*TEMPERATURE EFFECTS ON FREE RADICAL FORMATION AND ELECTRON MIGRATION IN IRRADIATED PROTEINS**

By Frank Patten and Walter Gordy

DEPARTMENT OF PHYSICS, DUKE UNIVERSITY

Communicated by Charles R. Hauser, June 15, 1960

From the first observation of electron spin resonance of irradiated proteins[1] in 1955 it was clear that the electron vacancy, or electron spin density, caused by the irradiation must be able to migrate through the protein from the multiple sites where the ionizing particles, or quanta, strike to the few sites such as the cystine sulfur where the spin density is finally detected. These original observations were carried out at room temperature. In the present work it is shown that such migrations do not, in fact, occur significantly at the temperature of liquid nitrogen, 77°K. Thus the migration of electron holes in the valence shell of proteins requires an activation energy, assistance from the molecular motions of excited vibrational or torsional oscillational states.

Studies similar to the ones reported here on the proteins have been made on most of the amino acids, many di- and tripeptides, and on the nucleic acids and their constituents. The results and conclusions drawn from them, which in many respects are similar to those for the proteins, will be published elsewhere. Here we show only the results on the few peptides and amino acids which give resonances like those of the proteins and which are essential for comparison with them. A preliminary report[2] on the present results was given at a meeting of the American Physical Society in 1959.

Experimental Procedure.—All measurements reported here were made at a microwave frequency of 9,000 Mc/sec. The samples were irradiated in a liquid nitrogen flask with a kilocurie cobalt 60 gamma ray source. Dosages of the order of 5 million r were employed. For the observations at 77°K the sample was inserted under liquid nitrogen into the tip of a liquid air flask made of low-loss glass which fitted into a hole in the microwave cavity.

Magnetic modulation was employed with a phase-sensitive amplifier tuned to the second harmonic of the modulation frequency. With the small amplitude modulation employed, this gave a response which represents the second derivative of the actual aborpption curve. The second derivative curve has its peak at the peak absorption of the resonance curve and minima on either side at approximately the half-power points of the actual absorption curve.

Some of the earlier curves obtained and shown here are on curved coordinate paper, whereas later curves are on rectangular coordinate paper. These differences cause no confusion and will be evident from the figures.

Results.—As found in the original observations[1] and elaborated in the accompanying paper, the electron spin resonance pattern indicates that unpaired spins usually occur at only one site in a given protein irradiated and observed at room temperature and at only two different sites for a variety of proteins. In contrast, we have found that if the proteins are irradiated at the temperature of liquid nitrogen (77°K) and their spin resonance observed at that temperature, without allowing the sample to warm up between irradiation and observation, distinguish-

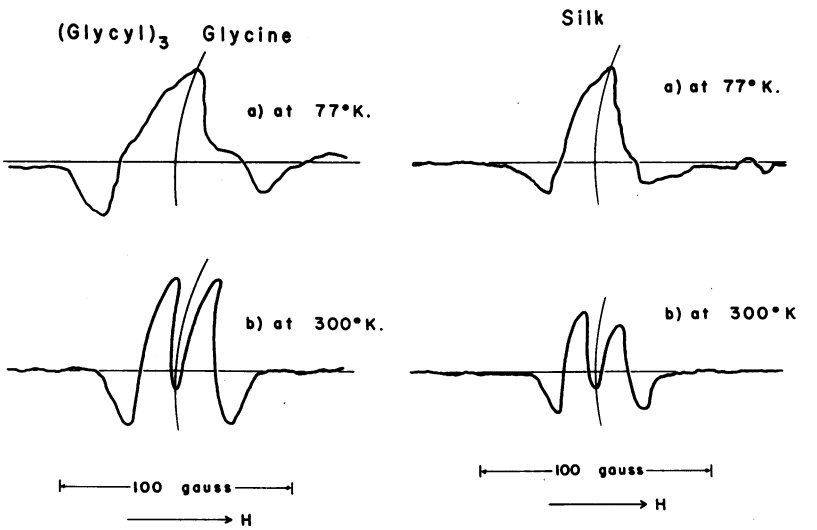


FIG. 1.—Electron spin resonance patterns (second derivative curves) of γ-irradiated silk compared with those of γ-irradiated $(glycyl)_3$ glycine at two temperatures. The top curves represent observation of the unwarmed sample immediately after irradiation at 77°K. The bottom curves were recorded about an hour after the same samples had been warmed to 300°K. The vertical lines mark the position for $g = 2.0036$ (the g factor of the DPPH reference signal).

