, to share with us some of the fun of trying to decode the complex microwave messages which we have been receiving from biological substances. You will be able, I hope, to decide for yourself what is definitely proved by the resonances, what is strongly suggested but not proved, and what is merely hinted.

1. *Nuclear Hyperfine Structure*

The hydrogen nucleus, with a relatively large magnetic moment, 2.79 nm, and nuclear spin of $\frac{1}{2}$, is abundant in all organic matter. The only other nucleus with a non-zero spin abundantly found in biochemicals is N^{14} ($I = 1$ and

$\mu_I = 0.40$ nm). Carbon, oxygen, and sulfur are of course also prominent constituents of biochemical matter, but their most abundant isotopes have zero nuclear spins and hence cannot interact with the electron spin. In strong resonances one might detect effects caused by C^{13} (spin $\frac{1}{2}$ and natural abundance 1.12 per cent) or S^{33} (spin 3/2 and natural abundance 0.74 per cent). For some

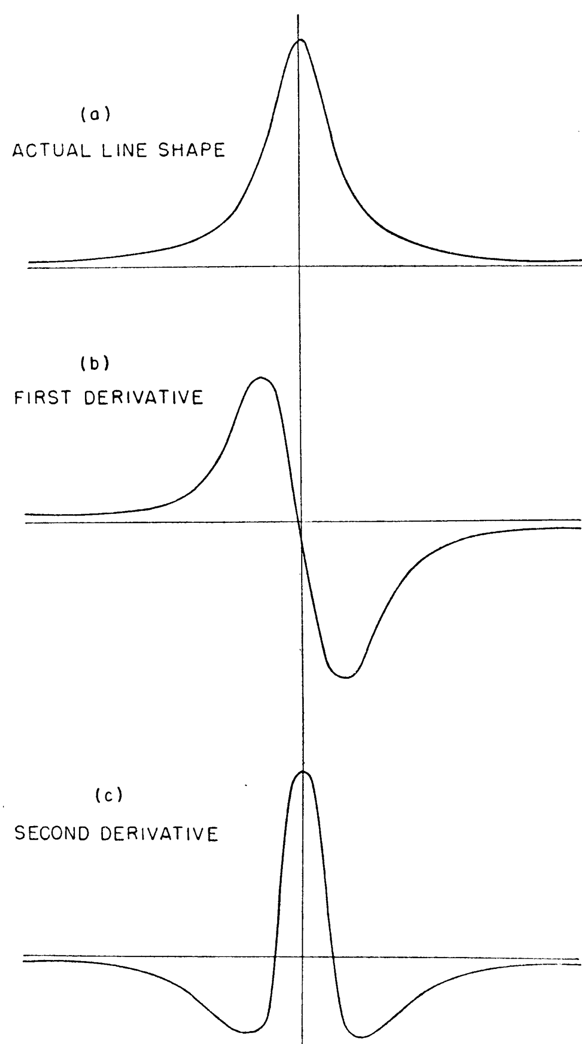

FIG. 1. Appearance of resonance signals as detected in various ways: (a) High fidelity. (b) First derivative curve obtained by small modulation of the resonance with a phase-sensitive receiver tuned to the modulating frequency. (c) Second derivative curve obtained by small modulation of the resonance with phase-sensitive receiver tuned to twice the modulation frequency.

substances one can obtain samples concentrated with C^{13}, S^{33} or O^{17}. Hyperfine structure of their nuclei thus obtained will greatly augment the information obtained from proton hyperfine structure, but it is fortunate for these studies that C^{13} is not the more abundant isotope of carbon. If hyperfine structure from all the nuclei were present at one time, the resulting pattern would often be unresolvable and its decoding thus more uncertain. As it is, there is seldom

any ambiguity about the identity of the nucleus which gives rise to the nuclear hyperfine structure of electron resonances in irradiated organic matter. Usually, it must be hydrogen. By substitution of deuterium for hydrogen, one should often be able to learn which hydrogens give rise to a particular splitting.

When the electron spin resonances of organic radicals are observed in the microwave region at frequencies of 30,000 Mc/sec, the corresponding magnetic field required is 10,700 gauss. A magnetic field of such strength is usually sufficient to produce the Paschen-Back effect, in which the $\boldsymbol{I} \cdot \boldsymbol{S}$ coupling is broken down and both $\boldsymbol{I}$ and $\boldsymbol{S}$ precess about the direction of $\boldsymbol{H}$. Under these conditions the resonance frequencies of the various components at constant field strength H_0 can be expressed as:

$$h\nu = g\beta H_0 + \sum_i A_i m_i \tag{3}$$

where A_i is the coupling constant of the electron for a particular nucleus i with spin I_i and where the magnetic quantum numbers have the values:

$$m_i = I_i, \quad I_i - 1, \cdots -I_i. \tag{4}$$

Usually the resonances are observed at a fixed frequency, ν_0, by variation of the d.c. magnetic field. The resonant field strengths for the various hyperfine components are then:

$$H = H_0 + \frac{1}{g\beta} \sum_i A_i m_i \tag{5}$$

$$= H_0 + \sum_i \Delta H_i m_i \tag{6}$$

The summation is again taken over all the coupling nuclei for each combination of the magnetic quantum numbers. All orientations of a given nucleus (all values of its m) are equally probable and independent of those of the other nuclei. In this expression $H_0 = h\nu_0/g\beta$ is the resonant field strength for the central component of the structure at the observation frequency, ν_0, or that for resonance if there were no nuclear perturbation; ΔH_i is the component separation (in magnetic field units) caused by a particular nucleus i. Obviously, $\Delta H_i = A_i/g\beta$. In these applications we can set g as equal to 2.00 and write:

$$\Delta H_i \text{ (gauss)} = A_i \text{ (Mc)}/2.80. \tag{7}$$

If all the coupling nuclei in a given free radical have the same coupling to the electron spin, one can define

$$T = \sum_i I_i, \tag{8}$$

and

$$m_T = T, \ T-1, \ T-2, \cdots, -T, \tag{9}$$

and can write equation (6) in the simpler form:

$$H = H_0 + (\Delta H) M_T \tag{10}$$

There will be $(2T + 1)$ components corresponding to the different values of M_T. This simplification is often possible in organic free radicals in solids where the coupling nuclei are all hydrogens. It is apparent that, where all

the equally coupling nuclei have the same spin, $T = nI$, and the total number N of components of the multiplet will be:

$$N = 2nI + 1 \tag{11}$$

or

$$n = \frac{N - 1}{2I} \tag{12}$$

Thus n equally coupling hydrogens ($I = \frac{1}{2}$) gives $n + 1$ components. The intensities of the components are proportional to the number of different combinations of the m_i's which give the same sum $\sum_i m_i$ or same value of M_T. Because all the $+\frac{1}{2}$ and $-\frac{1}{2}$ orientations of n hydrogens are equally probable and mutually independent, the intensities of a multiplet formed by equally coupling hydrogens will be gaussian.

The interaction constant A_i of the electron spin with the moment of a particular nucleus may contain both an isotropic and an anisotropic component. The isotropic component, the Fermi term, is independent of the orientation of the sample in the magnetic field and arises from the non-vanishing probability density, $\psi_0 \psi_0^*$, of the electronic wave function at the nucleus in question. Since only the s atomic orbitals are non-vanishing at the nucleus (radius $r = 0$), the presence of an isotropic coupling term for a particular atom in a molecule generally indicates s character in the bonding orbitals of that atom.

For an unpaired electron occupying wholly an s orbital of a particular atom, the coupling to the nucleus of that atom arises entirely from the non-vanishing density $\psi_0 \psi_0^*$ at the nucleus and has the value (17):

$$A_s = \frac{16}{3} \beta \beta_I g_I \psi_0 \psi_0^* = \frac{8}{3} \frac{hcg_I R \alpha^2 Z^3}{n^3} \tag{13}$$

where β is the Bohr magneton; β_I, the nuclear magneton; g_I, the g factor (μ_I/I) of the nucleus; h, Planck's constant; c, the velocity of light; R, the Rydberg constant; α, the fine structure constant; Z, the effective nuclear charge; and n, the total quantum number. For atomic hydrogen in the ground state, A is known to be 1420 Mc/sec. This value with equation (7) gives $\Delta H_i = 507$ gauss as the expected splitting for the atomic hydrogen doublet for the strong-field case ($H \gg \Delta H_i$). The non-isotropic components are zero because of the spherical symmetry of the s orbital. Thus the isotropic coupling to the nucleus of a particular atom gives a good measure of the s orbital contribution of that atom to the molecular wave function of the odd electron in a free radical.

