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An ESR Study of the Acid Dissociation of NH Protons.¹ 1. Linear Peptide Radicals and Related Radicals

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Abstract: Free radicals formed by the reaction of OH or O⁻ radicals with aliphatic compounds containing a peptide group (-CONH-) and structurally related compounds have been studied by the in situ radiolysis-steady state ESR method. Linear peptide radicals (-CONHĊ) from *N*-acetyl glycine, *N*-methylacetamide, *N*-ethylacetamide, *N*-methylpropionamide, ethyl methylcarbamate, *N*-methylformamide, *N*-acetylalanine, glycyglycine, and hippuric acid have been found to result from hydrogen abstraction from the C-H bond adjacent to the NH group. In strongly basic solutions significant changes occur in ESR parameters of six peptide radicals. These changes occur in α -, β -, δ -, or γ -proton coupling constants and can be interpreted in terms of the dissociation of the NH proton. The pK_a values for the NH proton dissociation have been determined to be 14.0 for CH₃CONHĊCOO⁻, 14.5 for CH₃CONHĊH₂, CH₃CH₂CONHĊH₂, and CH₃CH₂OCONHĊH₂, 14.6 for CH₃CONHĊHCH₃, and 13.3 for HCONHĊH₂. The pK_a values for the peptide radicals derived from *N*-acetyl glycine, *N*-methylacetamide, and *N*-methylformamide are respectively estimated to be 5.4 to 5.6, 4.9, and 5.2 units lower than those for the parent compounds. Since the pK_a value of CH₃NHĊHCOO⁻ from sarcosine appears to be larger than 17, the acidity of peptide radicals results from the electron-withdrawing effect of the carbonyl group adjacent to the NH group. Upon dissociation of the NH proton, α - and β -proton coupling constants decrease while δ - or γ -proton coupling constants increase. Analysis of the changes for four radicals suggests that dissociation is accompanied by a decrease in spin density on the α carbon by 0.09 to 0.12 while that on the γ carbon increases by 0.06 to 0.10. An accompanying slight increase in g factor is also observed upon the dissociation, indicating an increase in the spin density on the oxygen and/or nitrogen atom in the dissociated form. The changes in the ESR spectrum of the radical from *N*-acetyl glycine have been studied in considerable detail and the dynamics of the equilibrium CH₃CONHĊHCOO⁻ + OH⁻ \rightleftharpoons CH₃CON⁻ĊHCOO⁻ + H₂O have been investigated by means of the pH dependence of line width. The forward and reverse rate constants are found to be 8×10^8 and 1.6×10^7 M⁻¹ s⁻¹, respectively. The deviation of the rate of the forward reaction from the value expected under diffusion control is discussed. The coupling constants of the NH protons of peptide radicals are found to be accurately predicted by the following equation which includes not only the contribution from the spin density on the nitrogen atom but also the canceling effects of spin densities on the α - and γ -carbon atoms: $a^H(\text{NH}) = |25.0\rho_N - 0.6(\rho_{\alpha-C} + \rho_{\gamma-C})|$. Sigma radicals (ĊONH-) were also detected from three compounds with formyl groups. They were not, however, observable in the strongly alkaline region, probably because of hydrolysis of the parent molecule. With *N*-acetyethanolamine, the α -hydroxy radical was found rather than the peptide radical. Dissociation of the OH proton was observed in weakly basic solution.

The reactions of OH radicals with compounds which have the peptide linkage have been investigated by various workers using optical pulse radiolysis³⁻⁶ and ESR spectroscopy.⁷⁻¹² One of the most important radicals produced in these reactions is that resulting from hydrogen abstraction from the carbon atom adjacent to the peptide nitrogen, i.e., -CONHĊ-. This radical is sometimes called the peptide radical.⁶ Since the acid-base properties of the peptide radical¹³ may be of importance in understanding radiation damage to polypeptides and proteins in the α -helical configuration where hydrogen bonding, CO...HN, plays an important role, it is desirable to establish the acidity of NH protons of peptide radicals. Using optical pulse radiolysis, a number of pK values have been found for free radical intermediates formed by the reaction of OH radicals with linear and cyclic peptides.^{4,6} Several pK values are concluded to be due to the dissociation of the peptide protons. They are 9.6 for two cyclic peptide radicals⁶ and 10.9 to 12.1 for four linear peptide radicals.⁴ Little appears to be known regarding the acidity of linear peptide radicals with $pK > 13$. In an optical pulse radiolysis study⁴ it was speculated that

a few linear peptide radicals would dissociate peptide protons with pK values larger than 13 while ESR experiments¹¹ suggested that the radical from *N*-acetyl glycine (CH₃CONHĊHCOO⁻) might dissociate an acetyl proton instead of the NH proton. The present paper describes a detailed in situ radiolysis-ESR study¹⁴ of the acidity of some linear peptide and related radicals formed by hydrogen abstraction with OH or O⁻ radicals. Advantage is taken of the ability of ESR spectroscopy to follow the acid-base properties of a radical in a more direct way¹⁵ and over a wider acidity range¹⁶⁻¹⁸ than the optical pulse radiolysis method.

Experimental Section

Chemicals were of the highest grade available from Eastman, Aldrich, J. T. Baker, Sigma Chemical, Cyclo Chemical, Linde, and Matheson and were used without further purification. Water was distilled and freed from organic impurities by passing the vapor with oxygen through a silica oven at ~ 600 °C. Experiments were carried out by the in situ radiolysis-ESR method.¹⁴ Radicals were produced by irradiating an aqueous solution of the desired compound (5–10

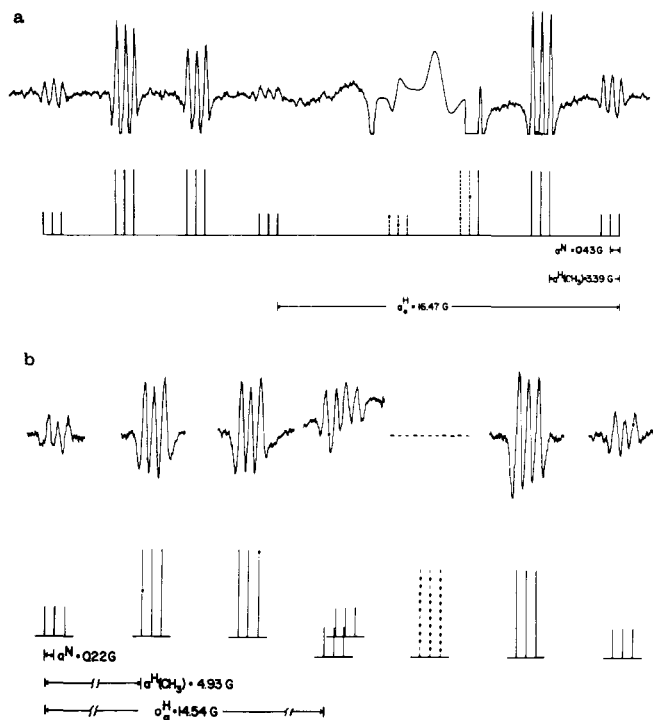


Figure 1. Second-derivative ESR spectra of the radical produced by the reaction of OH with *N*-acetylglycine: (a) at $H_{2-} = 13.73$ ([KOH] = 0.31 M) and, (b) at $H_{2-} = 16.01$ ([KOH] = 4.92 M). Spectra are displayed with the magnetic field increasing from left to right. The spectral lines corresponding to the dashed lines are hidden by the signal from the quartz cell. In (a) the lines in the third and fourth triplets from the down-field end of the spectrum are broadened by the dissociation process.

mM); reaction was with OH or O^- radical depending on pH [$OH + OH^- \rightleftharpoons O^- + H_2O$ ($pK = 11.85$)]¹⁹. Sample solutions were saturated with nitrous oxide in order to remove oxygen and to convert e_{aq}^- to OH. The irradiation by 2.8 MeV electrons was carried out directly in the ESR cavity. The solution flowed through the flat silica cell mounted in the cavity at about 1 ml/s. All measurements were made at about 20 °C.