ably different patterns are observed for different proteins. Furthermore, the spin resonance patterns for individual proteins are generally broad, unsymmetrical in shape, such as would result from superimposed patterns of a number of different radicals. Selected examples of these patterns are shown in the various figures.

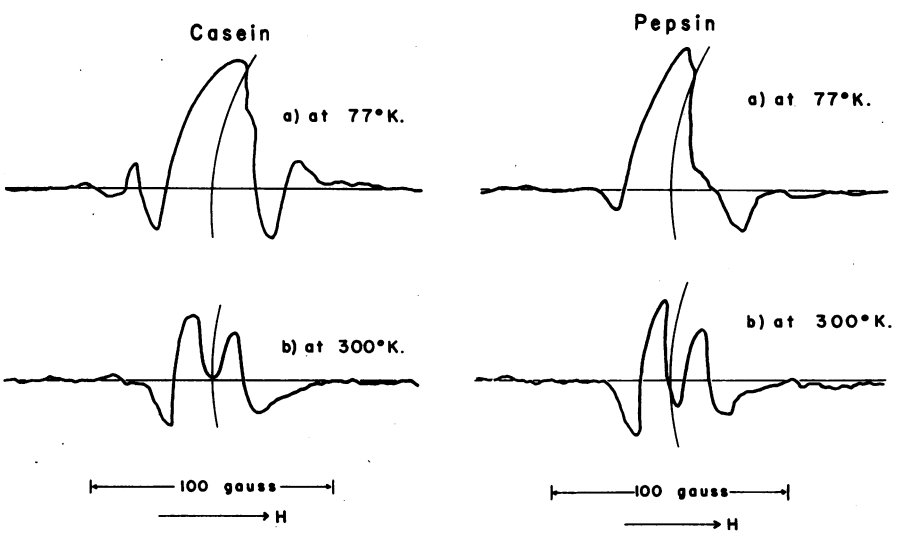
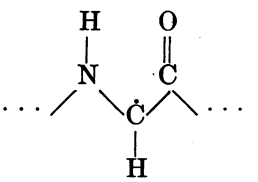


FIG. 2.—Electron spin resonance patterns (second derivative curves) of γ-irradiated casein and pepsin at two temperatures. The top curves represent observation of the unwarmed sample immediately after irradiation at 77°K. The bottom curves were recorded about an hour after the same samples had been warmed to 300°K. The vertical lines mark the position for $g = 2.0036$ (the g factor of the DPPH reference signal).

Figure 1 shows that $(glycyl)_3$ glycine and silk give noticeably different resonance patterns when the samples are irradiated and observed at 77°K. If the samples are then allowed to warm to room temperature the resonance patterns became essentially alike, both a doublet of 25 gauss spacing and a g factor of 2.0035 gauss. The samples, like others of this study, were of a powdered or polyoriented form. Similar results are shown for casein and pepsin in Figure 2. All the signals are noticeably different at 77°K, yet all become the same doublet at room temperature. For the pepsin there is a small amount of the cysteine-like resonance[3] superimposed which makes the intensity of the two components appear unequal.

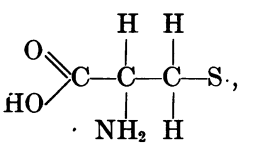
In the native proteins and feather quill (see accompanying paper by Gordy and Shields) the doublet resonance of the type observed here at room temperature has been shown to arise from a free radical of the form,



the electron spin density of which is concentrated mainly on the α-carbon in the peptide backbone structure. This is probably the most common type of free radical formed in the proteins which do not have significant cystine or cysteine residue. However, a difference in the oxygen effect on the doublet resonance in different proteins has been found[3,4] which suggests that there may be two forms of free radicals giving a similar doublet in the proteins, one which is readily attacked by oxygen and one which is not. The free radical shown above, which is thought to be of the type giving the doublets here, is relatively stable to exposure of the irradiated sample to air or oxygen.