An electron at a fixed distance from a nucleus i with non-zero spin will experience a magnetic field component arising from the magnetic moment of the nucleus. If the spin vectors of both the electron and the nucleus precess about the direction of an applied field H (this corresponds to the strong-field, Paschen-Back case), the non-vanishing field component at the electron, ΔH, caused by the nucleus, will lie along H and will have the value:

$$(\Delta H) = \left(\frac{m}{I}\right) \mu_I \beta_I \left(\frac{1}{r^3}\right) (3 \cos^2 \theta - 1) \tag{14}$$

where I, m and μ_I are the spin, magnetic quantum number, and magnetic moment (in nm units) of the nucleus, β_I is the nuclear magneton, r is the radius vector from the nucleus to the electron, and θ is the angle between r and H. Although the nucleus may be regarded as located at a fixed point within the molecule or crystal, the electron definitely cannot be so regarded. Hence, to find the averaged or effective $(\Delta H)_{\text{eff}}$ acting on the electron in a molecular orbital ψ, we must average the above quantity over the orbital ψ. Thus

$$(\Delta H)_{\text{eff}} = \left(\frac{m}{I}\right)\mu_I\beta_I \int \psi\left(\frac{1}{r^3}\right)(3\cos^2\theta - 1)\,\psi^*\,d\tau \quad . \tag{15}$$

Since the coordinates are separable, we can write this equation as:

$$(\Delta H)_{\text{eff}} = \left(\frac{m}{I}\right)\mu_I\beta_I\left\langle\frac{1}{r^3}\right\rangle_{\text{Av}}\langle 3\cos^2\theta - 1\rangle_{\text{Av}}, \tag{16}$$

where

$$\left\langle\frac{1}{r^3}\right\rangle_{\text{Av}} = \int \psi_r\left(\frac{1}{r^3}\right)\psi_r^*\,d\tau$$

and

$$\langle 3\cos^2\theta - 1\rangle_{\text{Av}} = \int \psi_\theta(3\cos^2\theta - 1)\psi_\theta^*\,d\tau \quad .$$

To attack such a problem one can assume, as is usually done in other calculations of molecular orbitals, that ψ is a linear combination of atomic orbitals, ψ_a, ψ_b, etc. We then readily get a part of the solution for we already know, at least to a fair approximation, $\left\langle\frac{1}{r^3}\right\rangle_{\text{Av}}$ and $\langle 3\cos^2\theta - 1\rangle_{\text{Av}}$ for electrons in various kinds of atomic orbitals. Expressions for these to various degrees of approximation together with coupling constants actually measured are available in standard texts on atomic spectra (17, 18). There is more to the problem than this, however. Although an overlap or cross term of the form $\psi_a(1/r^3)(3\cos^2\theta - 1)\psi_b^*$ may possibly be neglected, an electron in an atomic orbital of atom B might have a significant interaction with the nucleus of an adjacent atom A. It is thus necessary to include terms of the form:

$$\int \psi_b\left(\frac{1}{r_{ab}^3}\right)(3\cos^2\theta_{ab} - 1)\psi_b\,d\tau, \tag{17}$$

where r_{ab} and θ_{ab} are the coordinates of an electron on atom B referred to the nucleus of atom A as the origin. The values of these terms are sensitive functions of the hybridization of the atomic orbitals and of the direction of the projections of the major lobes of the hybridized orbitals. As we get greater skill in the procedure, these orientation-dependent coupling terms should give additional information about orbitals of radicals. Expressed in convenient numerical units equation (16) becomes:

$$\Delta H\,(\text{in gauss}) = 5.05\,\frac{m\mu_I}{I}\left\langle\frac{1}{r^3}\right\rangle_{\text{Av}}\langle 3\cos^2\theta - 1\rangle_{\text{Av}}, \tag{18}$$

where μ_I is in nm units and r is in Å.

In simple cases where single crystals can be prepared, it should be possible to measure $\langle 1/r^3 \rangle_{Av}$ for the interaction of an electron in atomic orbital of atom B interacting with the nucleus of another atom A. Such applications are made later in the discussion. If $\langle 1/r^3 \rangle_{Av}^{-\frac{1}{3}}$ is greater than the interatomic distance, the electron may be in a hybridized orbital of B which projects away from A. If it is less than the atomic distance, the electron may be in a hybridized orbital which projects toward A. In some instances $\langle 1/r^3 \rangle_{Av}$ may be so large that the field of the electron at the nucleus may be greater than the applied field. The nucleus would not then necessarily precess about the direction of H, and the above formula would not hold for all values of θ. It should still hold when θ equals zero or 90°, for then the field of the electron at the location of the nucleus would have, on the average, the same direction as H. If the cloud of the electron is symmetrical about the bond axis between A and B, the angle θ would, in effect, measure the orientation of the bond axis in the field H. For this case $\langle 3\cos^2\theta - 1 \rangle_{Av}$ equals 2 for $\theta = 0$ (bond axis parallel to H), and $\langle 3\cos^2\theta - 1 \rangle_{Av}$ equals -1 for $\theta = 90°$ (bond axis perpendicular to H). Thus the ΔH should have twice the value for θ equal to zero as that for θ equal to 90°. The dipole-dipole interaction of the electron with the nucleus averages to zero when the electron is entirely outside the nucleus and is moving in such a manner that its averaged density achieves spherical symmetry about the nucleus during its lifetime in a spin state.

Nuclear hyperfine structure of any type becomes independent of the magnetic field strength after the field becomes sufficiently strong to achieve the Paschen-Back case, which is assumed in the above treatment. Thus nuclear hyperfine structure can be readily distinguished from the splitting which arises from anisotropy in the g factor, discussed below, if measurements are made at two or more frequencies, both with strong fields. Although the direct-dipole type interaction with the nucleus varies with orientation in the field, it does not vary with the magnitude of the field after the strong field case is reached.

Figure 2 shows the type of hyperfine structure theoretically predicted for the strong field case for various radicals with equally coupling nuclei having spins of $\frac{1}{2}$ (H or F, for example). Figure 3 illustrates a few cases where the coupling of one or two of the nuclei differs from that of the others. It is apparent that these cases are easily distinguishable.

2. *Residual Spin-Orbit Coupling*

If the odd-electron density of a radical is largely concentrated on a non-s orbital of a single atom of a radical or is shared mainly by only two atoms, as it is on the —N—N— group of diphenyl picryl hydrazyl (DPPH), effects of spin-orbit interaction are not entirely negligible. The orbital momentum will be oriented by the strong electrical force of the chemical bond and will not be free to precess about the applied field. Bond-oriented orbital components will give rise to an observable anisotropy in the magnetic susceptibility and thus in the observed g factor. If the odd electron wave function is symmetric about a particular bond as in DPPH, the observed g factor will reflect this symmetry: if all such bonds in a given sample were oriented along the applied H, the $g_{\parallel}$ factor would differ from the $g_{\perp}$ observed when the bonds are all oriented

perpenidcular to H. For an arbitrary orientation θ of the bond axis with H, the observed g_θ factor would have the value:

$$g_\theta = \sqrt{g_{\parallel}^2 \cos^2 \theta + g_{\perp}^2 \sin^2 \theta} \quad (19)$$

In a sample in which the bond angles have arbitrary orientations in the field H such as would be true in a powder or polycrystalline sample, the