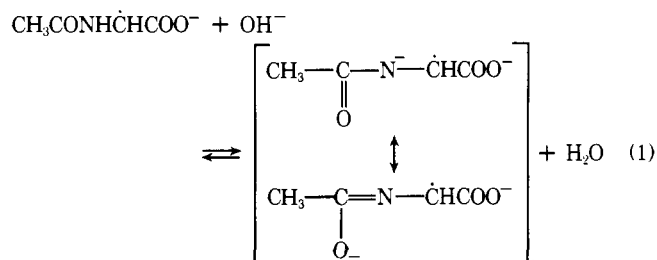
Solutions were buffered with potassium phosphate near neutral pH or made basic with potassium hydroxide (KOH). For solutions of pH < 13, pH measurement was carried out with a Beckman Model GS pH meter equipped with a Beckman E-2 type glass electrode. For more concentrated KOH solutions, the concentration of the base was determined by titration with a standard hydrochloric acid solution. Yagil's H_- or H_{2-} acidity function²⁰ was used (according to the charge of the radical under study) to provide an acidity scale above the pH scale for aqueous KOH. The acidity functions determined by Yagil using the ionization of indoles are the most well-defined among the few acidity functions available for concentrated aqueous KOH solutions.²¹ The higher the concentration of KOH became, the more difficult it was to obtain an ESR spectrum with good S/N ratio. This effect might be partly due to the decrease of cavity Q and the secondary reaction of impurities introduced by the large amount of KOH. Changes in the substrate such as hydrolysis might occur also in a highly basic solution.

Second-derivative presentation was used for the measurement of ESR line positions and first-derivative spectra were recorded for line width measurements. Where the separations among lines were small relative to the line width, it was not possible to determine the line width directly from the spectrum. In such cases the line widths were determined by synthesizing a spectrum with a sequence of line widths until a best fit to the experimental pattern was found. Spectrum synthesis was carried out with a Hewlett-Packard 9830A calculator equipped with a 9862A plotter. The line width measured directly from the spectrum or determined from such a synthesis was corrected for modulation broadening according to the procedure of Laroff and Fessenden²² who modified the correction curve of Wahlquist.²³ The calculated titration curves for the dissociation of NH protons were plotted using a Hewlett-Packard 9100A calculator with a 9125A plotter.

Results and Discussion

Radical from *N*-Acetylglycine. The spectrum of $CH_3CONH\dot{C}HCOO^-$ (I), which is well known from previous works,^{8,11} was observed during the irradiation of an N_2O saturated aqueous solution of 10 mM *N*-acetylglycine near neutral pH (8–11). The ESR parameters so determined are $a^N = 0.49$ G, $a^H(NH) = 1.28$ G, $a^H(CH_3) = 2.73$ G, $a_{\alpha}^H = 17.34$ G, and $g = 2.00331$ and are in good agreement with those reported by the previous authors.^{8,11} With an increase in the basicity of the solution the spectral lines became broader and were unobservable at pH values near 12.2. With further increase in basicity the spectral lines reappeared but in a different pattern from that observed in the neutral region. At pH 13.3 the spectrum showed a nominal triplet (1:1:1)–triplet (1:2:1)–doublet (1:1) pattern which is very similar to that observed at pH 13.5 by Neta and Fessenden¹¹ who suggested the radical structure $CH_2=C(O^-)NH\dot{C}HCOO^-$ to account for their spectrum (see Figure 9 in ref 11). In solutions of still higher potassium hydroxide concentration, however, another two sets of triplet lines emerged in an innermost region of the spectrum as shown in Figures 1a and 1b. The spectrum at $H_{2-} = 13.73$ shown in Figure 1a is a triplet–quartet–doublet pattern with the triplets near the center of the spectrum specifically broadened. The spectrum can be described by the ESR parameters of $a^N = 0.43$ G, $a^H(CH_3) = 3.39$ G, $a_{\alpha}^H = 16.47$ G, and $g = 2.00331$. These values are close to those obtained near neutral pH except that the NH proton splitting is absent. In the previous work,¹¹ the triplets near the center of the spectrum were not observed because of the line broadening so that the incorrect radical structure $CH_2=C(O^-)NH\dot{C}HCOO^-$ was suggested. Consequently, the hyperfine coupling constant obtained by subtracting $a_{\delta}^H (= 3.48$ G) from $a_{\alpha}^H (= 19.91$ G) in ref 11 corresponds to that of a_{α}^H reported here. The spectrum in Figure 1b, which was recorded at $H_{2-} = 16.01$, shows more clearly a triplet–quartet–doublet pattern with a normal intensity ratio (1:1:1–1:3:3:1–1:1) although the line positions have changed considerably from the conditions of Figure 1a. The ESR parameters here are $a^N = 0.22$ G, $a^H(CH_3) = 4.93$ G, $a_{\alpha}^H = 14.54$ G, and $g = 2.00336$. The behavior of the spectrum described above (the broadening effect and the line shifts) can be fully understood in terms of an exchange of the NH proton with water molecules and the acid dissociation of the NH proton of the radical $CH_3CONH\dot{C}HCOO^-$. The broadening of all the spectral lines around pH 12 is mainly due to exchange with the water and the specific line broadening around $H_{2-} = 13.5$ is associated with the acid dissociation. These broadening effects will be discussed fully later in the context of the kinetics of the process.

