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An ESR Study of the Acid Dissociation of NH Protons.[1] 2. Cyclic Peptide Radicals and Related Radicals

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Abstract: Free radicals formed by the reaction of OH or O^- radicals with alicyclic compounds containing a peptide group (-CONH-) and structurally related compounds have been studied by the in situ radiolysis-steady state ESR method. Eight cyclic peptide radicals resulting from hydrogen abstraction from the C-H bond adjacent to the peptide group have been observed. They are divided into two groups on the basis of the skeletal structure of the radicals: >Ċ-NH-CO- and >Ċ-CO-NH-. In basic solutions significant changes occur in the ESR parameters of these radicals which can be interpreted in terms of the dissociation of the NH proton in a peptide group. The pK_a values for the NH proton dissociation have been determined to be in the range of 7.6 to 13.6 for eight cyclic peptide radicals from 5-methyl-2-pyrrolidinone, 2-pyrrolidinone, 2-pyrrolidone-5-carboxylic acid, hydantoin (for both first and second NH proton dissociations), 1-methylhydantoin, 2-thiohydantoin (second dissociation only), succinimide, and 2,5-piperazinedione (first dissociation only) and 10.9 for a related cyclic radical from 2-oxazolidone. These pK_a values are considerably lower than those for corresponding linear peptide radicals, partly because π-electron density on the nitrogen atom decreases in cyclic peptide radicals with a more delocalized π-electron system. Within each group of cyclic peptide radicals with >Ċ-NH-CO- or >Ċ-CO-NH- the same trend of changes in ESR parameters was observed upon the dissociation of peptide proton. In the first group with structure similar to linear peptide radicals the g value, and the γ- or δ-proton and nitrogen coupling constants, increase while α- and β-proton coupling constants decrease upon the dissociation. Apparently 0.07 to 0.09 of spin density flows from the α-carbon atom to the peptide carbon atom after NH proton dissociation in the three pyrrolidinone radicals. In the second group the g values and nitrogen coupling constants decrease upon dissociation and no remarkable changes in α-proton coupling constant are observed, suggesting that local rearrangement of π-electron distribution occurs in the dissociating peptide group upon the dissociation of peptide proton.

In a previous paper,[4] it was reported that nine linear peptide radicals (-CONHĊ<) formed by hydrogen abstraction with OH or O^- radicals were studied by the in situ radiolysis-steady state ESR method.[5] Significant changes in the ESR parameters occurred in strongly basic solutions and these changes could be interpreted in terms of the dissociation of a peptide proton. The pK_a values for the NH proton dissociation were determined to be 13.3 to 14.6 for six linear peptide radicals.

In this paper an ESR study of the acid dissociation of NH protons has been extended to include cyclic peptide and related radicals. In a spectrophotometric pulse radiolysis study[6] the pK_a value for the NH proton dissociation was determined to be 9.6 for two cyclic peptide radicals from glycine anhydride and alanine anhydride. It was demonstrated that the pK_a value (9.6) is less than that of the corresponding linear peptide radicals because of the effect of cyclization and resonance delocalization.[6] General decrease of pK_a values for the NH proton dissociation was found in linear peptide radicals from $-(CONHCHR)_n-$ with increasing n and the decrease might be correlated with the effect of cyclization.[4,7]

In the present paper a detailed *in situ* radiolysis ESR study of the acidity of several cyclic peptide and related radicals is described. These radicals are formed by hydrogen abstraction with OH or O^- radicals. It should be pointed out that the ESR method has many advantages for following an acid-base equilibrium, such as ability to identify and relate the acid and basic forms of a radical directly and independence of radical yields, side reactions, or impurities.[8]

Experimental Section

The experimental arrangement and procedures for the observation of cyclic peptide and related radicals were essentially the same as described previously.[4] All materials were obtained from commercial sources and used without further purification. To provide an acidity scale above the pH scale, Yagil's H_- or H_2- acidity function[9] was used for concentrated aqueous potassium hydroxide solutions. Since these acidity functions were determined from the ionization of indoles,[9] they are quite appropriate for the structurally related cyclic compounds studied in this work.

When the ESR spectrum for the pure basic form could not be observed, the limiting value a_B of hyperfine coupling constants for the basic form was determined by an extrapolation using eq 1

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

where a is the observed hyperfine coupling constant at H_x (the value of the acidity function or pH of a solution), K_a is the acid dissociation constant, and a_A is the limiting value for the acid form. Using the limiting values a_A and a_B, pK_a values were determined from eq 2.

