*HYDROGEN-ADDITION RADICALS FORMED IN THE AROMATIC RINGS OF AMINO ACIDS, POLYAMINO ACIDS, AND PROTEINS**

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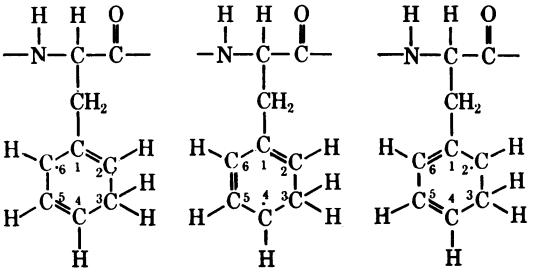
Since the original detection of electron spin resonance (ESR) signals from free radicals formed by ionizing irradiation in the amino acids and proteins,[1] numerous ESR studies of these and other powdered peptides have been made.[2-10] The free radicals that have been identified in these studies have been formed for the most part by the breakage of a $C_\alpha H$ bond to leave the electron spin concentrated mainly on C_α, or by breakage of a bond to a sulfur atom to leave the spin density concentrated on S. In this work we have obtained proof of the formation of secondary radicals by hydrogen-addition reactions on the aromatic ringed groups of phenylalanine, tyrosine, and tryptophan.

We have produced the H-addition radicals by subjecting powdered samples to thermal hydrogen atoms produced in a gaseous discharge or by exposing them to γ-irradiation and have observed their ESR spectra at a frequency of 9300 Mc/sec. The H-bombardment method was used earlier to produce H-addition radicals in the nucleic acid bases.[11-13] Snipes and Schmidt[10] bombarded a number of amino acids with thermal H atoms but reported radicals formed by H abstraction only.

L-Phenylalanine and Poly-L-Phenylalanine.—Figure 1 shows the ESR spectra obtained for L-phenylalanine and poly-L-phenylalanine after exposure to thermal hydrogen atoms at 300°K and at 77°K, respectively. In both patterns there is a strong signal in the central region like that reported earlier[2, 8] for samples that had been subjected to ionizing irradiation. In addition, there are quartet patterns on either side of the central region that are part of the spectra of radicals formed by H-addition on a *meta*-carbon of the ring. The radical can be described approximately as a hybrid of the following three valence-bond states:



The theoretical ESR pattern of such a radical is represented by the bars in Figure 1. The wider triplet splitting of 47 gauss is due to the equivalent couplings of the two H nuclei of the $C_{(3)}H_2$ group, and the quartet substructure (splitting of 11 gauss) arises from equivalent coupling by the H nuclei of the $C_{(2)}H$, the $C_{(4)}H$, and the $C_{(6)}H$ groups. A molecular orbital treatment of the spin density distribution for such a radical is given in a later section. One might conjecture that

H-addition can occur on the *ortho-* or *para-* rather than on the *meta-*carbon, but the hyperfine pattern predicted from molecular orbital theory for radicals formed by *ortho-* or *para-*addition does not agree with the observed pattern.

After finding evidence for the H-addition radicals in the H-bombarded samples, we re-examined the spectra of γ-irradiated samples and found components in their ESR signals that are due to the same H-addition radicals. This is shown most clearly in the bottom curve of Figure 1 for poly-L-phenylalanine, which has

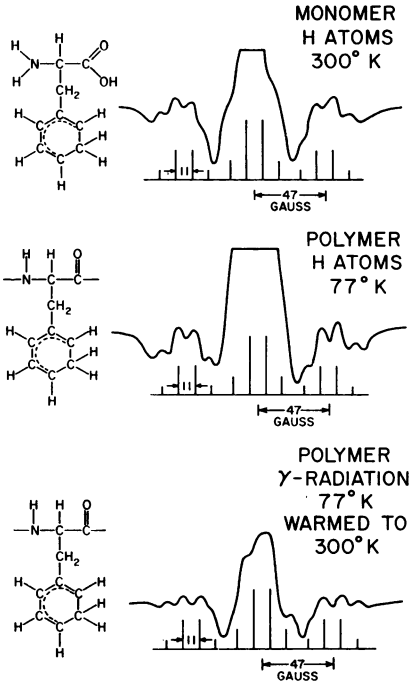


FIG. 1.—ESR spectra for radicals formed in L-phenylalanine by H atoms at 300°K, in poly-L-phenylalanine by H atoms at 77°K, and in poly-L-phenylalanine by γ-radiation at 77°K after warming to 300°K. In these spectra and in those which follow, the radicals have the usual organic radical g-value close to that of the free electron.