The dependence of the ESR parameters on the basicity of the medium was examined in the range from pH 8.0 to $H_{2-} = 16.90$ (7.39 M in KOH concentration). Especially large changes with basicity were found in the α -proton and methyl-proton coupling constants as shown in Figure 2a. The result has been analyzed in terms of the acid dissociation equilibrium expressed by eq 1. If the interchange between the



acid form (A) and the base form (B) is rapid, which is the case here, the observed hyperfine coupling constant, a , represents

the weighted average of the two forms. For a given solution the acid dissociation constant can be calculated by

$$\begin{aligned} \text{p}K_a &= H_x + \log (f_A/f_B) \\ &= H_x + \log [(a - a_B)/(a_A - a)] \quad (2) \end{aligned}$$

where H_x is the value of the acidity function or pH of the medium, a_A and a_B are the coupling constants for the acid and base forms, respectively, and f_A and f_B refer to the fraction of each ($f_A + f_B = 1$). On this basis a coupling constant is expected to change with basicity according to

$$a = [a_A + a_B \times 10^{(H_x - \text{p}K_a)}] / [1 + 10^{(H_x - \text{p}K_a)}] \quad (3)$$

The procedures for determining $\text{p}K_a$ values and related problems have been described in more detail elsewhere.¹⁸ In the case of an *N*-acetylglycine radical, the observed hyperfine coupling constants at pH 11.17 and H_{2-} 16.90 can be regarded as a_A and a_B , respectively, because they were obtained at basicities far from the $\text{p}K_a$ value of 14. The values of a_A and a_B are shown in Table I. The calculation by eq 2 using four experimental points within one unit of the half-neutralization point gave $\text{p}K_a$ values of 14.08 ± 0.16 from the α -H coupling constant and 13.99 ± 0.10 from the methyl proton coupling constant. The $\text{p}K_a$ value for the NH proton dissociation has been thus determined to be 14.0 ± 0.2 . The concentration of KOH at $H_{2-} = 14.0$ is 0.51 M. Also shown in Figure 2a are the solid curves calculated using eq 3. The agreement between the experimental points and the calculated curve is satisfactory for both of the coupling constants. The result is summarized in the first row of Table I.

Radicals from *N*-Methylacetamide, *N*-Ethylacetamide, *N*-Methylpropionamide, and Ethyl Methylcarbamate. ESR spectra observed below neutral pH from *N*-methylacetamide, *N*-ethylacetamide, *N*-methylpropionamide, and ethyl methylcarbamate have shown the presence of only one radical species in each case, i.e., $\text{CH}_3\text{CONH}\dot{\text{C}}\text{H}_2$ (II), $\text{CH}_3\text{CONH}\dot{\text{C}}\text{HCH}_3$ (III), $\text{CH}_3\text{CH}_2\text{CONH}\dot{\text{C}}\text{H}_2$ (IV), and $\text{CH}_3\text{CH}_2\text{OCONH}\dot{\text{C}}\text{H}_2$ (V), respectively. While there is no ambiguity in the identity of the latter three, the radical from *N*-methylacetamide could also be that produced by hydrogen abstraction from the acetyl group. The choice of assignment in this case is, however, supported by the similarity in coupling constants and in their pH dependence (see below) to the other three radicals (III, IV, and V) in this study as well as by the previous arguments.^{3,7-9,11} While the spectrum of radical III in acid form showed a 0.88-G splitting due to the NH proton, the spectra for II, IV, and V lacked the NH proton splittings and, therefore, their values must be smaller than the line width of each line, 0.2 G (see Table I).

The dependence on basicity of the ESR parameters for radical II was examined from pH 1.3 to $H_{2-} = 16.31$ (7.94 M in KOH concentration). The parameters remain constant up to pH 13 and are in good agreement with those previously reported^{9,11} but slightly different from those at a higher temperature.⁷ Above pH 13 the α -proton coupling constant showed a large decrease and the methyl proton showed a large increase upon dissociation of the NH proton while the nitrogen showed a slight decrease. Since it was not possible to observe the ESR spectrum for the base form alone because of the difficulty in spectral detection at a basicity higher than $H_{2-} = 16.3$, the limiting values for the base form were determined by an extrapolation using the following equation.

$$a = a_B + (1/K_a)(a_A - a) \times 10^{-H_x} \quad (4)$$

A linear plot of a against $(a_A - a) \times 10^{-H_x}$, using the method of least squares, gave the value for a_B as an intercept: 16.73 G for the α proton and 6.77 G for methyl protons. As shown in Table I, these values are very close to the value actually observed in the most basic medium which allowed spectral de-

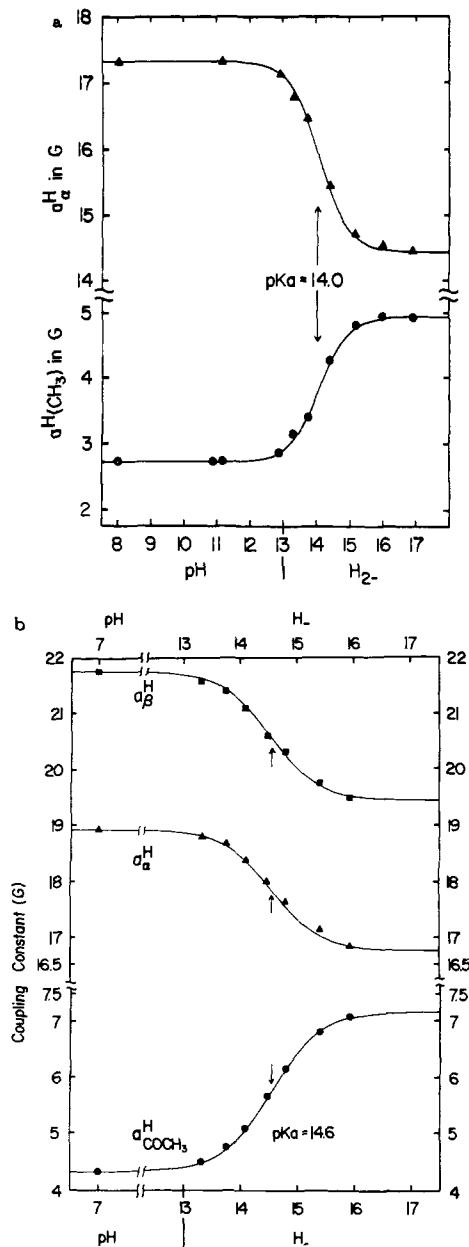


Figure 2. The hyperfine coupling constants as a function of basicity (a) for radical I from *N*-acetylglycine and (b) for radical III from *N*-ethylacetamide. The solid curves are calculated from the data in Table I under the assumption that the ESR spectra represent the weighted average of the concentrations of the acid and base forms of the radical.

tection. Using the limiting values (a_A observed and a_B calculated) for the α -proton and methyl-proton coupling constants, eq 2 gives $\text{p}K_a$ values of 14.52 ± 0.20 and 14.37 ± 0.02 , respectively. All the results for radical II are included in Table I.

Radical III has shown adequate signal intensity in spite of the large number of hyperfine splittings which produce 192 lines in the acidic form and 96 lines for the basic form. About 50 of the 96 lines could be detected and the ESR parameters were deduced up to a basicity of $H_{2-} = 15.93$ (6.74 M in KOH concentration). Changes in the coupling constants of the α , β -methyl, and acetyl-methyl protons are plotted against the basicity in Figure 2b. The $\text{p}K_a$ value was determined to be 14.66 ± 0.12 from hyperfine coupling constants of the α proton, 14.55 ± 0.12 from those of the β -methyl proton, and 14.54 ± 0.06 from those of the acetyl-methyl proton by the same procedure used for radical II.