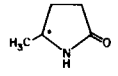
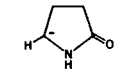
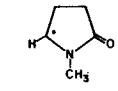
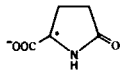
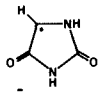
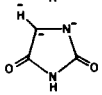
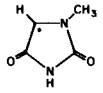
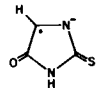
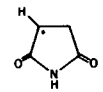
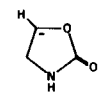
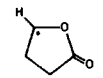
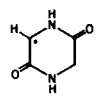
$$pK_a = H_x + \log [(a - a_B)/(a_A - a)] \quad (2)$$

The titration curves for the dissociation of NH protons were calculated and plotted with a Hewlett-Packard 9100A calculator and a 9125A plotter, according to eq 3.

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

The calculation of the peak-to-peak width in a first derivative ESR spectrum was carried out on a Hewlett-Packard 9830A calculator.

Table I. ESR Parameters[a] for the Acid and Basic Forms of Cyclic Peptide Radicals and Related Radicals and Their Dissociation Constants

		Acid form							Basic form						
		Hyperfine coupling constants, G							Hyperfine coupling constants, G						
Radical in acid form		N–H	N	N	α-H	β-H	γ-H	g factor	N	N	α-H	β-H	γ-H	g factor	pK_a
I		<0.2	1.80		CH_3 20.28	26.32	3.60	2.002 68	(2.00		CH_3 18.98 (18.90	23.10 22.84	6.77)[b] 7.00)[c]	2.002 85	13.6 ± 0.2
II		0.76	1.68		16.42	28.62	3.92	2.002 85	1.75		16.36	24.78	7.31	2.002 92	13.3 ± 0.2
III		CH_3 0.27	1.71		15.93	28.12	4.26	2.002 88				*d*			
IV		1.87	0.22			24.21	2.86	2.003 47	0.32			19.53	5.35	2.003 59	12.7 ± 0.2
V		3.08	1.08	1.47	14.75			2.003 75	1.33	1.99	11.43			2.003 76	7.6 ± 0.1
V			1.33	1.99	11.43			2.003 76	(1.25	1.41 (1.40)[c]	11.46)[b]			2.003 68	13.5 ± 0.2
VI		CH_3 3.40	1.70	1.56	14.14			2.003 77	1.66	1.10	13.85	CH_3 3.32		2.003 61	8.5 ± 0.2
VII			(0.32	1.95 (2.03)[c]	10.14)[b]			2.005 99	0.37	1.71	10.06			2.005 63	13.1 ± 0.2
VIII		1.46	0.42		20.64	29.46		2.003 32	0.99		20.41	26.70		2.003 21	9.5 ± 0.2
IX		1.55	<0.2		13.79	35.02		2.002 91	<0.2		15.29	34.51		2.003 06	10.9 ± 0.2
X					15.92	32.03	0.37	2.002 93				*e*			
XI		2.48 2.26	0.51	1.53	17.16	δ-H 8.58		2.003 32	0.54	1.58	14.82	δ-H 12.49	N–H 2.12	2.003 38	9.6 ± 0.1

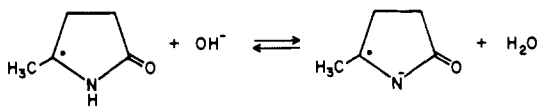
[a] The hyperfine coupling constants are given in Gauss and are accurate to ±0.03 G. The *g* factors were measured relative to the peak from the silica cell and are accurate to ±0.000 05. Second-order corrections have been made. [R. W. Fessenden, *J. Chem. Phys.*, 37, 747 (1962)]. The nomenclature for protons (e.g., α-H, 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. [b] Values observed in the most basic or acidic medium which allowed spectral detection. [c] Determined by extrapolation using eq 1. These values were used for obtaining pK_a values by eq. 2. [d] ESR parameters did not change within experimental errors up to $H_- = 13.61$. [e] Possibly $^-OCHCH_2CH_2COO^-$ radical was formed in basic solution, see text.

Results and Discussion

In this work ESR spectra of eight cyclic peptide radicals and three related radicals were observed and analyzed. The structures (I to XI) and ESR parameters of these radicals are summarized in Table I.