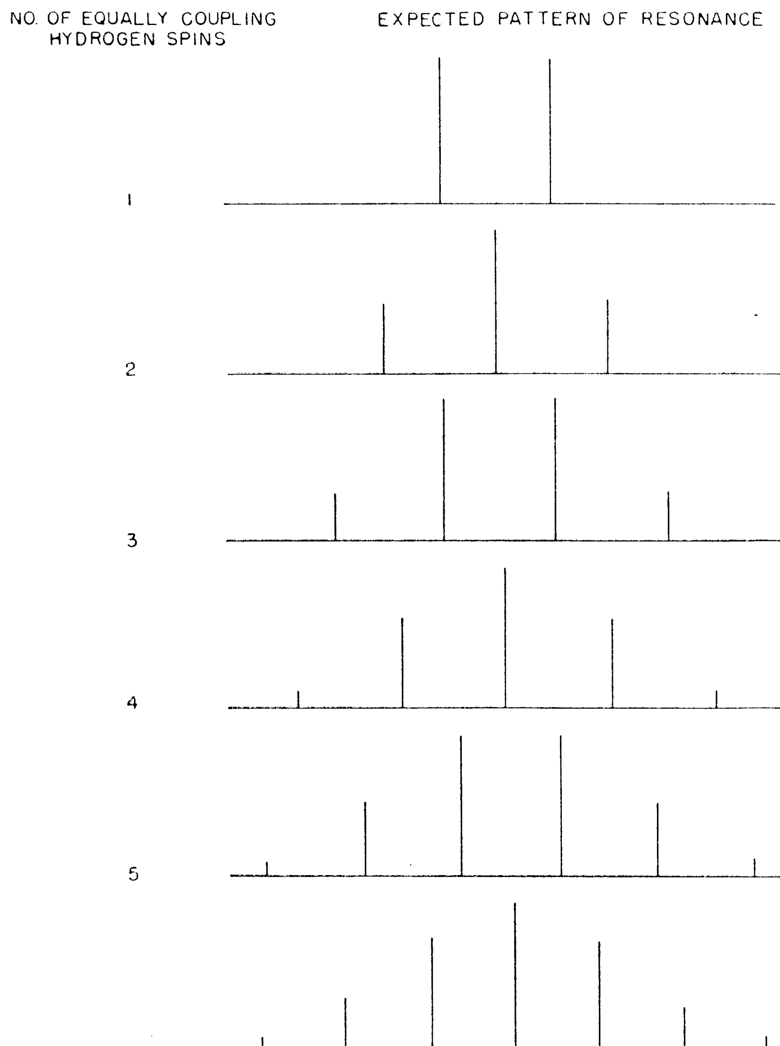

FIG. 2. Types of hyperfine structure predicted for strong-field case for various radicals having different numbers of equally coupling hydrogens or other nuclei of spin ½.

resonance absorption would spread over all values of the field intermediate between that corresponding to the resonance value for $g_{\parallel}$ and $g_{\perp}$. The $g_{\perp}$ would apply for any orientation of H in a plane perpendicular to the bond axis, whereas the $g_{\parallel}$ value would apply only for H along the bond axis. Thus for random orientations in the polycrystalline samples, the $g_{\perp}$ value has the greater weight, and the resonance has an asymmetric form with the highest

peak corresponding to the $g_{\perp}$ value. Such a resonance will have a shoulder or shelf on one side with the edge of the shoulder corresponding to $g_{\parallel}$. First derivatives of an asymmetric resonance arising from an anisotropic g factor in x-irradiated cystine are shown in Fig. 4 for three different observation frequencies. That the apparent structure in these curves is due to anisotropy

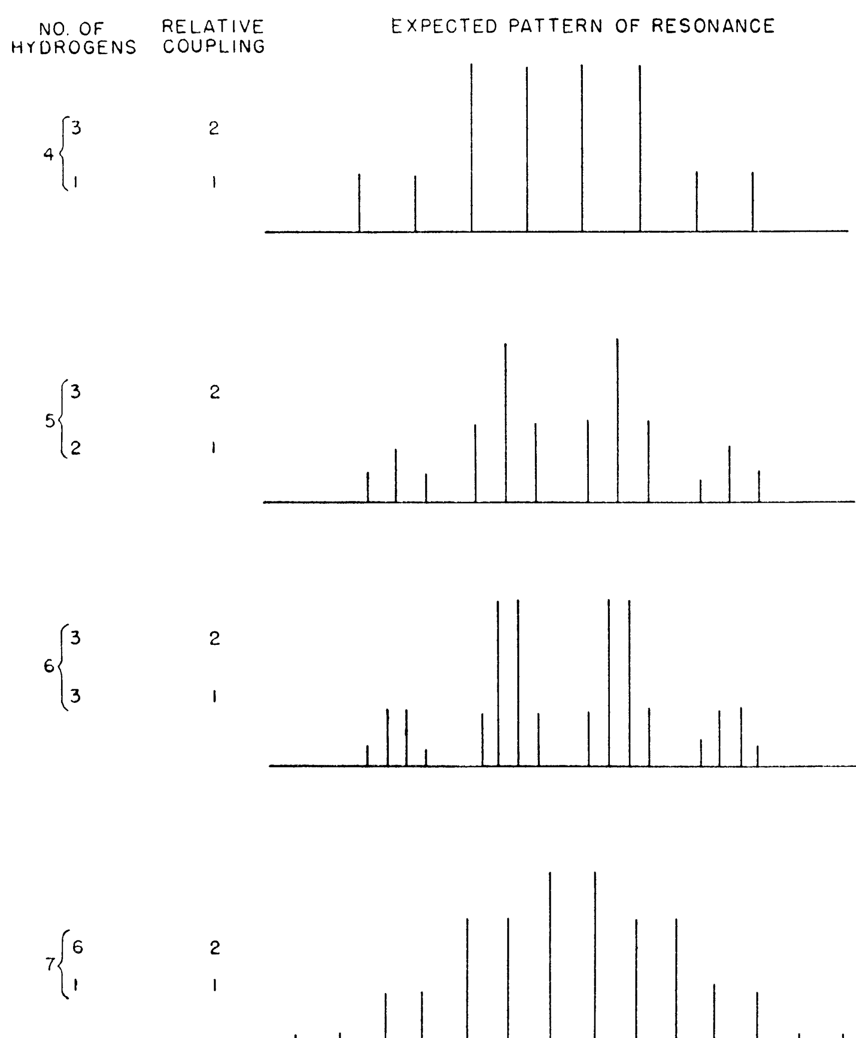

FIG. 3. Some illustrative theoretical hyperfine patterns of radicals with two sets of H nuclei (or other nuclei of spin ½). All nuclei of one set have the same coupling, but those of the two sets differ as indicated.

in the g factor has been established by measurements on a single crystal of cystine at different orientations in the field (19). Such curves show some differences depending upon amount of modulation, variations in natural line widths, degree of anisotropy in g, as well as variations in observation frequency or H value. Nevertheless, there should always be a bend point in the derivative curves corresponding to the H for resonance at $g_{\perp}$ and a lesser one for $g_{\parallel}$. This fortunate circumstance allows measurement of $g_{\parallel}$ and $g_{\perp}$ even in polycrystalline samples. The identity of these bend points, if in doubt, can usually

be established by variation of the modulation amplitude and observation frequency. The outermost bend points will in general correspond to $g_{\parallel}$ and $g_{\perp}$.

III. FREE RADICALS IN IRRADIATED AMINO ACIDS AND SIMPLE PEPTIDES

The work of our group at Duke University has revealed that the isotropic *s*-orbital contributions of hydrogen atoms in aliphatic hydrocarbon radicals are very significant and that they give rise to hyperfine structure in the spin resonance of these radicals which is frequently of the order of 100, and sometimes as much as 200, gauss. This coupling is an order of magnitude greater than that generally found for the aromatic ringed radicals (14, 15, 20) which can be prepared chemically and observed in solution. Furthermore, the first measurements indicated, and later work on single crystals (21) confirmed, that the isotropic *s*-orbital coupling to the hydrogen nuclei in aliphatic hydrocarbon radicals is generally much greater than the orientation-dependent, dipole-dipole component. This very fortunate circumstance makes possible detection and often identification of the aliphatic hydrocarbon radicals made by irradiation of solid matter in the polycrystalline powder and even in impure biological solids. In other words, it seems possible with microwave spectroscopy to 'fingerprint' many of the common radicals produced within solid matter by irradiation. I need not emphasize the usefulness of such a set of fingerprints for the study of radiation damage.

There are two important factors which we believe to be mainly responsible for the reduction of the anisotropic nuclear coupling in hydrocarbon radicals. One of these is the spreading of the odd electron density over a large molecular orbital so that there is no appreciable fraction of the total density near a particular nucleus. The other is the twisting, turning, tunneling, tumbling, or other motion of the radicals, or their parts, within the solid cages in which they are trapped. The first is generally more important for large radicals than for small ones, and the latter is generally more important for room temperature and elevated temperatures than for lower ones.

These properties of aliphatic free radicals and their remarkably long lifetime within solids were not predicted by theory. The conclusions were forced upon us from the experimental evidence for them. Furthermore, this pronounced isotropic interaction through the *s*-orbitals immediately gives much information about the electronic wave functions and structure of hydrocarbon radicals. The large coupling to the H nuclei in the CH_3 radical (total spread of quartet 70 gauss) indicates that this radical is not planar. Amazingly, the characteristic pattern of the ethyl free radical, C_2H_5, is a symmetrical sextet (or approximately so) of about 130 gauss spread. This indicates equivalent, or nearly equivalent, coupling to the electron spin of all five protons.