The ESR spectrum of radical IV was observed to a basicity of $H_{2-} = 15.62$ (5.63 M in KOH concentration). The changes

Table I. ESR Parameters^a for the Acid and Base Forms of Linear Peptide Radicals and Related Radicals and Their Dissociation Constants

| Radical in acid form | Acid form ^b | | | | | | Base form | | | | | | [KOH] corresponding to pK _a , M |
|--|------------------------------|------|-------|------------------------------|----------------------------|----------|------------------------------|--|--|--|-----------------------|-----------------|--|
| | Hyperfine coupling constants | | | Hyperfine coupling constants | | | Hyperfine coupling constants | | | Hyperfine coupling constants | | | |
| | N-H | N | α-H | β-H | Other proton | g factor | N | α-H | β-H | Other proton | g factor | pK _a | |
| I CH ₃ CONHCHCOO ⁻ | 1.28 | 0.49 | 17.34 | | CH ₃ CO, 2.73 | 2.003 31 | 0.17 | 14.44 | | CH ₃ CO, 4.92 | 2.003 40 | 14.0 ± 0.2 | 0.51 |
| II CH ₃ CONHCH ₂ | <0.2 | 2.22 | 19.00 | | CH ₃ CO, 4.02 | 2.002 71 | 1.95 ^c | 16.79, ^c 16.73 ^d | | CH ₃ CO, 6.74, ^c 6.77 ^d | 2.002 75 ^c | 14.5 ± 0.2 | 1.97 |
| III CH ₃ CONHCHCH ₃ | 0.88 | 2.83 | 18.93 | CH ₃ , 21.77 | CH ₃ CO, 4.32 | 2.002 68 | 2.72 ^c | 16.84, ^c 16.72 ^d | CH ₃ , 19.49, ^c 19.43 ^d | CH ₃ CO, 7.07, ^c 7.18 ^d | 2.002 71 ^c | 14.6 ± 0.2 | 2.25 |
| IV CH ₃ CH ₂ CONHCH ₂ | <0.2 | 2.18 | 18.98 | | -CH ₂ CO-, 3.45 | 2.002 69 | 1.99 ^c | 16.77, ^c 16.63 ^d | | -CH ₂ CO-, 4.97, ^c 4.07 ^d | 2.002 70 ^c | 14.5 ± 0.2 | 1.97 |
| V CH ₃ CH ₂ OCONHCH ₂ | <0.2 | 0.88 | 17.99 | | -CH ₂ O-, 0.60 | 2.002 70 | 0.76 ^c | 17.11, ^c 16.99 ^d | | -CH ₂ O-, 0.55 ^c | 2.002 82 ^c | 14.5 ± 0.1 | 1.97 |
| VI HCONHCH ₂ | 0.43 | 2.63 | 19.04 | | HCO, 4.99 | 2.002 75 | 2.59 ^c | 17.11, ^c 16.73 ^d | | HCO, 7.67, ^c 8.25 ^d | 2.002 75 ^c | 13.3 ± 0.1 | 0.19 |
| VII CH ₃ NHCHCOO ⁻ | 5.39 | 7.29 | 12.25 | | CH ₃ , 7.29 | 2.003 39 | | | | | | >17 | |

^aThe hyperfine coupling constants are given in Gauss and are accurate to ±0.03 G. The g factors were measured relative to the peak from the silica cell and are accurate to ±0.0005. Second-order corrections have been made [R. W. Fessenden, *J. Chem. Phys.*, **37**, 747 (1962)]. The nomenclature for protons (e.g., α, β-H, etc.) is with respect to the site of the trivalent carbon atom as is usual in ESR spectroscopy. Throughout this paper absolute values for hyperfine coupling constants are used unless specified otherwise. ^bRadicals I, II, VI, and VII in the acid form have been studied previously. See ref 8 and 11 for I, ref 7, 9, and 11 for II, ref 7 and 9 for VI, and ref 8 for VII. ^cValues observed in the most basic medium which allowed spectral detection. These values do not necessarily represent the fully dissociated form but may include a slight contribution from the acid form. ^dDetermined by extrapolation using eq 4. These values were used for obtaining pK_a values by eq 2. ^eNo contribution from the base form was observed below H₂ = 16.0.

in the spectrum of radical IV upon dissociation were found to be quite similar to those of radicals II and III and the pK_a value was determined to be 14.50 ± 0.15 from the H₋ dependence of the α-proton coupling constant and 14.46 ± 0.08 from the methylene-proton coupling constant. The comparison of the pK_a values for radicals II-IV indicates that the acidity is little affected by methyl substitution on either end of CH₃CONHCH₂.

The main radical species observed and identified in an electron-irradiated aqueous solution of ethyl methylcarbamate was CH₃CH₂OCONHCH₂ (V). ESR parameters were assigned between pH 6.31 and H₋ = 15.54 (5.36 M in KOH concentration). The triplet (1:2:1) splitting of about 0.6 G was attributed to a long-range hyperfine coupling of the methylene protons across the -HNCOO- group. The pK_a value for the dissociation of the NH proton was determined to be 14.48 ± 0.04 from the change of the α-proton coupling constant using the same procedure as mentioned above. The pK_a value of radical V is in the same range as that of the structurally related radical IV.

Radicals from N-Methylformamide. Around neutral pH, two radical species were identified from N-methylformamide as reported by Yonezawa et al.⁹ One is a π radical (VI, HCONHCH₂) and the other is a σ radical (XIV, ·CONHCH₃). The ESR parameters are shown in Tables I and III, respectively. These radicals were not, however, observable in the strongly alkaline region, probably because hydrolysis of the parent molecule occurred; the σ radical was not detected above pH 12.5 nor was the π radical above H₋ = 14.0. ESR lines of an additional radical appeared in the spectrum at pH 12.5 and their intensity increased in more strongly basic solutions such that they became more intense than those of radical VI at H₋ > 13.7. Above H₋ = 14.1, only these new lines consisting of a triplet (1:2:1)-triplet (1:1:1)-triplet (1:2:1) pattern were found. This ESR spectrum can be ascribed to ·CH₂NH₂ radical produced by the reaction of O⁻ with methylamine formed as a result of hydrolysis. ESR parameters of ·CH₂NH₂ are a_α^H = 15.03, a^N = 5.00, a^H(NH₂) = 4.33 G, and g = 2.002 77 which are in good agreement with those reported previously by Neta and Fessenden.¹¹ From the ESR parameters of the π radical VI observed below H₋ = 14.00 (0.80 M in KOH concentration) the limiting values for the base form which are shown in Table I, were estimated using equation 4. Subsequently, the pK_a value for the dissociation of the NH proton was determined to be 13.30 ± 0.04 from hyperfine coupling constants of the α proton and 13.32 ± 0.02 from those of the formyl proton.

Radical from Sarcosine. The radical produced in a neutral solution of sarcosine is CH₃NHCHCOO⁻ (VII) as assigned by Paul and Fischer.⁸ The spectrum remained essentially the same even at KOH concentrations of 5 M (H₂ = 16.0) except for a slight increase in α-proton coupling constant (12.25 to 12.40 G) and a slight decrease in NH proton coupling constant (5.39 to 5.16 G). Although the reason for these slight changes is not clear it has been concluded that the radical VII does not dissociate its NH proton in the basicity region examined. Therefore, the pK_a value must be larger than 17.