Radicals from 5-Methyl-2-pyrrolidinone, 2-Pyrrolidinone, and 2-Pyrrolidone-5-carboxylic Acid. The ESR spectra observed during the electron irradiation of N_2O saturated aqueous solutions of 10 mM 5-methyl-2-pyrrolidinone near neutral pH showed a triplet (1:1:1)-triplet (1:2:1)-quartet (1:3:3:1)-triplet (1:2:1) pattern. The radical species which gave this ESR pattern is ascribed straightforwardly to structure I in Table I. The hyperfine splitting of the NH proton was not observed and it seems to be less than 0.2 G. The equivalence of the two β- and γ-proton coupling constants suggests planarity of the ring rather than rapid interconversion since one can reasonably assume planarity of the peptide bond and the trivalent carbon atom.

With an increase in the basicity of sample solutions, the observed ESR lines shifted and the ESR parameters changed; the hyperfine coupling constants of the β and methyl protons decreased and those of the γ proton and nitrogen increased. These changes in ESR parameters can be fully explained in terms of the acid dissociation equilibrium between the acid and the basic forms of 5-methyl-2-pyrrolidinone radical.



The dependence of the ESR parameters on the basicity of the medium is shown in Figure 1a. Hyperfine coupling constants for the basic form of this radical were determined by an extrapolation using eq 1. As shown in Table I, these calculated values are close to the observed values at the highest basicity (H_- = 14.75 for 2.68 M KOH) available for the detection of the ESR spectrum.

Using the limiting values (a_A observed and a_B calculated) for γ-, methyl-, and β-proton coupling constants and seven experimental points within one unit of the half-neutralization point, pK_a values for the NH proton dissociation were calculated to be 13.56 ± 0.18, 13.58 ± 0.08, and 13.62 ± 0.19, respectively, using eq 2. The pK_a value has been thus determined to be 13.6 ± 0.2. The concentration of KOH at H_- = 13.6 is 0.4 M. Three sigmoid curves in Figure 1a are calculated from the nature of the acid dissociation equilibrium assuming that the ESR spectra of 5-methyl-2-pyrrolidinone radical represent the weighted average of the concentrations of the acid and basic forms of this radical. The agreement between the experimental points and the calculated curves is satisfactory for each coupling constant.

The ESR spectrum observed during the irradiation of a neutral 2-pyrrolidinone (2-pyrrolidone) solution showed the presence of only one species and has been assigned to the cyclic peptide radical II. The presence of only one set of coupling constants in the range of β-proton coupling constant, the comparatively low g value, and the fact that OH radical is an electrophile favor the radical II over the other two possible radical species. The general tendency of the change in ESR parameters upon the NH proton dissociation also supports this identification as described later. Shiga et al.[10] suggested tentatively that the structure of the 2-pyrrolidinone radical produced by a Ti^{3+}-H_2O_2 redox system is radical II. They assigned hyperfine coupling constants as follows: a_α^H = 16.3 G and a_β^H = 29.1 G, although a hyperfine splitting of the NH group was not resolved well.[10] These values agree well with those reported here.

Changes in the ESR parameters of 2-pyrrolidinone radical have been recorded over the entire range of the dissociation

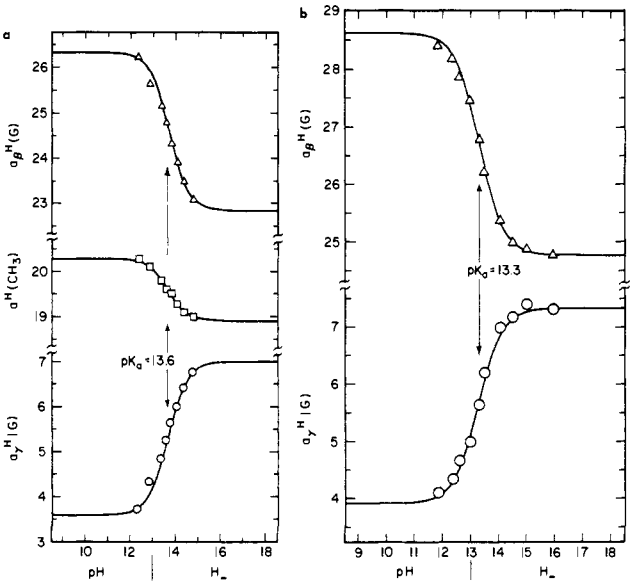


Figure 1. The hyperfine coupling constants as a function of basicity (a) for radical I from 5-methyl-2-pyrrolidinone and (b) for radical II from 2-pyrrolidinone. The solid curves are calculated from the data in Table I using eq 3 based upon the nature of the acid dissociation equilibrium.

equilibrium (up to H_- = 15.96 for [KOH] = 6.86 M) and especially large changes with basicity were found in the β- and γ-proton coupling constants as shown in Figure 1b. The pK_a value for the NH proton dissociation was determined to be 13.31 ± 0.08 from the β-proton coupling constants and 13.20 ± 0.14 from the γ-proton coupling constants.