Fig. 5 illustrates some characteristic hyperfine patterns of hydrocarbon radicals produced by x-irradiation of some simple peptides. Compare these with the theoretical patterns for different numbers of equally coupling protons in Fig. 2. Similar patterns have been obtained by irradiation of amino acids (4) and other compounds (6, 22, 23) with x-rays and with ultraviolet light (24).

Figs. 6 and 7 illustrate somewhat more complex resonances.

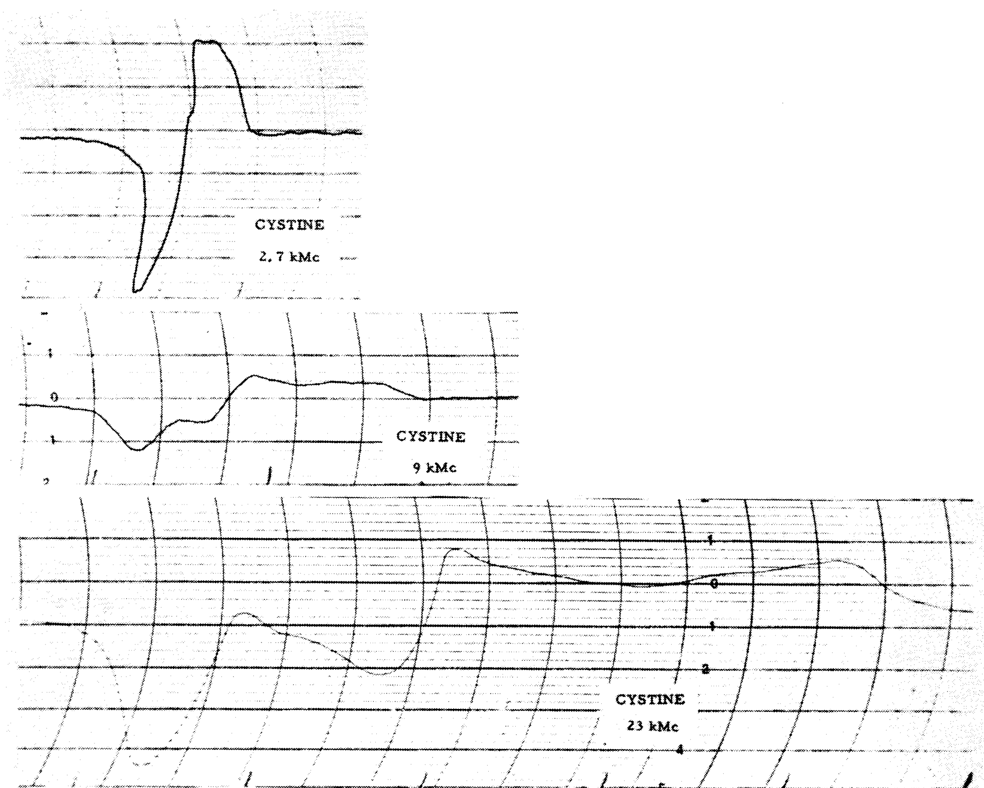

FIG. 4. First derivative curves at different frequencies of powdered cystine after x-irradiation in a vacuum. The markers at the base are 68 gauss apart. The top curve for 2.7 kMc requires a magnetic field of 960 gauss; the middle curve at 9 kMc, one of 3200 gauss; the bottom curve at 23 kMc, one of 8200 gauss.

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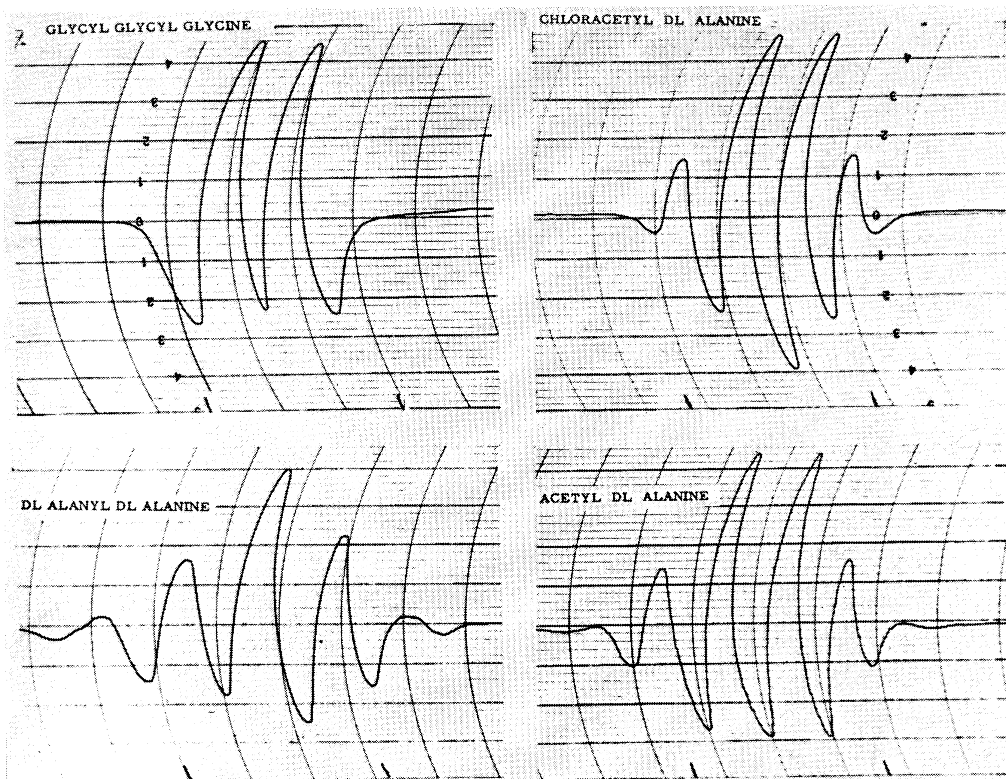


Fig. 5. Some illustrative patterns of resonances of x-irradiated peptides (second derivative curves). The markers at the base are spaced 68 gauss apart. The observation frequency is 9 kMc. From G. McCormick and W. Gordy (5).)

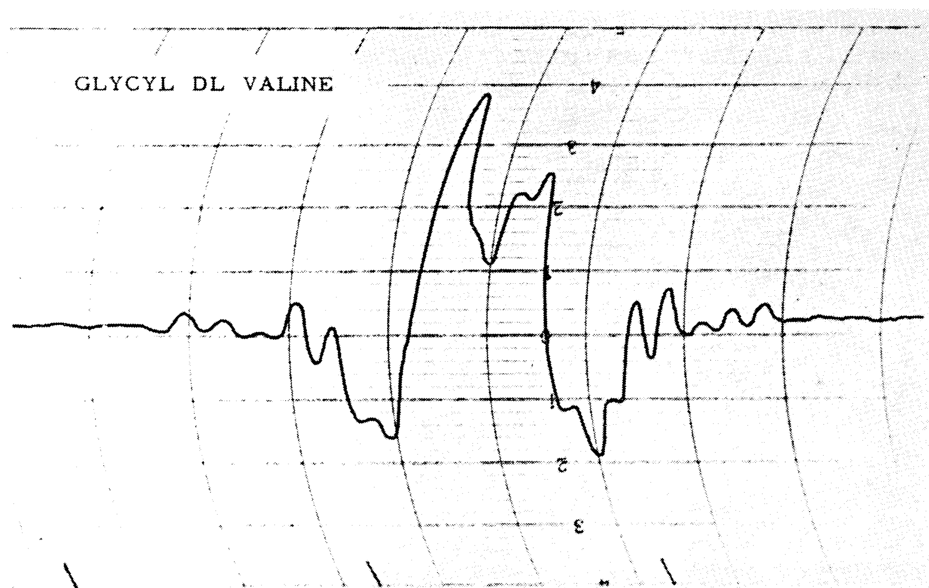


Fig. 6. Hyperfine pattern (second derivative curve) of the radical produced by x-irradiation of glycyl DL-valine. Marker spacing is 68 gauss. Observation frequency, 9 kMc. (From G. McCormick and W. Gordy (5).)