Discussion of the pK_a Values and the Changes in ESR Parameters upon Dissociation of the NH Proton. The pK_a values for the dissociation of the NH proton of linear peptide radicals (I-VI) have been found to be lower than 14.6 while the radical from sarcosine (VII) which lacks the carbonyl group adjacent to NH has a pK_a value larger than 17. This clearly shows that the acidity of the former radicals results from the electron-withdrawing effect of the carbonyl group adjacent to the NH group as already seen in the corresponding parent molecules, i.e., amides and amines. The pK_a values for the dissociation of the NH proton of N-acetyl glycine²⁴ and N-methylacetamide²⁵ have been estimated to be 19.4 and 17.7, respectively.

Table II. Calculated Spin Densities and NH Proton Hyperfine Coupling Constants

| Radical | Calculated spin densities in acid form | | | | $ a^H(\text{NH}) $, G ^d | | Calcd spin densities ^e in base form | | |
|---------|--|----------------------------|---|---------------------|-------------------------------------|------|--|----------------------------|---|
| | $\rho_{\alpha\text{-C}}^a$ | $\rho_{\gamma\text{-C}}^b$ | $\rho_{\alpha\text{-C}} + \rho_{\gamma\text{-C}}$ | ρ_{N}^c | Calcd | Obsd | $\rho_{\alpha\text{-C}}^a$ | $\rho_{\gamma\text{-C}}^b$ | $\rho_{\alpha\text{-C}} + \rho_{\gamma\text{-C}}$ |
| I | 0.720 | 0.093 | 0.813 | 0.071 | 1.29 | 1.28 | 0.599 | 0.170 | 0.769 |
| II | 0.788 | 0.137 | 0.925 | 0.015 | 0.18 | <0.2 | (-0.121) | (+0.077) | (-0.044) |
| III | 0.785 | 0.147 | 0.932 | -0.009 | 0.78 | 0.88 | 0.694 | 0.231 | 0.925 |
| IV | 0.788 | 0.118 | 0.906 | 0.014 | 0.19 | <0.2 | (-0.094) | (+0.094) | (0.000) |
| VIII | 0.670 | 0.119 | 0.789 | 0.042 | 0.58 | 0.57 | 0.694 | 0.245 | 0.939 |
| IX | 0.729 | 0.113 | 0.842 | 0.065 | 1.12 | 1.13 | (-0.091) | (+0.098) | (+0.007) |
| | | | | | | | 0.690 | 0.173 | 0.863 |
| | | | | | | | (-0.098) | (+0.055) | (-0.043) |

^a Estimated by using eq 5 for radicals I–IV and IX and by using eq 6 (where α is used instead of γ) for radical VIII. ^b Estimated by using eq 6 for radicals I–III and VIII and by using eq 7 for radicals IV and IX. ^c Estimated by using eq 11. ^d Absolute values in G. Calculated by using eq 13. ^e Changes in spin densities upon dissociation of the NH proton are shown in parentheses.

They were obtained from the rate constants of the base-catalyzed exchange reaction of the NH proton using the Eigen scheme.²⁶ However, the pK value for the proton dissociation from H_2O was taken as 15.8 in the former reference²⁴ (although it was not described explicitly), while it was taken as 14 in the latter one.²⁵ Using the pK value of 15.75²⁶ and necessary data in the latter paper,²⁵ pK_a values of parent compounds could be recalculated to be 19.6 for *N*-acetyl glycine, 19.4 for *N*-methylacetamide, and 18.5 for *N*-methylformamide. These values are about 5 to 5.5 units higher than the pK_a values of radicals I, II, and VI derived from such compounds. Consequently, one can say that the loss of a hydrogen atom from the α position to the NH group enhances the acidity by about 5 to 5.5 units in the pK_a value.

It should be noted that there are some remarkable changes in ESR parameters upon the dissociation of the NH proton. In all radicals, the α -proton coupling constant decreased upon dissociation while the hyperfine coupling constants of protons beyond the peptide bond increased indicating a decrease of spin density on the α -carbon atom and an increase on the γ -carbon atom (that is a peptide carbon atom). The spin density on the α -carbon atom, $\rho_{\alpha\text{-C}}$, can be estimated from the observed hyperfine coupling constant of the α proton using McConnell's relation

$$a_{\alpha}^H = Q_{\text{CH}^H} \rho_{\alpha\text{-C}} \quad (5)$$

in which the parameter Q_{CH^H} was chosen empirically to be 24.1 G, that is, the averaged value of Q_{CH^H} in alkyl radicals with C_α substituents H and COOH .^{27,28} The hyperfine coupling with a δ proton across the peptide group from the radical carbon could be explained in terms of hyperconjugation with unpaired spin density localized in the $p\pi$ orbital on the C_γ atom. Consequently, the spin density on the γ -carbon atom, $\rho_{\gamma\text{-C}}$, was estimated from the observed hyperfine coupling constant of $\text{C}_\gamma\text{-CH}_3$ protons using the well-known relation

$$a^H(\text{C}_\gamma\text{-CH}_3) = Q_{\text{CCH}_3^H} \rho_{\gamma\text{-C}} \quad (6)$$

where the parameter $Q_{\text{CCH}_3^H}$ is 29.3 G.²⁷ When the hyperfine coupling constants of $\text{C}_\gamma\text{-CH}_2$ methylene protons were observed, the same equation as eq 6 is applicable if it is assumed that

$$a^H(\text{C}_\gamma\text{-CH}_2) = 29.3 \rho_{\gamma\text{-C}} \quad (7)$$

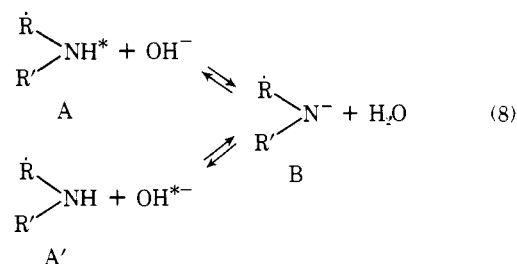
a similar hyperconjugative mechanism is present and that the methylene group is free to rotate around the $\text{C}_\gamma\text{-CH}_2$ bond.

From the values of a_A and a_B listed in Table I, spin densities, $\rho_{\alpha\text{-C}}$ and $\rho_{\gamma\text{-C}}$, of radicals I–IV, were obtained using eq 5–7. The result is summarized in Table II. Changes in the spin density upon dissociation are also expressed in parentheses in Table II. It becomes clear that after dissociation of the NH proton the spin density on the α -carbon atoms decreases by

0.09 to 0.12, while that on the γ -carbon atoms increases by 0.06 to 0.10. Apparently, 0.06 to 0.09 of spin density flows from the α - to the γ -carbon atoms. For all radicals except VI the g factor increases significantly upon dissociation of the NH proton. This fact suggests a greater spin density on the oxygen and/or nitrogen atoms in the dissociated forms.

Kinetics of the Acid–Base Equilibrium of the Radical from *N*-Acetyl glycine. The line broadening observed in the spectrum of radical I from *N*-acetyl glycine was used to measure the rate constant of the removal by hydroxide ions of the NH proton of the radical (reaction 1 in the forward direction). A first-derivative ESR spectrum of good quality was obtained throughout the basicity range of pH 8 to $H_{2-} = 15$ (except near pH 12) and the peak-to-peak line widths were determined by the procedure described in the Experimental Section.