From an irradiated aqueous solution of 1-methyl-2-pyrrolidinone, a similar ESR spectrum to that of radical II was observed. It has been ascribed to radical III on the same ground as discussed for radical II. The ESR parameters of the radical III at pH 8.83 are shown in Table I and they did not change within experimental errors up to H_- = 13.61 ([KOH] = 0.40 M) indicating that there is no dissociable group (such as an NH group) in this radical. A comparison of the ESR parameters of radical III with those of the acid form of radical II shows that a_α^H and a_β^H decrease while a_γ^H increases. It means that π-electron spin density flows apparently from an α-carbon atom to a carbonyl carbon atom upon the substitution of a methyl group for a peptide proton.

The ESR spectrum obtained from an irradiated aqueous neutral solution of 10 mM L-2-pyrrolidone-5-carboxylic acid (DL-pyroglutamic acid) is shown in Figure 2a. Since this ESR spectrum does not have hyperfine splitting in the range of α-proton coupling constant, it has been attributed to radical IV referring to the identification of radicals I to III.

With an increase in the basicity of the solution, the spectral lines became broader and moreover the specific line broadening effect and remarkable line shifts were observed in the ESR spectra of radical IV between pH 11 and 14. Especially around pH 13, all ESR lines except the outermost groups at both higher and lower fields could not be observed. The behavior of the ESR spectrum described above is similar to that observed in a linear peptide radical of *N*-acetylglycine[4] and it can also be fully understood in terms of an exchange of the NH proton with water molecules and the acid dissociation of the NH proton of radical IV. An ESR spectrum reproduced in Figure 2b shows a typical example of the specific line broadening effect observed at H_{2-} = 13.80 ([KOH] = 0.36 M). This broadening effect will be discussed later in some detail.

The ESR spectrum of the basic form of L-2-pyrrolidone-5-carboxylic acid radical obtained at H_{2-} = 14.97 ([KOH] = 2.06 M) is also shown in Figure 2c. Hyperfine coupling