been γ-irradiated at 77°K and then warmed to 300°K. The source of the H atom that is added to the ring in the γ-irradiated sample is not definitely known, but it is evidently released by the radiation, possibly from a peptide chain carbon in the formation of a primary radical which may give rise to the superimposed absorption in the central part of the spectrum. The fact that there is no evidence for H-addition radicals in γ-irradiated L-phenylalanine supports the view that the H atoms come from the peptide chain.

Strangely, signals from the H-addition radicals in the H-bombarded monomer and the γ-irradiated polymer could be observed at room temperature, while those of the H-bombarded polymer disappeared before the sample reached 300°K. The reason for this difference is not entirely clear.

Upon exposure of the irradiated samples to air, the ESR signals formed by either H atoms or γ-rays are converted to those of the peroxide radical R-O-O, as has been previously found in many irradiated biochemicals.

L-Tyrosine and Poly-L-Tyrosine. The structure of poly-L-tyrosine is like that of poly-L-phenylalanine except that an OH is substituted for an H on

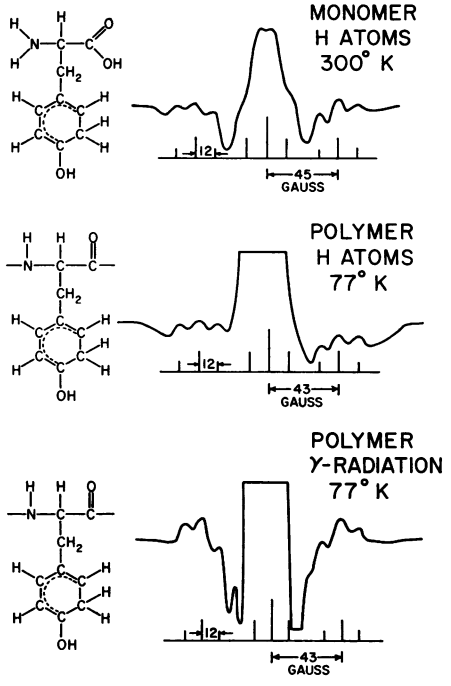


FIG. 2.—ESR spectra for radicals formed in L-tyrosine by H atoms at 300°K, in poly-L-tyrosine by H atoms at 77°K, and in poly-L-tyrosine by γ-irradiation at 77°K.

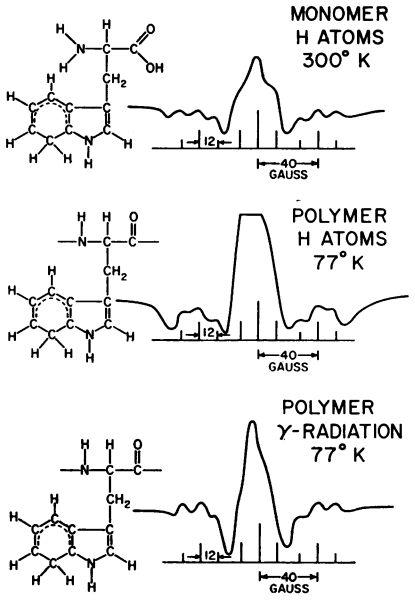


FIG. 3.—ESR spectra for radicals formed in DL-tryptophan by H atoms at 300°K, in poly-DL-tryptophan by H atoms at 77°K, and in poly-DL-tryptophan by γ-irradiation at 77°K.