The treatment of the data follows that of Laroff and Fessenden.²² They treated broadening effects due to exchange and dissociation separately in their study on the kinetics of the dissociation equilibrium of the OH protons in α -hydroxyalkyl radicals. The reaction involved here is hydroxide ion catalyzed and may be expressed by



where the asterisk denotes a particular spin state of the NH proton of the radical. Although a complete analysis requires the treatment of three species, A, A', and B, in one model, it is rather complex. The separation of the effects of exchange and dissociation makes it possible to use a much simpler "two site-jump" theory. Since the situation encountered here is quite similar to that in the α -hydroxyalkyl radicals,²² the simpler treatment should be valid.

The broadening of all the lines from the acid form of the radical encountered around pH 12 can be treated as the result of only the exchange of the NH proton between A and A'. Since the equilibrium is to the left at this pH region, species B is present in only a small concentration and thus acts merely as an intermediate in the exchange. The uppermost diagram in Figure 3 illustrates the spectral lines of the acid form at the slow exchange limit. As the exchange rate increases with increase in hydroxide ion concentration, these lines broaden and the doublets due to the NH proton eventually collapse to a single line at the center of each former doublet as shown in Figure 3b. The maximum line width possible is equal to the

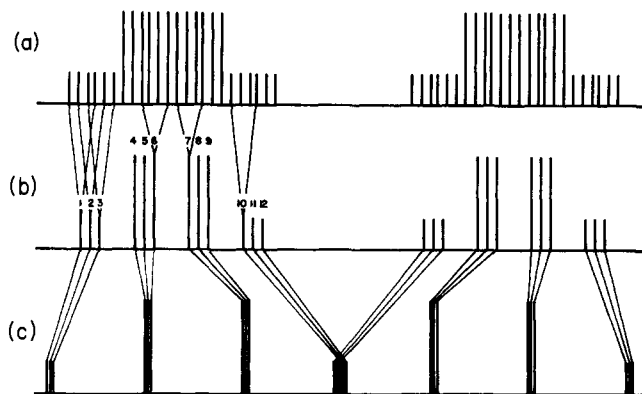
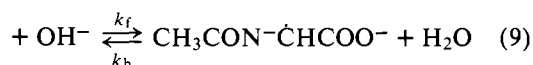


Figure 3. Correlation diagram for the lines in the spectrum of Radical I: (a) for the acid form, (b) for the acid form without NH splitting, and (c) for the base form.

original NH splitting (1.28 G). With further increase in hydroxide ion concentration, the equilibrium shifts to the right, and the concentration of the base form (B) becomes significant. In such a situation a further broadening can occur as the result of the interchange of the radical back and forth between the acid and base forms. The lower part of Figure 3 illustrates the correlation between the lines of the acid form, which has already lost the NH proton splitting, and the base form. Since the shift in line position differs for individual lines the magnitude of the broadening is different for each line.

The absorption line shape function for intermediate rates of exchange derived by Gutowsky and Holm²⁹ was used to calculate line widths from both effects. The calculation of its first derivative and the peak-to-peak width was carried out on a Hewlett-Packard 9830A calculator using an assumed value as described below for the rate constant for removal of the NH protons by OH⁻ ions. A factor of 0.5 was included to take account of the fact that only one-half the exchanges would invert the NH proton spin. In the calculation of line widths due to the dissociation the relative ratio of the two forms of the radical was determined on the basis of $pK_a = 14.0$ obtained above. The sum of the contributions from the natural width and from the exchange and the dissociation processes should give the observed line width.

The line widths determined by experiment for lines 6, 7, and 10 (see Figure 3) are plotted against the basicity of the medium in Figure 4. The large peak in the region of pH 11–12.5 is due to the exchange and is common to all lines. Line 6 (see Figure 3 for numbering to lines) shifts only 0.09 G down field upon dissociation and has no contribution to its line width from the dissociation process. Lines 7 and 10 shift up field by 2.73 and 4.92 G upon dissociation, respectively. For these lines the contribution from the dissociation is evident as seen from the second peak (or shoulder) in the region around pH 13.5. Curves shown in Figures 4a–c are calculated using the values of 6, 8, and $10 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ for the forward rate constant, k_f , respectively.



The value of $1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ gives the best fit for the main peak while the value of $6 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ seems to give the best fit for the second peak. The rate constant has been thus determined to be $(8 \pm 2) \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$. A possible reason for the difficulty in obtaining a value which gives a good fit for both peaks is that the rate constant may change with ionic strength. The backward rate constant $k_b = 1.6 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ has been obtained from the forward rate constant and the pK_a value.

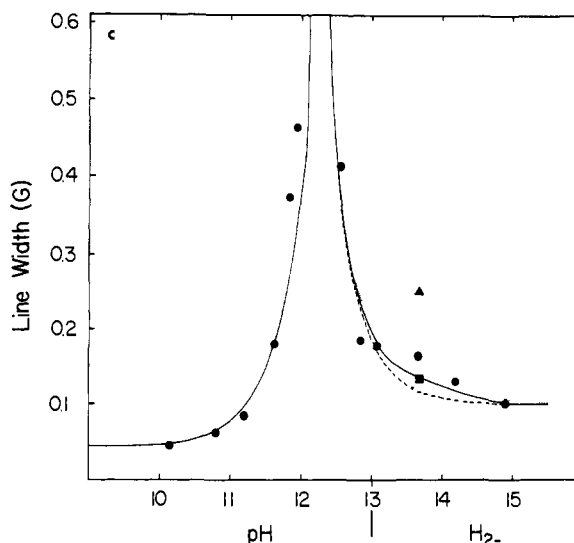
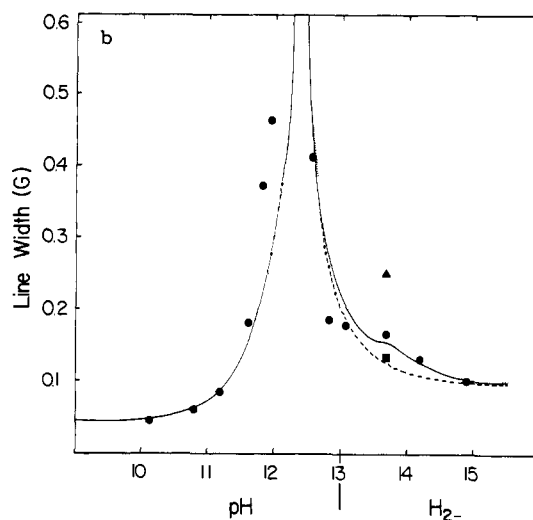
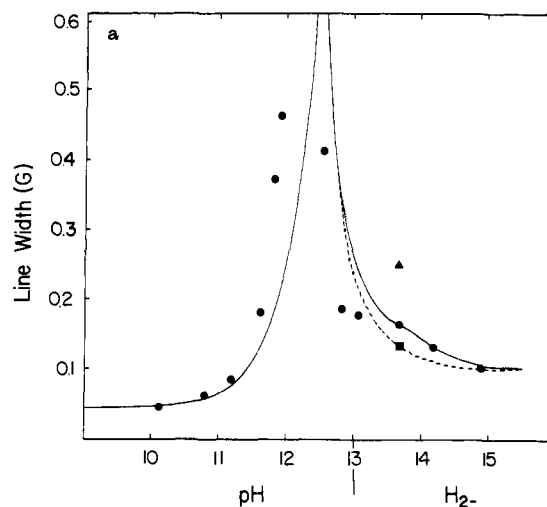






Figure 4. Line width against basicity for the lines 7 (●), 6 (■), and 10 (▲) in the ESR spectrum of radical I. Curves are calculated for the three lines (— for line 7, --- for line 6, and -·-·- for line 10) using forward rate constants of (a) 6×10^8 , (b) 8×10^8 , and (c) $1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, respectively.