The results for acetylglycine, shown in Figure 3, provide an interesting exception to the observation that the free radicals formed at 77°K are different from those formed at room temperature. This exception helps us to understand why the others are different. It will be discussed later.

Figures 4, 5, and 6 are illustrative of the material which gives the cysteine-like resonance when irradiated at room temperature. The resonance patterns of samples of irradiated cystine and cysteine are essentially alike at room temperature. Both probably arise from the same free radical,



which would be formed by the breaking of the S—H bond in cysteine and the S—S bond in cystine. The slight variations in the resonance patterns are thought to arise from differences in the medium rather than from differences in the chemical form of the free radical. L-cysteine and DL-cystine, however, give noticeably different resonances when they are irradiated and observed at 77°K before warming. Compare Figures 4 and 5. Likewise, the proteins which give this resonance

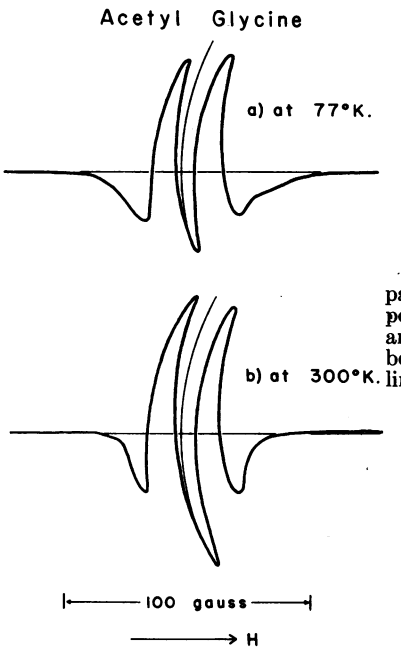


FIG. 3.—Electron spin resonance patterns (second derivative curves) of powdered acetyl glycine, γ-irradiated and observed (a) at 77°K and (b) after being warmed to 300°K. The vertical lines mark the position for $g = 2.0036$.

of the common type at room temperature give characteristically different patterns when irradiated and observed at 77°K. When the samples are warmed to room temperature, the patterns are converted to those of the cystine-like resonance. They retain this character after the samples are cooled again to 77°K.

Nature of Free Radicals Produced by Ionizing Radiations at Low Temperatures.—From the shape of their resonances we conclude that when proteins are irradiated

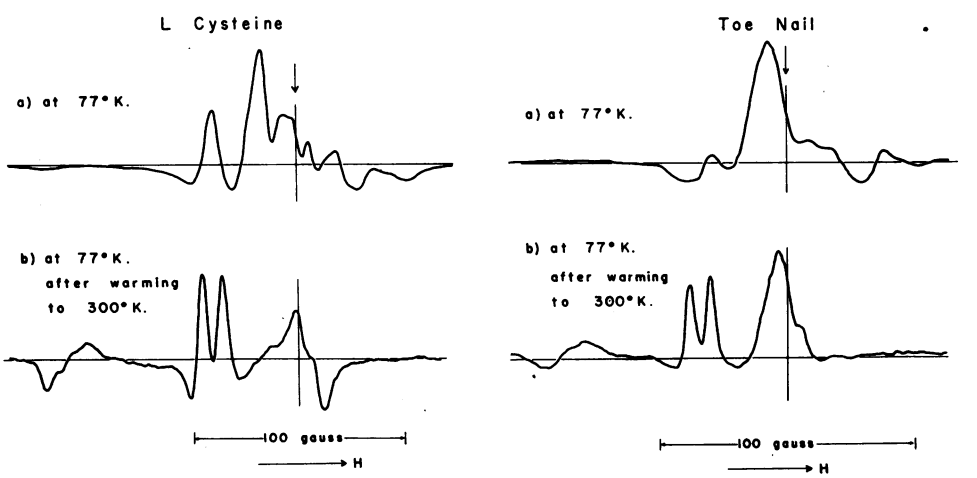


FIG. 4.—Electron spin resonance patterns (second derivative curves) of γ-irradiated cysteine and toe nail. The top curves were obtained after irradiation at 77°K without allowing the sample to warm. The bottom curves were obtained from the same samples at the same temperature but after they were allowed to warm to room temperature and remain so for about an hour before being recooled to 77°K. The arrows mark the position for $g = 2.0036$.