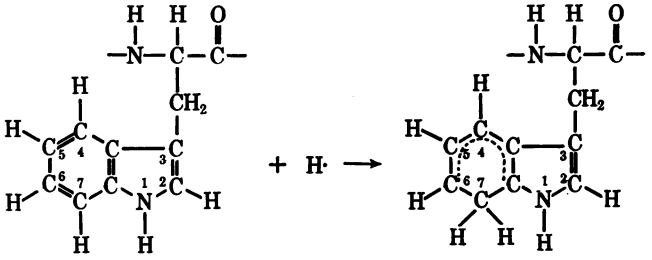
$C_{(4)}$ (Fig. 2). The observed ESR pattern for samples exposed to H atoms has a strong component which conforms to that expected for the radicals (Fig. 2) in which H addition occurs at the *meta*-position.

Figure 2 shows the patterns for L-tyrosine (at 300°K) and for poly-L-tyrosine (at 77°K) with bars to indicate the ESR pattern expected for the H-addition radical. The H-addition radicals in the H-bombarded polymer could not be observed at 300°K. All H-addition radicals in tyrosine samples decayed to a weak, broad resonance when the samples were exposed to air. The larger triplet splitting of 43 (or 45) gauss is caused by the $C_{(3)}H_2$ group on the ring; the subtriplet splitting of 12 gauss, by the $C_{(4)}H$ and the $C_{(6)}H$ protons. Note that the replacement of the $C_{(4)}H$ proton by OH has changed the substructure from a quartet in phenylalanine to a triplet in tyrosine. This change confirms the choice of the *meta*-position as the site of H-addition in both phenylalanine and tyrosine.

After identifying the pattern of the H-addition radical in the H-bombarded samples, we were able to produce similar patterns when we subjected the samples to γ-irradiation (Fig. 2). As for phenylalanine, the H atoms released by the irradiation from some other part of the molecules became attached to the aromatic rings. There are stronger components from other radicals that are like

those reported by Drew and Gordy,[8] who failed to detect the weaker components from the H-addition radicals. Efforts to find H-addition radicals in the γ-irradiated monomer were not successful.

DL-Tryptophan and Poly-DL-Tryptophan.—Figure 3 shows the spectra for powdered samples of DL-tryptophan and of poly-DL-tryptophan after exposure to H atoms. As in the compounds described above, the similarity of the ESR spectra for the monomer and polymer indicates that the observed radicals are formed on the aromatic rings. Theoretical calculations described later indicate that the components corresponding to the bars arise from a radical formed by H-addition to $C_{(7)}$ of the tryptophan side group by the following reaction.



The H-addition radical formed by H-bombardment of poly-DL-tryptophan, like that in poly-L-tyrosine, is not stable at room temperature. Its pattern was observable at 200°K, but it decayed quickly into a weak singlet as the sample was warmed to 300°K. In contrast, the pattern for the monomer DL-tryptophan was readily detectable at 300°K (Fig. 3). The same spectra were observed in the monomer at 77°K and at 300°K. However, the pattern changed into a singlet and decayed rapidly upon exposure of the sample to air.

The H-addition radicals for poly-DL-tryptophan were observable in γ-irradiated samples both at 77°K (Fig. 3) and at 300°K. The fact that the H-addition radicals formed in the γ-irradiated poly-DL-tryptophan were observable at room temperature, whereas the H-addition radicals formed by H-bombardment were not, may be caused by the degradation of the polymer by the irradiation. The H-addition radicals were not identified in the earlier experiments on the monomer or the polymer. Upon exposure to air, the signals of both the monomer and the polymer of tryptophan, like tyrosine, decayed to a broad, unresolved resonance, probably arising from the peroxide radical R-O-O.