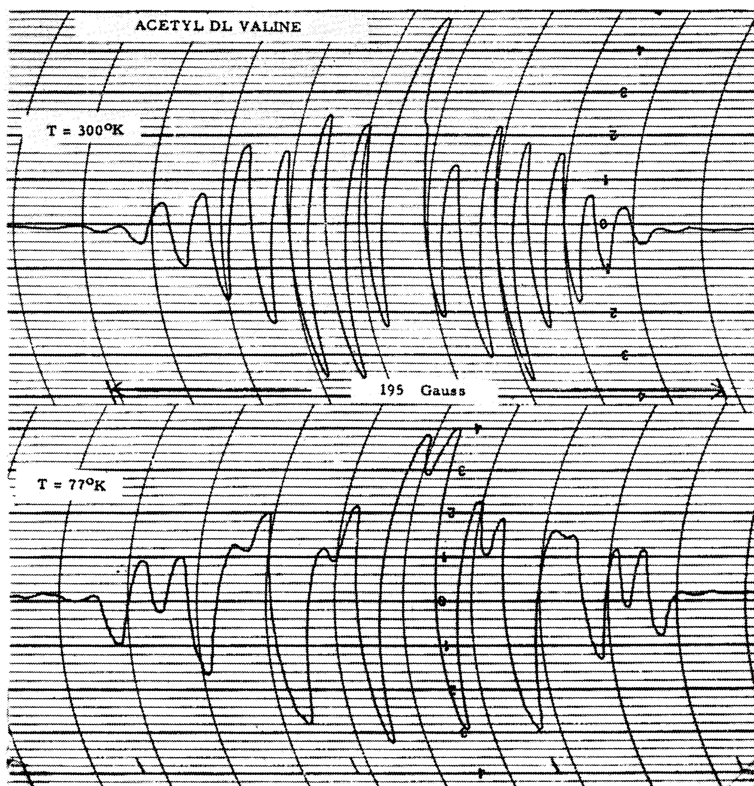


Fig. 7. Hyperfine pattern (second derivative curve) of the radical produced by x-irradiation of acetyl DL-valine. Marker spacing, 68 gauss. Observation frequency, 9 kMc. (From G. McCormick and W. Gordy (5).)

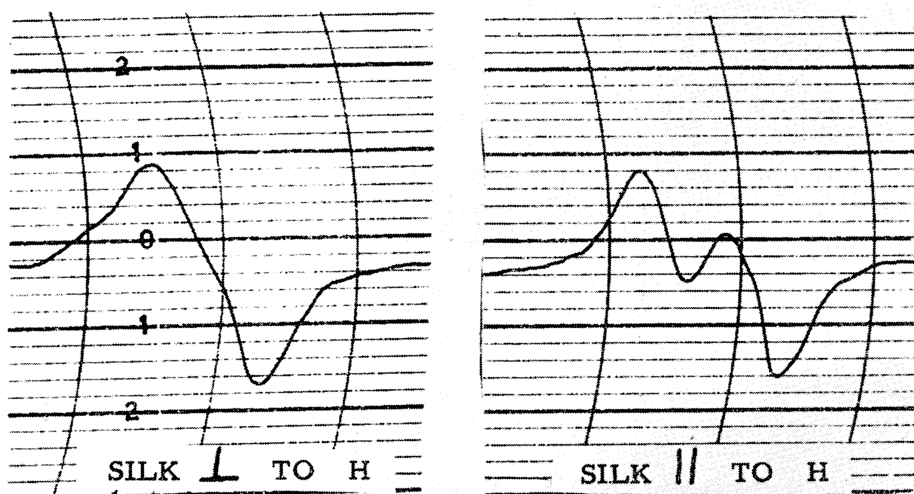


Fig. 8. Resonances (first derivative curves) obtained for x-irradiated silk with strands oriented parallel and perpendicular to the magnetic field. The observation frequency is 23 kMc. (From W. Gordy and H. Shields (8).)

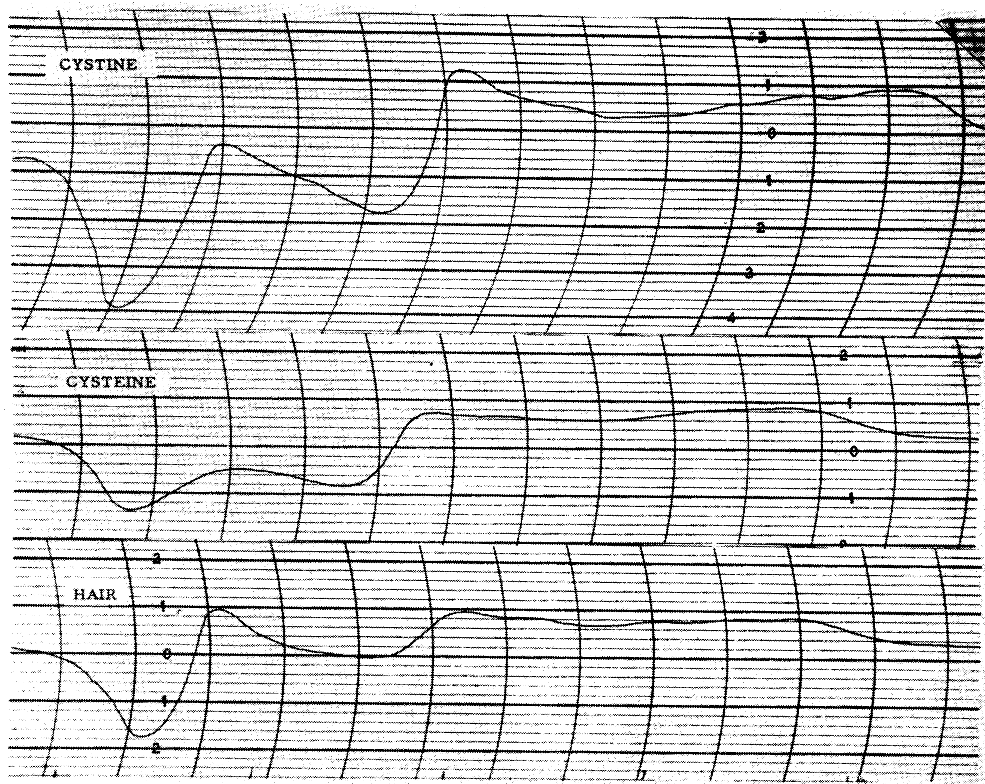


FIG. 9. Resonances (first derivative curves) of x-irradiated hair compared with similar resonances for cystine and cysteine. Marker spacing at base, 68 gauss. Observation frequency, 23 kMc. (From W. GORDY and H. SHIELDS (8).)

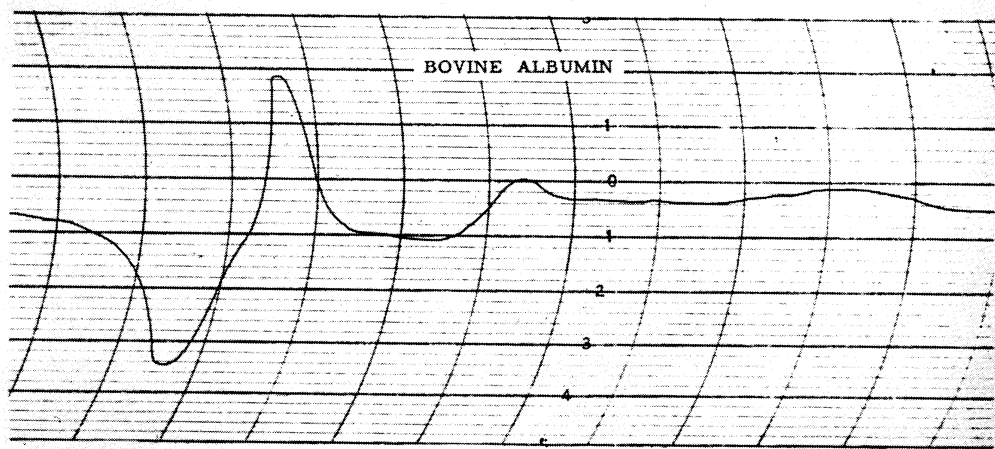


FIG. 10. Resonance (first derivative curve) of bovine albumin which represents a combination pattern of the glycyl-glycine (or silk) doublet and cysteine (or hair) resonance. Observed at 23 kMc. (From W. GORDY and H. SHIELDS (8).)