The theory summarized by Eigen²⁶ predicts that the forward rate should be diffusion controlled since the pK_a difference is negative, $\Delta pK_a = pK_a(\text{radical I}) - pK_a(\text{H}_2\text{O}) = 14.0 - 15.75 \approx -1.7 < 0$. The observed value is significantly lower than this prediction. However, since the pK_a difference is not very large a deviation from the ideal case is possible. The negative charge

Table III. ESR Parameters^a of Other Aliphatic Radicals Obtained from Peptide Bond Containing Compounds and Structurally Related Compounds

| Compd | pH | Radical ^b | g factor | Hyperfine coupling constants | | | | | | | |
|------------------------------------|---------------------|----------------------|---|------------------------------|------------|----------------------------|-------|--------------|-------------------------------|-----------------|------------------|
| | | | | α -H | β -H | N | NH | Other proton | | | |
| <i>N</i> -Acetylalanine | 10.18 | VIII | CH ₃ CONH \dot{C} (CH ₃)COO ⁻ | 2.003 46 | | CH ₃ , 19.64 | 1.15 | 0.57 | CH ₃ CO, 3.49 | | |
| Glycylglycine | 5.68 | IX | H ₃ N ⁺ CH ₂ CONH \dot{C} HCOO ⁻ | 2.003 33 | 17.57 | | 0.73 | 1.13 | -CH ₂ CO-, 3.30 | | |
| Hippuric acid | $H_{2-} =$ 15.52 | X |  | 2.003 33 | 13.34 | | 0.85 | | Ortho H, 1.08 | Meta H, 0.35 | Para H, 1.17 |
| <i>c</i> | 3.12 | XI |  | | | | | | Ortho H, 8.31 | Meta H, 2.63 | Para H, 12.27 |
| <i>c</i> | 3.12 | XII |  | | | | | | 8.94 | 2.65 | 12.74 |
| <i>c</i> | 3.12 | XIII |  | | | | | | 8.36 | 2.62 | 0.65 |
| <i>N</i> -Methylformamide | 12.31 | XIV | \dot{C} ONHCH ₃ | 2.001 60 | | | 22.13 | 1.91 | CH ₃ , 0.97 | | |
| <i>N</i> -Formylglycine | 2.94 | XV | \dot{C} ONHCH ₂ COO ⁻ | 2.001 62 | | | 23.24 | 1.35 | CH ₂ , 0.76 | | |
| <i>N,N'</i> -Methylenebisformamide | 5.90 | XVI | \dot{C} ONHCH ₂ NHCOH | 2.001 64 | | | 21.46 | 1.18 | CH ₂ , 1.61 | | |
| <i>N</i> -Acetylethanolamine | 6.48 | XVII | CH ₃ CONHCH ₂ \dot{C} HOH | 2.003 01 | 16.50 | 14.56 | 7.28 | 0.53 | OH, 0.53 | | |
| | 12.74 | XVIII | CH ₃ CONHCH ₂ CHO ⁻ | 2.003 50 | 13.74 | 13.74 | 7.92 | 0.62 | | | |

^a See footnote *a* of Table I. ^b Radicals IX and XIV in the acid form have been studied previously. See ref 10 and 12 for IX and ref 9 for XIV. ^c Because of intensity differences of high- and low-field lines only the high-field lines could be measured; consequently, no $>CHOH$ splitting could be obtained, and no *g* factor was determined. The nomenclature for protons (e.g., ortho, meta, and para H) is with respect to the carbon atom substituted with the OH group.

on CH₃CONH \dot{C} HCOO⁻ cannot account for the retarded rate because this effect can decrease the rate constant by only a factor of 2–3.²⁶ It seems that the rather small rate constant obtained here is not common to all the peptide radicals because for the radicals from glycine anhydride and alanine anhydride the reaction rates are close to being diffusion controlled⁶ as is expected from the more negative value of ΔpK_a , -6.1. This view may also be supported by the fact that values of the rate constants of the base-catalyzed exchange reaction of peptide hydrogen in various peptide molecules vary over three orders of magnitude: 2.7×10^6 (for *N*-acetylglutamate) to 1.0×10^{10} (for glycylglycineamide) M⁻¹ s⁻¹.²⁴

Other Linear Peptide Radicals and Related Radicals. Radicals obtained from other compounds containing peptide bonds are summarized in Table III. Linear peptide radicals VIII, IX, and X were identified from *N*-acetylalanine, glycylglycine, and hippuric acid (*N*-benzoylglycine), respectively. ESR parameters of radical IX are in good agreement with those reported previously.^{10,12} Radicals VIII and X are assigned here for the first time. ESR spectra of VIII and IX could not be detected clearly in stronger alkaline region probably because of the hydrolysis of substrates.

Major radical species from hippuric acid were hydroxycyclohexadienyl radicals XI–XIII at pH < 12.7 and the linear peptide radical X at $H_{2-} > 13.5$. At higher pH, where the OH radical is rapidly converted into the O⁻ anion radical, addition of O⁻ to aromatic compounds is relatively slow so that in many cases hydrogen abstraction dominates.³⁰ Consequently, it is reasonable that the addition of OH radical to hippuric acid occurs mainly at lower pH, while hydrogen abstraction from the substrate takes place at higher pH. Radical X was assigned between $H_{2-} = 13.66$ and 15.52 (0.26 and 3.61 M in KOH concentration, respectively) and ESR parameters at $H_{2-} = 15.52$ are shown in Table III. There is no remarkable change of ESR parameters in these H_{2-} regions. Since the ESR spectra of the acid form of radical X could not be observed, the *pK_a* value for the dissociation was not determined. The ratio of proton coupling constants due to ortho, meta, and para protons is 1:0.32:1.08 and it is very similar to the ratio 1:

0.35:1.20 observed in benzyl radical.³¹ Radicals from aqueous solutions of hippuric acid below pH 12.7 were ascribed mainly to hydroxycyclohexadienyl radicals XI–XIII. Because of low intensity in the low-field lines, hyperfine coupling constants of *CHOH* proton and *g* values could not be obtained. Hyperfine coupling constants of ortho, meta, para, and hydroxyl protons at pH 3.12 are shown in Table III and are in good agreement with those of hydroxycyclohexadienyl radicals from benzoic acid reported by Eiben and Schuler.³² However, a small difference of ortho- and meta-proton coupling constants due to the asymmetric structure of radicals XI and XII was not observed in our experiments and the difference seems to be less than 0.2 G.