Spin Densities and Free Valences.—The experimentally observed proton couplings for the different radicals are listed in the first column of Table 1. The experimental spin densities obtained from these couplings are shown in the second column. The spin densities in the $2p$-orbital of the α carbon of the $C_\alpha H$ groups are obtained from the empirical relation

$$\rho_{C_\alpha} = \frac{a(H_\alpha)}{Q},$$

where Q has the value of 26 gauss. The spin density in the pseudo-atomic orbital of the $C_\beta H_2$ group, formed by a linear combination of the $1s$-orbitals of

TABLE 1. *Comparison of experimental and theoretical spin densities.*

Compound	Experimental coupling (gauss)	Spin Densities: Experimental	Spin Densities: Theoretical — Hückel's method	Spin Densities: Theoretical — McLachlan's SCF method
Phenylalanine				
$C_\alpha H$	11	0.42	0.28	0.35
$C_\beta H_2$	47	0.18	0.16	0.19
Tyrosine				
$C_\alpha H$	12	0.46	0.27	0.34
$C_\beta H_2$	43	0.17	0.15	0.17
Tryptophan				
$C_\alpha H$	12	0.46	0.32	0.39
$C_\beta H_2$	40	0.16	0.12	0.15

the two hydrogens in the hyperconjugation model, can be estimated from the coupling of each of the protons by

$$\rho_{H_\beta} = \frac{2a(H_\beta)}{508}.$$

The factor of 2 takes account of the fact that half the spin density of the pseudo-orbital is in each of the 1*s*-orbitals of the hydrogens.

Spin densities on the various atoms in the π system of the rings were calculated with Hückel molecular orbital theory[14] and with the McLachlan self-consistent field theory.[15] The LCAO molecular orbitals are obtained by diagonalization of the secular equation coefficients by an IBM 360 computer that employed the Jacobi rotation method described by Ralston and Wilf.[16] The heteroatom parameters for the methylene groups employed in the calculation were those of Levy;[17] the heteroatom parameters for oxygen and nitrogen were those of Pullman and Pullman.[18] McLachlan's empirical parameter λ was set equal to 1.0.

The resulting spin density values calculated with the Hückel and McLachlan methods are compared with the experimental values in Table 1. The spin densities calculated with the Hückel approximations are numerically equal to the charge densities of the unpaired electron. Since they are all positive and must be normalized to unity, they often predict a smaller over-all spread of the spectrum than is observed. By taking into account the difference of the interaction of the unpaired electron with π-electrons of the same spin and those of opposite spin, the McLachlan method predicts negative as well as positive spin densities. The agreement is seen to be relatively good for those spin densities calculated with the self-consistent field (SCF) method. This agreement provides confirmation of the assignment of the radicals, since no approximate agreement could be obtained with other conceivable radicals.

The sites of H-addition found from experimental evidence are also sites of relatively high free-valence values calculated with molecular orbital theory.[18] This is shown in Figure 4. In tyrosine the free valence is higher at the site of H-addition than at any other site on the ring. In the other two molecules, where the free valence at the site of addition is only about 4 per cent lower than the highest free valence on the rings, steric factors may be important in determining

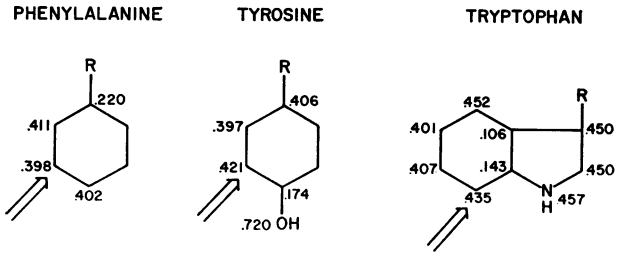
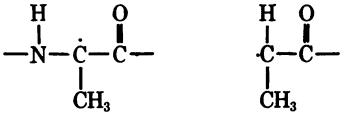


FIG. 4.—Free-valence values calculated by Pullman and Pullman.[18] Arrows indicate sites where H-addition is found to occur.

which of the sites of relatively high free valence an H atom can most easily reach. For H-addition to occur, evidently the site of addition must have a relatively high free valence, and steric factors must not prevent the H atom from assuming the position necessary for the formation of a normal C—H bond with an sp^3 hybrid orbital of the carbon.