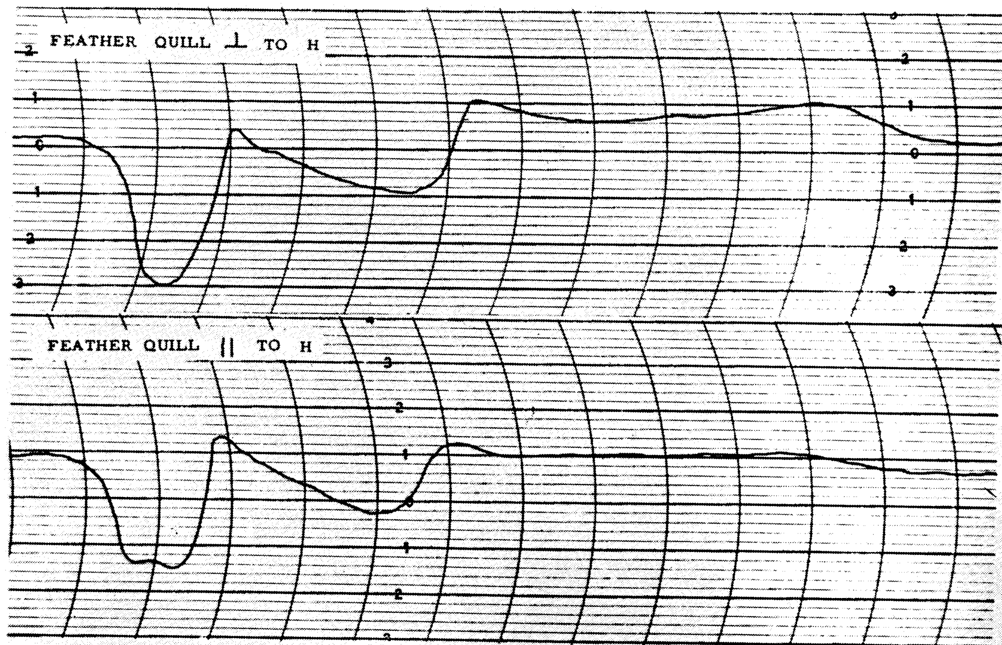


FIG. 11. Resonances (first derivative curve) of x-irradiated feather quill (of a goose) at 23 kMc for parallel and perpendicular orientation in the magnetic field. (From W. GORDY and H. SHIELDS (8).)

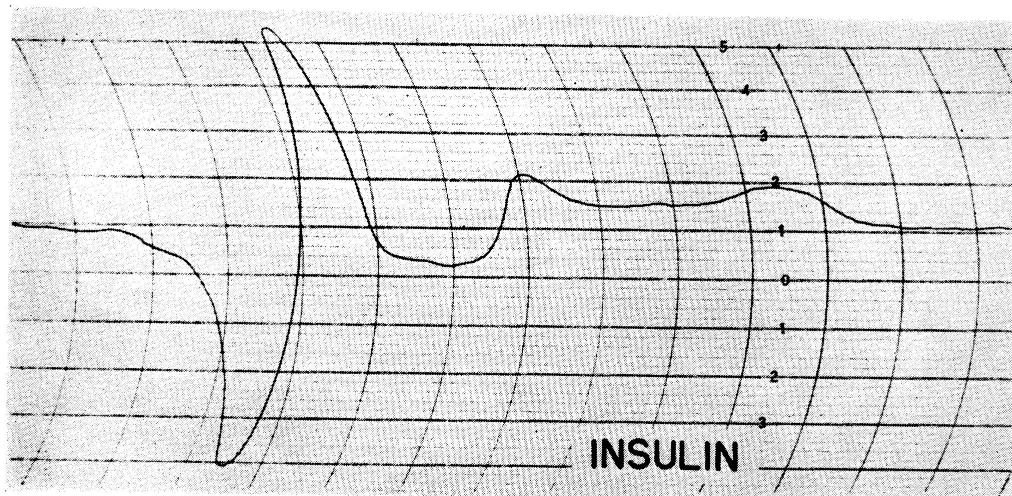


FIG. 12. Resonance (first derivative curve) of x-irradiated insulin observed at 23 kMc. (From W. GORDY and H. SHIELDS (8).)

Cholesterol, $C_{27}H_{45}OH$

X-rayed in vacuum
(indicating the top curve)

X-rayed in air
(indicating the bottom curve)

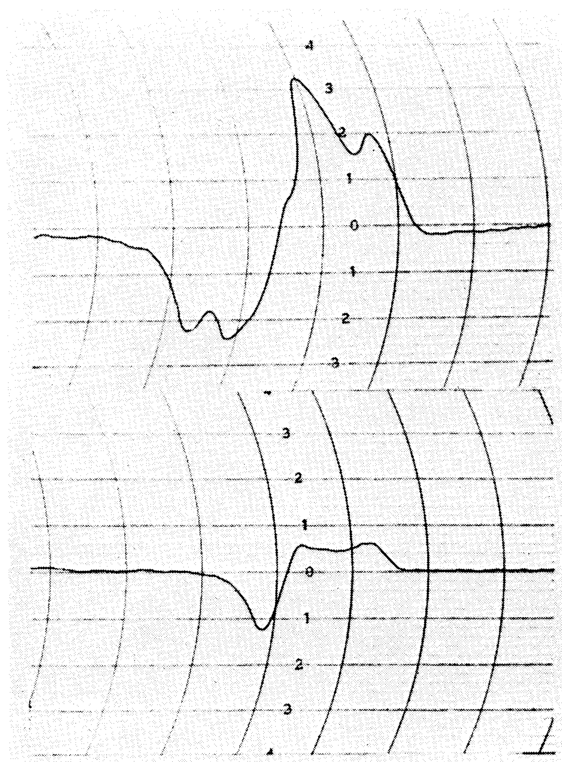


FIG. 13. Resonance (first derivative curve) of cholesterol at 2.7 kMc, x-irradiated in a vacuum (upper figure) and in air (lower figure). (From H. N. REXROAD and W. GORDY (24).)

There are far too many radicals already observed in irradiated amino acids and peptides to discuss them here. I should like to mention one more, however. The pattern of Fig. 7 for acetyl valine consists mainly of a set of nine symmetrical doublets spread over 200 gauss. There is another resonance near the center of the group which I ignore for the present discussion. Seemingly, the nine doublets must arise from eight equally-coupling protons and a ninth with coupling only about half as much as each of the eight at room temperature, and only about a fourth as much at liquid air temperature. This pattern requires an almost unimaginable radical. The odd electron must spread two-fifths of its total density in 1*s* orbitals of the eight equivalent hydrogens. This indicates a radical with a high concentration of hydrogens. It is difficult to design a radical with eight equally coupling hydrogens, especially with a ninth coupling differently. The $(CH_3)_3C$ radical would have nine equally coupling hydrogens which would be expected to give a hyperfine spread of the order of 200 gauss. If we should assume that one of the hydrogens in $(CH_3)_3C$ is replaced by a group RH with only one coupling hydrogen (such as OH) and one which does not noticeably disturb the coupling of the other two, we would have a radical which might account for the acetyl valine pattern of nine doublets.

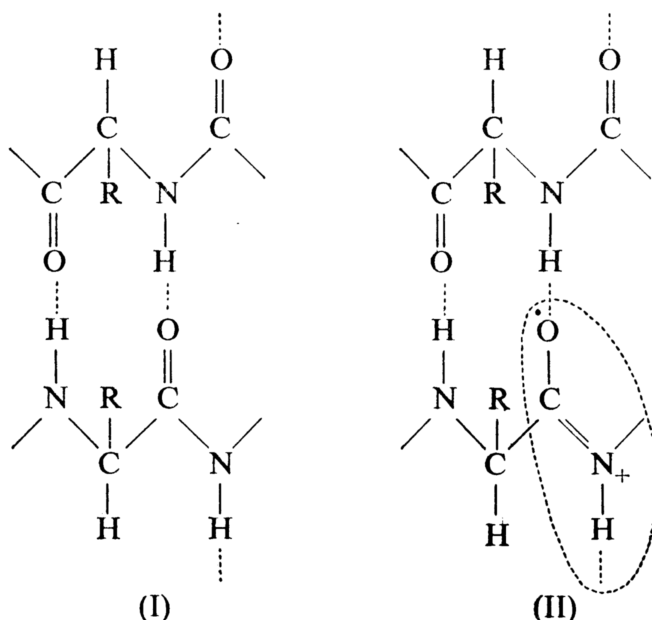
IV. RADIATION DAMAGE IN PROTEINS

In contrast to the varied hyperfine patterns found for the resonances of the x-irradiated amino acids and simple peptides, we have found mainly (but not exclusively) two patterns either singly or in combination for numerous proteins. One of these patterns consists of a simple doublet arising from interaction of the odd electron spin with a single proton spin. The other pattern is a field-dependent one like that of powdered or polycrystalline cystine, cysteine, or glutathione. Fig. 8 illustrates the first type; Fig. 9, the second; and Fig. 10 is a combination of the two patterns.

In our first papers on electron resonances in irradiated proteins (4), we suggested that the doublet pattern in the proteins might arise from an odd electron localized mainly on an oxygen joined by a hydrogen bridge as indicated in Structure II.