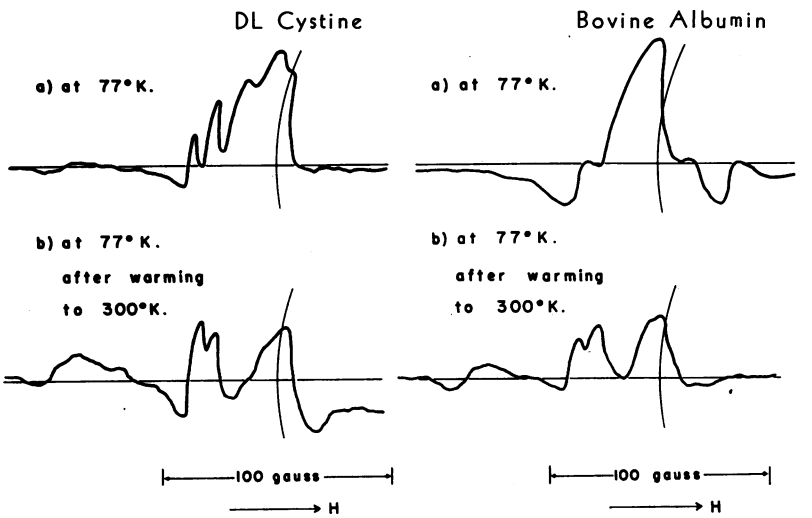


FIG. 5.—Electron spin resonance patterns (second derivative curves) of γ-irradiated bovine albumin compared with those of γ-irradiated DL cystine. The top curves were obtained after irradiation at 77°K without allowing the sample to warm. The bottom curves were obtained from the same samples at the same temperature but after they were allowed to warm to room temperature and remain so for about an hour before being recooled to 77°K. The vertical lines mark the position for $g = 2.0036$.

at temperatures as low as 77°K (and perhaps not so low) a variety of free radicals is produced, i.e., unpaired spins occur at many different sites on the molecule. When the proteins irradiated at the lowered temperature are allowed to warm to room temperature, these complex patterns are usually converted to the simple pattern of a single free radical, the one characteristic of that protein irradiated at

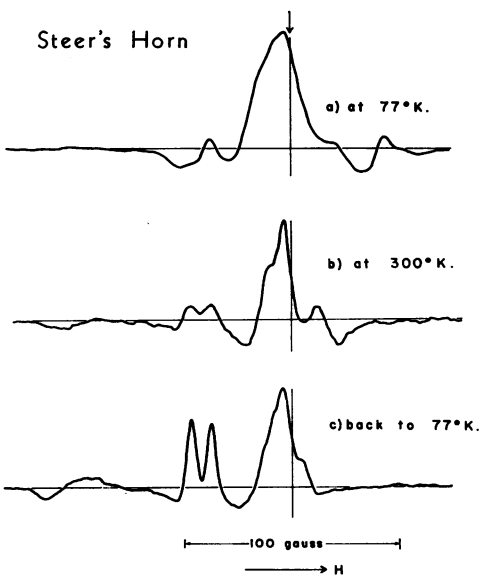


FIG. 6.—Electron spin resonance patterns (second derivative curves) of steer's horn (*a*) immediately after γ-irradiation at 77°K, (*b*) after warming to 300°K, and (*c*) after cooling again to 77°K. The arrow marks the position for $g = 2.0036$.

room temperature. Our observations were made after the samples had been at room temperature for an hour or more.