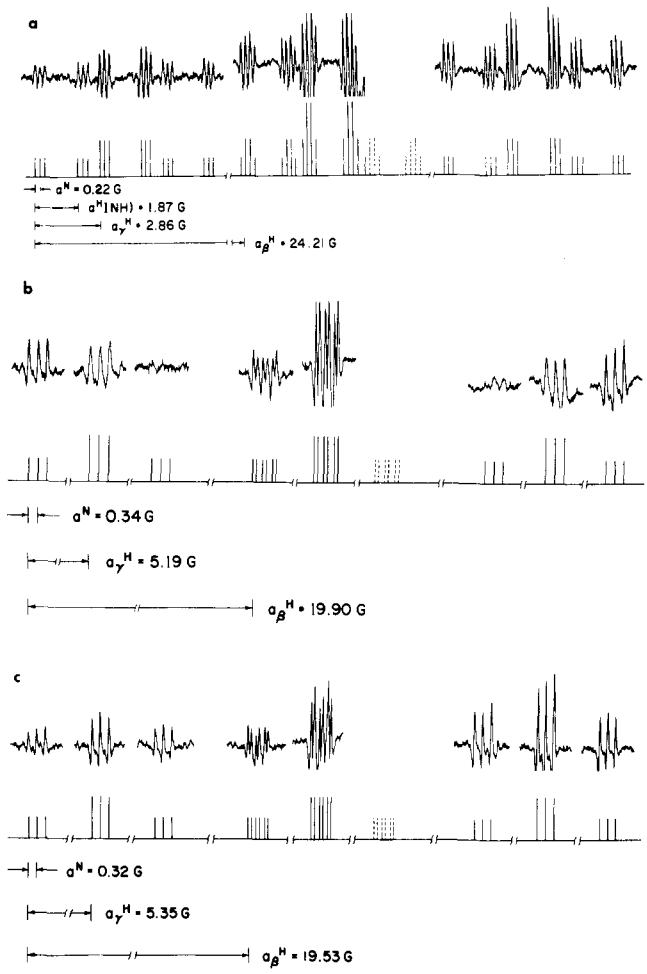


Figure 2. Second-derivative ESR spectra of the radical produced by the reaction of OH or O^- radicals with L-2-pyrrolidone-5-carboxylic acid (a) for the acid form at pH 7.20, (b) at H_{2-} = 13.80 ([KOH] = 0.36 M), and (c) for the basic form at H_{2-} = 14.97 ([KOH] = 2.06 M). ESR spectra are reproduced 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. Second-order splittings of about 0.2 G are also observed for the central groups. The splitting is expected to be 0.18 G in (a) from the theory of R. W. Fessenden, *J. Chem. Phys.*, **37**, 747 (1962). In (b) the lines in the second, third, seventh, and eighth triplets from the downfield end of the spectrum are specifically broadened by the acid-dissociation process of the NH proton of the radical.

constants of the γ-proton and nitrogen as well as the g value increase while the β-proton coupling constant decreases upon the NH proton dissociation as listed in Table I. Using the observed values of hyperfine coupling constants in acid and basic forms, pK_a values for the NH proton dissociation are estimated to be 12.71 ± 0.08 from a_γ^H and 12.78 ± 0.09 from a_β^H. As an ESR spectrum could not be observed actually around pH 12.7 (corresponding to the pK_a value), the calculation of the pK_a value was based upon the observed hyperfine coupling constants at H_{2-} = 13.56, 13.80, and 13.94.

Since the shift in line position differs for individual lines, the broadening effect due to the interchange between the acid and basic forms is different for each line. The calculated line widths for each center line of 9 nitrogen triplets (1:1:1) at H_{2-} = 13.80 are 0.13 (+2.00), 0.17 (+4.50), 0.22 (+6.99), 0.14 (−2.68), 0.12 (−0.19), 0.13 (+2.31), 0.23 (−7.36), 0.17 (−4.87), and 0.14 G (−2.37 G) from the downfield end. These values agree well qualitatively with the observed tendency in Figure 2b. Line shifts (in Gauss) are also indicated for each line in parentheses (+ for upfield shift upon the dissociation and − for downfield shift). In this calculation, both exchange and acid dissociation effects of the NH proton in radical IV are taken into account and the forward rate constant k_f for $-CONHC(COO^-)- + OH^- \rightarrow -CON^-\dot{C}(COO^-)- + H_2O$ is assumed to be 1×10^9 M^{-1} s^{-1}. The procedure for calculating line widths has been described in more detail previously.[4] The forward rate constant seems to be much less than that expected for diffusion-controlled reaction, though it should remain at $\sim 10^{10}$ M^{-1} s^{-1} judging from an estimated pK_a value, 12.7, and the negative pK_a difference, $\Delta pK_a = pK_a$ (radical IV) $- pK_a(H_2O)$ = 12.7–15.75 $\simeq -3$.[11,12] This strange situation is quite similar to the case of *N*-acetylglycine radical (estimated $k_f = 8 \times 10^8$ M^{-1} s^{-1} and pK_a = 14.0).[4] The negative charge on the carboxyl group adjacent to the radical center of both *N*-acetylglycine and L-2-pyrrolidone-5-carboxylic acid might retard the rate effectively here, while the reaction rates are close to being diffusion controlled for the radicals from glycine anhydride and alanine anhydride[6] with a lower pK_a value (9.6) and without a charged group.

The pK_a values for the NH proton dissociation in three pyrrolidinone radicals (I, II, and IV) are lower by 1.0 to 1.3 units than those in the structurally related linear peptide radicals from *N*-ethylacetamide, *N*-methylacetamide, and *N*-acetylglycine,[4] because the π-electron system in cyclic peptide radicals is somewhat more delocalized than that in linear ones. Upon substituting COO^- for CH_3 or H at the 5-position of pyrrolidinone radicals, the pK_a value decreases from 13.6 to 12.7 while the g value in the acid form increases from 2.002 68 to 2.003 47. Among the corresponding linear peptide radicals the same trend was observed; i.e., the pK_a value decreases from 14.6 to 14.0 while the g value in the acid form increases from 2.002 68 to 2.003 31.[4] Probably this trend means that the more a substituent becomes electron withdrawing, the more π-electron spin delocalizes (to a carbonyl oxygen atom) to induce the increase of g value and also to lead to somewhat decreased total π electron density on the peptide nitrogen atom, which decreases the pK_a value for NH proton dissociation.