Model I represents a structural section of the unirradiated β-keratin protein. The doublet structure, we thought, might arise from dipole-dipole interaction of the electron spin with the proton of the hydrogen bridge. Partly to test this hypothesis, H. W. SHIELDS and the author (25) have made observations on strands of irradiated silk directed along the applied magnetic field, and also on strands directed perpendicular to it. It is known from infrared and x-ray studies (26) that hydrogen bridges in silk lie approximately in a plane perpendicular to the direction of the silk strands. If we assume, for simplicity, that the odd electron density is symmetrically localized on the oxygen, the θ of equation (18) would measure the angle of the O—H axis with the magnetic field. Hence, when the silk strands are along the applied field, θ equals 90° for all hydrogen bridges, and the doublet splitting is the same for all radicals of the silk. Under these conditions one would expect a clear resolution of the doublet. When the silk strands are perpendicular to the applied field, the

hydrogen bridges have all orientations with the field from 0 to 180°. With this arrangement one would expect the individual components of the doublet to be broader and less well resolved than for the parallel case. These features are not completely in accord with the observed results on silk. The doublet splitting for $\theta = 0$ (parallel case) is found to be approximately 25 gauss, somewhat larger than that previously estimated from the polycrystalline material, and also significantly larger than that expected for the hydrogen bonded model.



Furthermore, the separation of the doublet seems to be greater for the parallel case.

It should be appreciated that what is proved for silk is simply that the radical formed is one in which the odd electron interacts with one and only one proton, and that this interaction is at least partly anisotropic. Later we hope to obtain more specific evidence from deuterium substitution in glycyl glycine, which appears to have the same doublet as that for silk.

Irradiated feather quill gives a composite pattern of a doublet and the cysteine-like resonance. However, the doublet is not as widely spaced as that for silk and is not resolved for a polyoriented sample. It has been found (19) that the strong component to the left of the cysteine-like resonance in feather quill (Fig. 11) is partially resolved into a doublet when the feather quill is arranged parallel to the applied magnetic field, whereas it has only about half the width of the unresolved resonance for the perpendicular orientation. Presumably the structure of the feather quill is that of the alpha helix of PAULING and COREY (27), with the helix axis along that of the quill. Interestingly, the cysteine-like component of the resonance is not orientation-dependent. We believe for reasons given later that this situation indicates that the S—S or the C—S bonds of the quill have many different orientations with respect to the quill axis.

A resonance found to be prominent in x-irradiated proteins which contain sulfur is like that of cystine, shown in Fig. 4. Biological substances such as hair (Fig. 9), hoof, horn, and feather have this as the predominant if not the

only pattern, despite the fact that the cystine or cysteine content is only a few percent. The fact that the same pattern, but one very different from any so far obtained from non-sulfur compounds, is observed for many sulfur-containing proteins and for cysteine, cystine, and glutathione convinces us that the odd electron giving these resonances is essentially localized on sulfur. Whether it is on a single sulfur or is shared by two sulfurs of the S—S link, as originally suggested, remains a question to be answered by later work. That the odd electron is localized mainly on one or two atoms is borne out by the large amount of residual spin orbit coupling evidenced by the anisotropy in the observed g factor, as already explained.

Because cysteine with only —SH sulfur gives the same type of resonance as cystine with —SS— sulfur, it is uncertain whether the electron which gives rise to the characteristic resonance of Figs. 4 and 9 is on a single S or is shared between two sulfurs to form a 'three-electron bond'. When the plus charge accompanying the odd character arrives at the S of the —SH of cysteine, it would probably 'shock' off either the naked proton to leave the neutral free radical RS·, or the H atom to leave RS^+, where R represents the part of the cysteine exclusive of the SH group. In the latter case, the H atom would escape through the lattice or react with something. (We have been unable to detect the free hydrogen radical at room temperature in any irradiated substances.) We do not know at this time which if either of these two events occurs. Interestingly, RS^+ is not a free radical, and no resonance would be detected for this case until further events had transpired. At room temperature, however, the molecules may flop about sufficiently to allow the RS^+ to react with the —SH of a neighbor and to release another H and form the same charged disulfide radical which has been postulated for irradiated cystine. The common patterns of cystine and cysteine might be thus explained. I should say, however, that the two patterns although alike are not identical: the resonance pattern of cysteine has a slightly greater over-all width than that of cystine, a variation which we believe arises from the difference in dielectric medium. If the radicals were different—if one were RS· and the other were $R \cdot (SS)^+ \cdot R$ —a much greater difference would be expected.

If the resonance in irradiated cysteine arises from RS· mentioned above, the resonance of cystine must arise from the same radical, which would result first from ionization of the cystine molecule and later from rupture of the S—S bond to leave RS· and RS^+. There is no evident mechanism by which the positive charge could disrupt the S—S bond other than the initial 'shock' of the sudden arrival of the charge. Such 'shock' effects can be anticipated from the FRANCK–CONDON principle (28). They would hardly be expected to break the S—S link, because its potential curve would be lowered and its bond length shortened by the removal of an anti-bonding electron. The positive charge would have no preference for either sulfur; and, if the S—S bond holds, the odd electron would be shared equally by both sulfurs to form an additional half-bond. The FRANCK–RABINOWITCH caging principle (28) would also tend to prevent the breaking of the S—S link by the 'shock' effect. The two S atoms are in a sense caged and hindered from flying apart by the large inert R groups to which they are attached. Any 'shock' energy would probably be dissipated as vibrational energy throughout the whole dimeric molecule. Such a charged

link would of course tend to attract other agents such as O_2 or H_2O which might later sever the bond or an electron which would restore the normal S—S link.

Although we are not yet certain whether the cystine or cysteine-like resonance arises from radicals of the type R—(S · · · S)$^+$—R or RS·, we are inclined to favor the latter. It would seem that the neutral radical would enjoy the longer life and hence be the more probable one to be detected. Furthermore, in the RSH compounds the formation of the RS· radical would require the simpler process. With the present information we are inclined to believe that R—(S · · · S)$^+$—R is the primary radical formed by ionization of the disulfide compounds but that the healing of the molecule through capture of an electron or later rupture of the charged link, probably by attraction of other groups, or molecules, may occur so rapidly that this charged radical is not the one detected, but rather the neutral radical R—S. However, our interpretations are still tentative. Because we consider the question an important one we are continuing to investigate it experimentally. Studies using S^{33} can clear up this uncertainty. What already seems established is that the odd electron giving rise to the pattern is essentially localized on the sulfur.

The large anisotropy in the g factor for the cystine-type resonance suggests the potential usefulness of this resonance for obtaining structural information about the proteins. Studies by SHIELDS and the author on single crystals of cystine (19) showed a resonance simpler and much narrower than that for polycrystalline cystine, and one which shifted position sensitively with orientation in the magnetic field. After this observation the same crystal was crushed up and found to give the resonance pattern characteristic of polycrystalline cystine, shown in Fig. 4. Observations (19) on strands of hair and on feather quill at various orientations in the d.c. magnetic field showed only the polycrystalline type of cystine resonance for all orientations. It is significant, we think, that the cystine-like resonance in these proteins is not orientation-dependent, for that fact gives convincing proof that the bonds to sulfur, either the C—S or S—S links, in the keratins are randomly oriented (in contrast to hydrogen bridges). We have also made measurements (19) on stretched and unstretched hair and found no significant variation in its cystine-like resonance pattern. In all cases it is like that of the polycrystalline cystine.

The resonance of x-irradiated insulin may exhibit a third type of protein resonance (cf. Fig. 12). It has the characteristic sulfur or cystine-like pattern but with a relatively sharp resonance superimposed (at the left of the pattern). Although it has the same g factor—that of the free electron spin—this component to the left seems too sharp to be classified as an unresolved doublet like that of feather quill or silk. Possibly this sharp component of the insulin resonance may arise from an electron trapped in one of the unsaturated ringed structures known to be on the side chains of this protein. The ringed structure may act as a sink or trap for the odd electron produced by ionizing radiation. We have found a similarly sharp resonance (29) for x-irradiated polystyrene, where the odd electron observed is believed to be trapped in the aromatic rings attached to the backbone structure.

Considering the varied patterns which we found for the resonances of the different amino acids and simple peptides, it was at first surprising to us that

the proteins gave such simple patterns with the same few features, described above, repeating so often either singly or together. We were forced to conclude that the electron hole or vacancy created by an ionizing quantum or particle at any given locality in the protein can move through the polypeptide chain until it reaches one of a few traps or sinks where it becomes lodged. One such low-energy trap we believe is sulfur. Both —SH and —S—S— groups are effective traps. Possibly the unsaturated rings of certain side chains are an important trap.