The changes which occur when the sample irradiated at 77°K is allowed to warm are not reversible when the temperature is again lowered to 77°K. In contrast, any changes of the room temperature patterns with the lowering of the temperature to 77°K are reversible. The conclusion drawn from this difference in reversibility is that the free radicals produced at low temperature are changed to chemically different ones upon warming, whereas those formed at room temperature remain in the same chemical form upon cooling. The reversible changes in the patterns can be attributed to effects of changes in the medium or the molecular motions but not to changes in the chemical form of the free radical.

In confirmation of the conclusion that unpaired spins occur at different sites when proteins are irradiated at 77°K, we have, with Raymond Patten, irradiated mechanical mixtures of the amino acid constituents of some of these proteins at 77°K and under similar conditions, without warming the sample, have observed a broad resonance resembling those of the proteins irradiated and observed at 77°K. In these mechanical mixtures we are reasonably certain to be observing many different species of free radicals at once, the resonances of which are superimposed to give the complex and unresolvable pattern which is observed.

From the superimposed and unresolved patterns at 77°K one could hardly hope to identify the specific free radicals giving the observed signals. Nevertheless, the fact that these different free radicals are converted to the single radical giving the resonance characteristic at room temperature indicates that the free radicals formed at low temperature are mainly the primary products of ionization, the electron holes in the valence cloud of the protein molecules, and the negatively charged molecules, or groups, which capture the electrons removed by the ionizing radiations. Probably the electron holes are unable to migrate effectively through the proteins at this temperature but remain trapped in localized groups or sections of the protein. Some of the electrons may be captured by impurities such as absorbed O_2.

If bonds were broken to form free radicals of a number of different chemical species at low temperature, it would seem improbable that all these free radicals could be converted to a single one, such as the oriented peptide free radical in silk or the cysteine free radical XS·, simply by an elevation of temperature to 300°K. No such conversion was found to occur for the mechanical mixture of amino acids mentioned above. On the other hand, if the observed unpaired spin were on the unbroken but ionized protein molecules, such a conversion to a single free radical could occur simply by a migration of the unpaired electron within the protein molecule to the point where a bond is most easily broken. This concept is in agreement with the previous interpretation of the mechanism for the production of a single electron spin site in a large protein molecule at room temperature. These results give additional support for this interpretation and show further that spin density can be trapped at different sites in proteins irradiated at low temperature. They also show that this type of "conduction" does not occur significantly at low temperatures.

Possible Mechanism for Formation of Characteristic Free Radicals of Irradiated Proteins.—The formation of the free radicals characteristic of irradiated proteins

at room temperature appears to occur in two separate phases. The first phase is essentially the ionization of molecules. This can be produced separately by irradiation at sufficiently low temperature. Molecular motions bring about the second phase as the irradiated sample is allowed to warm. The same stepwise mechanism probably occurs when the free radicals are produced by irradiation at room temperature, but too rapidly for observation of the ionized species.

The second phase starts with electron vacancies at different sites in otherwise complete protein molecules and with negative charges (the dislocated electrons) trapped at other sites on the protein molecules or on the impurity molecules. As the temperature is raised and the molecular motions increase, the electron vacancies (holes in the valence cloud) migrate, perhaps to a glycine residue where one of the protons of the α-carbon is lost to form the peptide free radical already described. The proton might then capture one of the trapped electrons and escape through the lattice or might react with something. If the vacancy migrates to the RS—H of the cysteine residue, the proton H^+ would be lost from the SH to form the characteristic RS· radical. If it migrates to the cystine residue to form the same free radical, an RS^+ would also be formed, which might in turn capture one of the trapped electrons to form a similar free radical. In the latter case the S—S bond might often be reformed. This healing process would account for our observation that the cystine resonance is noticeably weaker than that of cysteine for the same dosage.

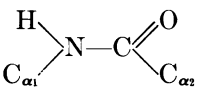
For the formation of the characteristic room temperature free radicals from the ionized molecules two conditions must seemingly be met: (1) the charges or electron holes must be able to migrate in the molecule; and (2) the atom or group broken off when the free radical is formed must be able to move away so that the bond is not immediately reformed. The molecular motions accompanying the temperature increase probably assist both of these processes.