It might be interesting to point out that the γ-proton coupling constants of radicals I to IV in acid form (2.86 to 4.26 G) are larger than that of cyclopentyl radical (0.50 G)[13] and the β-proton coupling constants of I to IV (24.21 to 28.62 G) are smaller than that of cyclopentyl radical (35.11 G).[13] The larger γ- and smaller β-proton coupling constants clearly indicate a greater ease of the delocalization of spin density propagation through the peptide bond than through a saturated carbon chain.

Radicals from Hydantoin, 1-Methylhydantoin, and 2-Thiohydantoin. ESR spectra observed from irradiated hydantoin solution around neutral pH have shown the presence of two radical species. Both ESR spectra consist of two kinds of nitrogen triplet (1:1:1) and a proton doublet (1:1). Hyperfine coupling constants are different for the two radicals. Moreover, one spectrum has a small proton doublet (1:1) and the other does not have this splitting. The ratio between these two species changes with pH. Consequently these ESR spectra are ascribed to the superposition of the spectra of the acid and basic forms of hydantoin radical. As predicted by Laroff and Fessenden,[12] the pK_a value of the dissociation is less than 10 for such a slow dissociation process. From the ratios of both forms as a function of pH, the pK_a value for the NH proton dissociation is determined directly to be 7.63 ± 0.10. The NH proton with this pK_a value is assigned to that adjacent to an α-carbon atom judging from the general tendency of changes in ESR parameters upon the dissociation, which will be discussed later in this text. The structures of the hydantoin radical in acid and basic forms are thus considered to be V and V′, respectively. The two nitrogen hyperfine constants of 1.08 G in V and 1.33 G in V′ might be ascribed to a nitrogen atom adjacent to an α-carbon atom because a_N of 1.99 G in V′ is ascribed to another nitrogen as described below and a_N seems to increase

from the general tendency observed upon dissociation of an NH proton adjacent to an α-carbon atom.

With the increase in basicity up to H_{2-} = 15.52 ([KOH] = 3.60 M), the ESR spectrum of radical V′ with one dissociated proton still showed a change especially in a_N and g values. This change can be explained in terms of the second acid dissociation of the NH proton located across a C=O group from an α-carbon atom. From the observed value of nitrogen hyperfine coupling constant a_N (1.99 G) in this NH group at pH 11.58 and estimated a_N value (1.40 G) in the basic form with two dissociated NH protons, the pK_a value for the second NH proton dissociation is determined to be 13.45 ± 0.21. The second pK_a value is higher by 5.9 units than the first pK_a value. The difference between pK_a values for two NH protons is comparable to that for two OH protons of $C_6H_5\dot{C}(OH)_2$ radical (pK_a values are 5.3 and 12.0)[14] and of p-$HN^+C_5H_4\dot{C}(OH)_2$ radical (−0.17 and 6.3).[15]

The superposition of two radical species was also observed from irradiated aqueous solutions of 1-methylhydantoin in weakly alkaline region. Both radical species have closely similar ESR patterns consisting of two inequivalent nitrogens, three methyl protons, and one α proton, suggesting they can be attributed to undissociated (VI) and dissociated forms with respect to an NH proton. The nitrogen coupling constants of 1.56 G in the acid form and 1.10 G in the basic form should come from a nitrogen atom of the NH group because of its considerable change on the NH proton dissociation. Those of 1.70 G in acid and 1.66 G in basic forms are due to the nitrogen atom in the N-CH_3 group and the slight decrease in a^N upon the dissociation is exactly consistent with that in a^H (CH_3) from 3.40 to 3.32 G.

From the ratios of the two radical species, the pK_a value for this dissociation is estimated to be 8.52 ± 0.18. Upon the dissociation the g value and a^N in the NH group decrease and $a_\alpha{}^H$ does not change remarkably. This is the same situation as observed for the second dissociation of an NH proton in hydantoin radical. However, the pK_a value of 1-methylhydantoin radical is lower by 5.0 units than that for the corresponding dissociation of hydantoin radical because the second dissociation in the latter radical is impeded by the first dissociation.