Furthermore, we must postulate that there are effective traps for the electrons knocked away in the ionization process since these do not always seem to be able to return readily to fill the hole. Because they have not given recognizable resonances, we do not speculate on the negative traps. For most of them, the resonances may be too broad for detection.

V. PROTECTIVE MECHANISMS

I am sure that there are many who have suspected that some proteins when ionized can hold together and conduct the electron hole to certain side-chain groups such as the sulfur link. I think that I have heard Professor E. C. Pollard, of Yale, and members of his group express such views. However, from my brief and sketchy acquaintance with the literature in this field I surmise that this question has been a highly debatable one. In the microwave resonances we have a new and perhaps more direct type of evidence in favor of the migration of the electron holes to certain side-chain groups.

Now that there is new evidence for effective resistance to the breaking of the polypeptide backbone of the proteins by ionization, it is interesting to speculate on the reasons why this is true. If one of the electrons of a localized, covalent bond between two atoms were suddenly removed, the two atoms might—according to Franck–Condon principle—become dissociated while trying to adjust to the new and shallower potential curve with the longer equilibrium distance commensurate with the 'one-electron bond'. It might be supposed that the Franck–Rabinowitch caging would help to prevent any two atoms of a protein chain faced with such an emergency from becoming dissociated. However, the evidence which we have obtained for the migration of the electron vacancy to a sink in the side chains indicates that a particular bond of the polypeptide chain does not have to face the Franck–Condon catastrophe because the bonds are not in a strict sense localized. If we imagine that charge density equivalent to a single electron is removed completely from the localized region of two adjacent atoms along the main chain, we must, at the same time imagine that this charge density is restored quickly, before the atoms have time to move significantly apart, by the flow of electronic charge from a side chain group such as the S—S link. It might be better to think of the ionization as taking place only at these sites where the electron vacancy is detected. A molecular chain or polymer which can conduct a hole out to a non-essential side-chain sink or to a point where a simple recapture of an electron restores the *status quo* has, in effect, a built-in, remarkably effective method of self-protection from radiation damage. Such polymers have a high survival value in a world where ionizing radiations are ever present.

Although our measurements were made in dry—reasonably dry—samples, it seems likely that the same transfer of an electron hole to low-energy sites, such as the side-chain sulfur, would take place in the proteins of living systems. The better mobility of charges in the more fluid systems should only speed up the recapture of an electron and hence the recovery of the system. Of course the attack on the charged radicals such as —(S—S)$^+$— by molecules like H_2O would also be speeded up in the living systems, but in the living systems the electron recovery might well be the more rapid. Even if a break in the S—S bond should occur, this might be less damaging and more easily healed than a break in the polypeptide trunk line.

We seem to be proposing here a self-protective mechanism which would prevent almost any radiation damage whatever to proteins. This is not true for several reasons, one of which is that not all proteins have —S—S— links in their side chains. There are other traps for the 'hole' where bonds are probably broken as postulated for silk, or for the sulfhydryl group, where the hydrogen atom or proton is believed to be freed. A free hydrogen atom could cause trouble in the living system, even though it could be temporarily spared from the S—H group of the protein. Moreover, not all damage to proteins in the living systems is due to the direct ionization of the protein which we have been discussing here. Much of the damage (30) is thought to be done by radicals such as H, OH, and OOH produced by radiation in the inter-penetrating fluid, which later attack and damage the protein. These are the so-called indirect effects.

About the time of our initial experiment on the proteins, a very significant experiment of an entirely different kind was in progress by ELDJARN, PIHL, and SHAPIRO (31) which indicated that the indirect effects are probably not as significant as had been previously thought, and that a high degree of protection could be achieved by previously converting the —SH groups in proteins to —S—S— links. Their experiments are of a chemical nature and employ tagged sulfur (S^{35}) in cysteamine ($NH_2C_2H_4SH$). I shall not attempt to give the details of their experiments but merely to connect their results with ours. The interdependence of the two apparently different types of results has been pointed out in an interesting paper by EHRENBERG and ZIMMER (32). Our results indicate that any ionization of a protein which contains S—H groups would always tend to dissociate the —SH group through the migration of the 'hole' or positive charge to the S. Because of the large cross-section of the proteins there would be a large release of H atoms by this mechanism unless there were many competing —S—S— links or other traps in the protein to protect the —SH. The experiment of ELDJARN *et al.* would seem to 'protect' the —SH group by first destroying it! By carrying the hydrogen away peacefully in a harmless molecule they prevent its being released by the irradiation as a damaging free radical. Later, after the upheaval is past, it can be restored peacefully if needed.

Our results, as well as those of ELDJARN *et al.*, suggest that some agents may exert their protective effects by becoming temporarily attached through a chemical bond to the protein or other thing which they protect. Cystine, glutathione, or other agent which gives up electrons easily is needed for protection against the damaging effects of positive holes. Cysteine, glutathione

in the reduced form, or other —SH agents may exert their protective effects by forming an —S—S— link with an —SH of the protected molecule, as ELDJARN *et al.* proved for cysteamine. Electron sinks which collect the electrons knocked out of the holes, and thus prevent them from causing damaging reactions, would also be protective agents. The most desirable electron storage tank would be a molecule which would accept the electron without itself becoming dissociated, would hold it loosely, and would give it up easily when it was needed elsewhere.

I should like to add that the electron sources (traps for electron holes) attached to side chains are not necessarily restricted to protective action against direct hits: they may also protect from some of the indirect effects. Certain free radicals produced in the medium around the protein might exert their damage simply by stealing away an electron from some point in the protein. This would of course be replaced by an electron borrowed from the protective group, just as if the electron had been removed by irradiation. The effects of ionized O_2 or H_2O—if there are such things—would be, I suppose, to ionize the protein when they came near it. An OH radical might react with the protein molecule, or it might simply ionize the protein and form OH^-. I do not know which would happen in a particular case. I simply wish to illustrate a possible unrecognized protective mechanism against indirect action of the radiation. Some specialists on radiation effects evidently believe that the damage to the protein of the cells is due mainly to the indirect effect of radicals produced in the medium around the protein molecules and that the protective action of such agents as cystine or glutathione is entirely the elimination of these radicals before they get to the protein. I do not mean to imply that such effects and the mechanism proposed to protect against them are not very important. What seems clear is that protection is also needed against direct hits as well, and if possible against those radicals or charges produced in the medium which survive long enough to reach the protein. Because of the ability of the protein to transfer a charge, it now seems possible to provide this type of protection too. In fact it has already been achieved in some measure by ELDJARN *et al.*

The protective mechanism which I have proposed is strikingly related to enzyme activity. Pollard's group at Yale, and perhaps others, have been making experiments which show, I believe, that a single hit in a large enzyme molecule by an ionizing particle is enough to destroy the enzyme activity of that molecule. This is not strange if the sensitive sites for the enzyme activity are synonymous with the sinks or sources for electrons about which we have been talking, and the ability of the enzyme molecule to conduct a hole or excitation is required for enzyme action.

I like to think of the protective agents which are described here as enzymes which prevent reactions. I know, of course, that the normal function of an enzyme is to cause reactions. Some enzymes, so I understand, exert their catalytic action by accepting spare electrons for a time and giving them up again later. In the vivid language of Professor Henry Eyring, they take over the unnecessary children (electrons) during the divorce proceedings and give them back after the remarriages have taken place. One kind of 'protective enzyme' supplies children to prevent divorces (broken bonds) and then later recovers children indistinguishable from those given up (electrons all). Another

kind provides temporary abode for the disrupted children to prevent their disturbing the neighbors. Our living systems probably have already built in both types of protective agents in sufficient quantity to provide reasonably good protection from ionizing radiation encountered in normal living of the past. For the future we may need to add some.

I have not space to discuss radiation damage to other substances—such as fatty acids, nucleic acids, and hormones—for which our group has obtained many spin resonance data similar to that described here. I have not space to discuss the important effects of oxygen on radiation damage to molecules, about which we have obtained information from spin resonance, of the type shown in Fig. 13. I hope that I have described enough of the results to convince you—and the biologist who rode with me in the car—that microwave electron-spin resonance is an important new way of 'seeing into' biological things.

ACKNOWLEDGEMENTS

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Without implying that he is to any degree responsible for any misinterpretations I may have made, I wish to acknowledge with thanks some stimulating and enlightening discussions with Dr. JAMES FRANCK during the preparation of this paper.

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