Chemical Protection—Random Copolymers.—From these studies it is evident that the aromatic rings of phenylalanine, tyrosine, and tryptophan in the side groups of proteins are capable of capturing hydrogen atoms released by ionizing radiation and thus might prevent the H atoms from causing other, more serious, secondary radiation damage to the proteins. To obtain more specific evidence of this form of chemical protection we have studied a few random copolymers which contain these aromatic groups. Figure 5 shows that the random copolymers having equal numbers of L-alanine and L-tyrosine residues give only the poly-L-tyrosine ESR signal when subjected to gaseous H atoms. Likewise, the random copolymers having equal numbers of L-glutamic acid and L-tyrosine residue show only the poly-tyrosine pattern.

The ESR pattern for poly-L-alanine which has been exposed to H atoms at room temperature is unlike that for γ-irradiated poly-L-alanine at the same temperature and is believed to arise from a radical such as (II), which is formed by the breakage of the polypeptide bond.



If this is true, the tyrosine rings that act as scavengers of H atoms tend to prevent the breakage of the polymer chain at an alanine residue. As was previously shown,[10] γ-irradiation of poly-L-alanine at room temperature produces signals from radicals of structure (I) only, although signals from radicals similar to (II) are also produced at reduced temperatures.

The Proteins.—Although H-addition radicals produced in the aromatic side groups have not been previously identified in the proteins, it is evident that they should be formed in those proteins that have significant components of phenyl-

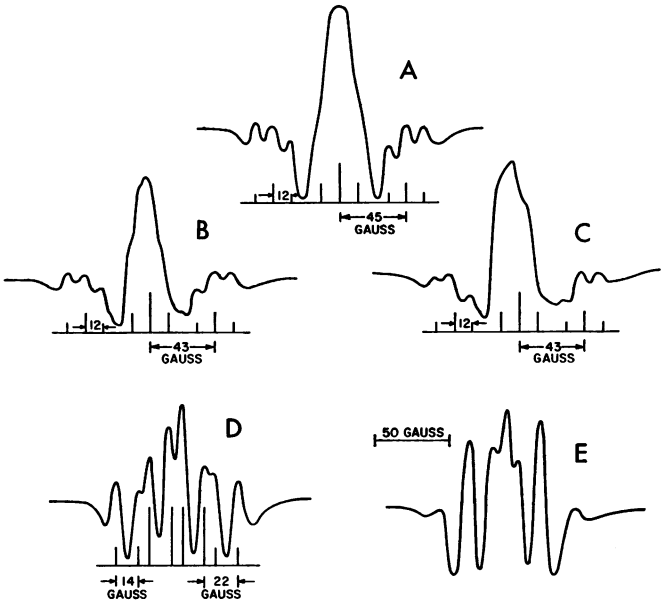


FIG. 5.—ESR spectra for radicals formed by H atoms at 77°K in a random copolymer of equal numbers of L-alanine and L-tyrosine, a random copolymer of equal numbers of L-glutamic acid and L-tyrosine, and the polymers of their constituents.

(*A*) L-tyrosine; (*B*) random copolymer, alanine: tyrosine; (*C*) random copolymer, glutamic acid: tyrosine; (*D*) poly-L-alanine; (*E*) poly-L-glutamic acid.

anine, tyrosine, and/or tryptophan. We have therefore examined the ESR spectra produced by H-bombardment of a few selected proteins having significant components of these residues. Figure 6 shows the signals observed for carboxypeptidase A, which resembles most the pattern obtained for poly-L-phenylalanine, and the signals observed for insulin and papain, for which there is definite evidence for the H-addition radicals found in H-bombarded poly-L-tyrosine. Radicals are probably formed on the tryptophan rings also, but the tryptophan content is small in these proteins, and the ESR patterns of its H-addition radicals are indistinguishable from those of tyrosine. Carboxypeptidase A has 5 per cent phenylalanine, 6 per cent tyrosine, and 2 per cent trypto-