From the substrates with formyl groups, σ radicals XIV, XV, and XVI were observed predominantly and linear peptide radicals were not obtained except for the case of *N*-methylformamide in which both σ and π radicals were formed. These σ radicals have characteristic low *g* values and large nitrogen coupling constants.⁹ In the reaction of *N*-acetylethanolamine with hydroxyl radical, a hydrogen atom is abstracted from the C–H bond adjacent to the hydroxyl group, as is the case for most aliphatic alcohols and the α -hydroxyl radical (XVII) is assigned instead of the peptide radical. In stronger base, ESR parameters of the radical XVII change into those of XVIII due to the dissociation of OH proton^{14,22} as shown in Table III. From *N,N'*-dimethylformamide (CH₃NHCOCONHCH₃), a peptide radical was not observed and $\cdot CH_2NH_2$ radical was identified at $H_{2-} = 13.0$ to 14.0 as is the case in strongly alkaline solution of *N*-methylformamide. The ESR parameters were as follows: $a_{\alpha H} = 15.08$, $a^N = 4.96$, $a^H(NH_2) = 4.36$ G, and $g = 2.00276$. Hydantoic acid (*N*-carbamoylglycine) and *N*-2-hydroxyethylformamide gave only poorly resolved ESR spectra of the intermediate radicals formed in the reaction with hydroxyl radical.

Discussion of the Hyperfine Coupling Constants Due to Nitrogen and the NH Proton. In this work hyperfine coupling constants of nitrogen and NH protons were determined accurately for the acid forms of several peptide radicals. The nitrogen coupling constant a^N is rather small, <2.83 G. The

observed value of a^N has been explained quantitatively using the following equation,

$$a^N = Q_N^N \rho_N + \sum_i Q_{CN^N} \rho_{i-C} \quad (10)$$

where ρ_N is the π -electron spin density on the nitrogen atom, ρ_{i-C} is that on the carbon atoms adjacent to the nitrogen, and Q_{CN^N} is of opposite sign to Q_N^N . It is considered that a^N becomes small in the peptide radicals because the contribution to a^N from ρ_N is canceled by the negative contribution from ρ_{i-C} through the σ - π interaction.^{8,10} From the observed values of a^N , spin densities on nitrogen $2p\pi$ orbital can be estimated using eq 11 with parameters $Q_N^N = 25.0$ and $Q_{CN^N} = -2.8$ G, assuming that the sign of a^N is negative.⁸

$$\rho_N = \{2.8(\rho_{\alpha-C} + \rho_{\gamma-C}) + a^N\}/25.0 \quad (11)$$

The values of the parameters Q_N^N and Q_{CN^N} are in a reasonable range because the nitrogen hyperfine coupling constants of some peptide radicals have been explained using almost the same parameters.^{8,10} The calculated values of $\rho_{\alpha-C}$ and $\rho_{\gamma-C}$ are obtained as stated above in this text. The estimated values of ρ_N are listed in Table II with a maximum value of 0.07. In radical III there is a possibility that negative spin density exists on the nitrogen atom as pointed out by Paul and Fischer⁸ for $\text{HCON}(\text{CH}_2\text{CH}_3)\dot{\text{C}}\text{HCH}_3$ radical.

There has previously been little discussion of the NH proton coupling constant, $a^{\text{H}}(\text{NH})$, of peptide radicals. Judging from the calculated values of ρ_N , the observed values of $a^{\text{H}}(\text{NH})$ do not follow the simple McConnell's relationship.¹²

$$a^{\text{H}}(\text{NH}) = Q_{\text{NH}^{\text{H}}} \rho_N \quad (12)$$

In an attempt to explain the values of $a^{\text{H}}(\text{NH})$, canceling contributions to $a^{\text{H}}(\text{NH})$ by the spin densities on C_α and C_γ are assumed. (It should be noted that these spin densities are quite large, amounting in total to 0.8–0.9). Consequently, an additional term is introduced to eq 12 so that the empirical eq 13 is proposed for obtaining $a^{\text{H}}(\text{NH})$ of the peptide radicals.

$$a^{\text{H}}(\text{NH}) = |Q_{\text{NH}^{\text{H}}} \rho_N + Q_{\text{CNH}^{\text{H}}}(\rho_{\alpha-C} + \rho_{\gamma-C})| \quad (13)$$

The parameters $Q_{\text{NH}^{\text{H}}}$ and $Q_{\text{CNH}^{\text{H}}}$ have opposite signs. They were adjusted to give the best fit to observed values of $a^{\text{H}}(\text{NH})$ using estimated values of ρ_N and $\rho_{\alpha-C} + \rho_{\gamma-C}$ from Table II. The best values are $Q_{\text{NH}^{\text{H}}} = 25.0$ and $Q_{\text{CNH}^{\text{H}}} = -0.6$ G. The value of $Q_{\text{NH}^{\text{H}}}$ is comparable to the value which might be used in the simpler eq 12 and happens to be the same as Q_N^N . Calculated values of $a^{\text{H}}(\text{NH})$ are in excellent agreement with the observed values of $a^{\text{H}}(\text{NH})$ as shown in Table II.

Conclusion

In the present work, the dependence on basicity of the hyperfine coupling constants of the linear peptide radicals ($-\text{CONH}\dot{\text{C}}<$) produced by hydrogen abstraction by OH or O^- was examined and analyzed in terms of the dissociation equilibrium 1. The equilibrium constants for the dissociation of NH protons were determined and the values were expressed as $\text{p}K_a$ for six peptide radicals using Yagil's acidity function. The $\text{p}K_a$ values were found to be 13.3–14.6. It should be pointed out that the loss of a hydrogen atom from the C–H bond adjacent to the NH group enhances the acidity of the radical over that for the parent molecule by about 5 to 5.5 units in $\text{p}K_a$ value. Upon dissociation, α - and β -proton coupling

constants decreased markedly, δ - or γ -proton coupling constants increased significantly, and g factors increased slightly. Apparently, spin density flowed from the α -carbon atom to the γ -carbon atom.

In the ESR spectra of the radical ($\text{CH}_3\text{CONH}\dot{\text{C}}\text{HCOO}^-$) from N -acetylglycine, line-broadening effects and line shifts were observed with change of pH. These effects could be fully understood in terms of the exchange of the NH proton with water molecules and the acid dissociation of the NH proton of the radical. The dynamics of equilibrium 1 were investigated by means of the ESR line broadening associated with the dissociation and values for the forward and reverse rate constants were obtained.

Hyperfine coupling constants due to NH protons of peptide radicals were discussed in detail using spin densities on the nitrogen and α - and γ -carbon atoms calculated from the observed hyperfine coupling constants. An experimental eq 13 was proposed to estimate NH proton coupling constants in which a canceling contribution from the total spin density on the carbon atoms adjacent to the nitrogen was introduced. The proposed eq 13 explained the observed values very well. Several related radicals including hydroxycyclohexadienyl radicals from hippuric acid and three σ radicals ($-\text{CONH}\dot{\text{C}}<$) were identified and discussed.

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