It is interesting that both of these conditions are apparently met in acetylglycine even at 77°K. In this substance the doublet characteristic at room temperature is produced by irradiation at 77°K directly, without warming. See Figure 3. It is evident that condition (1) would be met in this molecule at low temperature because its atoms, except for the methyl hydrogens, lie in a single plane.[5] The unpaired electron of the ionized molecule should thus be in a π orbital which would spread over the entire molecular plane. On the other hand, the methyl hydrogens out of the plane would cause a looseness of the crystal lattice which might allow a proton to escape from the broken CH bond even at low temperatures.

In contrast to the behavior of acetylglycine, Henriksen and Pihl[6] have found that the spin resonance of glutathione changes with time even after irradiation at room temperature, and we have found the same to be true for glycine and for β alanine. Thus, for simple molecules particularly, one might expect structural differences to cause considerable differences in the temperature range over which the phenomena observed here might occur.

Temperature Effects on Electron Hole Migration in Proteins.—The results of the present study indicate that the migration of the electron hole in the valence shell of ionized proteins requires activation energy and is assisted by the molecular motions. It seems probable that molecular motions mainly assist the holes in the

electron cloud of the ionized protein to come in from the various points on side chains where they may be produced and to pass by the α-carbon bend points of the polypeptide chain. The unpaired spin should experience no difficulty in moving from one α-carbon to the next across the planar peptide group,



since here it would probably be in the π orbital which would have significant density over all the plane. This π orbital density would spread to the α-carbons through the mechanism of hyperconjugation. Whether the electron spin density could migrate past an α-carbon to the next peptide plane would depend on the relative orientation of the two planes, as discussed in the accompanying paper. If the two planes were orthogonal, this migration should have the highest resistance. The migration resistance should thus depend upon the manner in which the proteins are coiled. Where the migration is hindered by an unfavorable orientation of the two planar groups, it should be assisted by the slow torsional motions (in opposite phase) of the molecules in excited torsional vibrational states.

Likewise the passage of the unpaired spin by the CH_2 groups in the side chains must occur through the mechanism of hyperconjugation. This mechanism would be assisted by the ability of the side group to twist or turn into the most favorable orientation for this hyperconjugation.

Recently Augenstine *et al.*[7] have observed a very interesting variation of thermoluminescence with temperature in irradiated proteins which seems to correlate with these electron spin resonance results.

Possible Protein Analysis by Electron Spin Resonance.—The evidence that γ-irradiated proteins give characteristically different electron spin resonance patterns when irradiated and observed at low temperatures suggests the possibility of using electron spin resonance combined with ionizing irradiations as a new method for analysis of the amino acid residue in proteins. This method seemed to be ruled out by the early observations at room temperature which revealed patterns for only two or three residue, but the results at 77°K indicate that it merits exploration. It may not prove to be an accurate method, but it would be relatively simple and rapid once due comparisons and calibrations are made.

* This research was supported by the United States Air Force through the Air Force Office of Scientific Research and Development Command, and by the Office of Ordnance Research, Department of the Army. Reproduction in whole or in part is permitted for any purpose of the United States Government.

[1] Gordy, W., W. B. Ard, and H. Shields, these PROCEEDINGS, **41**, 983 (1955).

[2] Patten, F., and W. Gordy, *Bull. A.P.S.*, **4**, No. 4, 290 (1959).

[3] Gordy, W., and H. Shields, *Radiation Research*, **9**, 611 (1958).

[4] Rexroad, H. N., and W. Gordy, these PROCEEDINGS, **45**, 256 (1959).

[5] Carpenter, G. B., and J. Donohue, *J. Am. Chem. Soc.*, **72**, 2315 (1950)

[6] Henriksen, T., and A. Pihl, *Nature* (London), **307**, 185 (1960).

[7] Augenstine, L. G., J. G. Carter, D. R. Nelson, and H. P. Yockey, *Radiation Research, Suppl.*, **2**, 19 (1960).