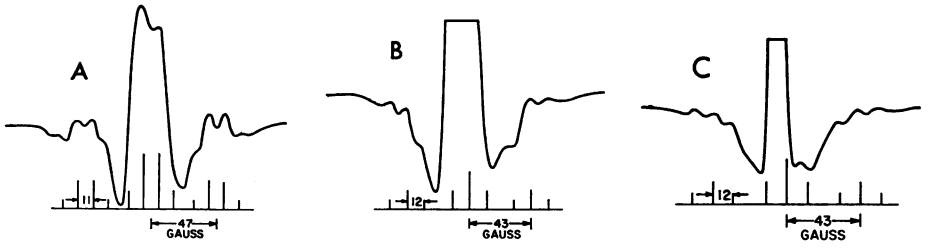


FIG. 6.—ESR spectra for radicals formed in (*A*) carboxypeptidase A, (*B*) insulin, and (*C*) papain proteins by H atoms at 77°K.

phan; insulin has 6 per cent phenylalanine, 7 per cent tyrosine, and 0 per cent tryptophan; and papain has 2 per cent phenylalanine, 7 per cent tyrosine, and 2 per cent tryptophan. Possibly these H-addition radicals have important roles in radiation protection and in other protein functions.

Other Polyamino Acids.—We have also studied the ESR spectra of 11 other polyamino acids.[21] Most of them show radicals formed by H abstraction, but a large 49-gauss triplet pattern for poly-L-histidine shows evidence for H-addition on the five-membered ring. Because of the poorly resolved substructure of the spectrum, the site of the addition is not yet certain. The radicals formed in these other polyamino acids will be discussed in a later publication.

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

[2] Shields, H., and W. Gordy, *J. Phys. Chem.*, **62**, 789 (1958).

[3] Patten, F., and W. Gordy, these PROCEEDINGS, **46**, 1137 (1960); Patten, F., and W. Gordy, *Radiation Res.*, **14**, 573 (1961).

[4] Box, H. C., H. G. Freund, and K. Lilga, in *Free Radicals in Biological Systems*, ed. M. S. Blois *et al.* (New York: Academic Press, 1961), pp. 239–248.

[5] Gordy, W., and H. Shields, "Electron spin resonance investigations of the proteins" (Brussels Symposium, April 1961), in *Proc. Royal Acad. Belg.*, **33**, 191 (1961).

[6] Zimmer, K. G., and A. Müller, "New light on radiation biology from electron spin resonance studies," in *Current Topics in Radiation Research*, ed. M. Ebert and A. Howard (Amsterdam: North-Holland Publishing Co., 1961), vol. 1, pp. 1–47.

[7] Henriksen, T., T. Sanner, and A. Pihl, *Radiation Res.*, **18**, 147, 163 (1963).

[8] Drew, R. C., and W. Gordy, *Radiation Res.*, **18**, 552 (1963).

[9] Schoffa, G., *Elektronenspinresonanz in der Biologie* (Karlsruhe, Germany: Verlag G. Braun, 1965).

[10] Snipes, W., and J. Schmidt, *Radiation Res.*, **29**, 194 (1966).

[11] Herak, J. N., and W. Gordy, these PROCEEDINGS, **54**, 1287 (1965).

[12] Heller, H. C., and T. Cole, these PROCEEDINGS, **54**, 1486 (1965).

[13] Holmes, D. E., L. S. Myers, Jr., and R. B. Ingalls, *Nature*, **209**, 1017 (1966).

[14] Hückel, *Z. Physik*, **70**, 204 (1931); *ibid.*, **72**, 310 (1931).

[15] McLachlan, A. D., *Mol. Phys.*, **3**, 233 (1960).

[16] Ralston, A., and H. S. Wilf, *Mathematical Methods for Digital Computers* (New York: John Wiley and Sons, Inc., 1966).

[17] Levy, D. H., *Mol. Phys.*, **10**, 233 (1966).

[18] Pullman, B., and A. Pullman, *Quantum Biochemistry* (New York: Interscience Publishers, 1963).

[19] Lichter, J. J., and W. Gordy, these PROCEEDINGS, **60**, 794 (1968).

[20] Tristran, G. R., and R. H. Smith, in *The Proteins*, ed. H. Neurath and K. Bailey (New York: Academic Press, 1963), 2nd ed., vol. 1, pp. 46–50.

[21] Liming, F. G., Jr., and Walter Gordy, *Bull. Am. Phys. Soc.*, **13**, 619 (1968).