From irradiated aqueous solutions of 2-thiohydantoin ESR spectra could be observed only above pH 12.70. Comparatively large g values were also observed due to the existence of a conjugated π-electron system including a sulfur atom. Significant changes in one nitrogen coupling constant and the g value occurred in these basicity regions. As compared with a structurally similar radical from hydantoin, the change of a^N mentioned above is considered to result from the second dissociation of the NH proton located across the C=O group from the α-carbon atom in radical VII. The value of a^N in the basic form was obtained from an ESR spectrum at H_{2-} = 15.21 ([KOH] = 2.76 M). The value of a^N in the acid form can be calculated using eq 1 and it is estimated to be 2.03 G. The pK_a value for the second NH proton dissociation is determined to be 13.15 ± 0.11, which is similar to that obtained from hydantoin radical V′ as expected from the structural similarity. The reason why an ESR spectrum of 2-thiohydantoin radical cannot be observed below pH 12.70 is still unclear. Since an ESR spectrum of hydantoin radical was observed near the neutral region, the existence of a sulfur atom might induce some change in the reaction rate of the NH proton exchange.

Radicals from Succinimide and 2-Oxazolidone. From an electron-irradiated neutral solution of succinimide, the cyclic peptide radical VIII is produced by hydrogen abstraction from a methylene group by OH radical. The ESR parameters in acid form agree well with those reported by Livingston and Zeldes (a^N = 0.40 G, a^H(NH) = 1.43 G, $a_\alpha{}^H$ = 20.58 G, $a_\beta{}^H$ = 29.57 G, and g = 2.003 39).[16] The ESR spectrum observed above pH 10 has been assigned to the basic form of succinimide radical. Between pH 9 to 9.5 the ESR spectra of the acid and basic forms coexist as shown in Figure 3 because the dynamics of the dissociation process is slow. In Figure 3 a higher field portion of the ESR spectrum of succinimide radical at pH 9.00 is shown. The pK_a value for the NH proton dissociation has been determined to be 9.45 ± 0.10 by the direct measurement of the relative intensity ratio of acid and basic forms. This pK_a value is in the same range as that for the parent molecule (9.5).[17,18]

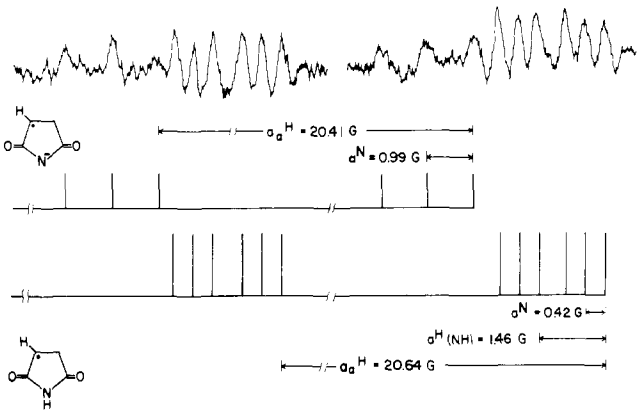
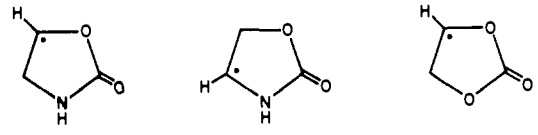


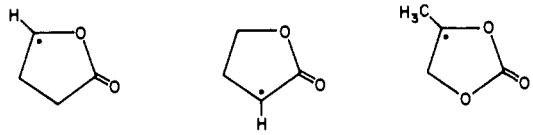
Figure 3. A higher field portion of the ESR spectrum of a succinimide radical obtained at pH 9.00. The observed spectrum is the superposition of the discrete spectrum of acid and basic forms.

The ESR spectrum observed from a neutral 2-oxazolidone solution showed the existence of only one radical species and has been analyzed to have the ESR parameters of $a_\alpha{}^H$ = 13.79 G, $a_\beta{}^H$ = 35.02 G, a^H(NH) = 1.55 G, and g = 2.002 91. The radical could be IX or IX′. Very recently, Zeldes and Livingston[19] reported a structurally similar radical IX″ derived from ethylene carbonate. The ESR parameters of radical IX″ were assigned to be $a_\alpha{}^H$ = 14.17 G, $a_\beta{}^H$ = 35.30 G, and g = 2.003 02. The ESR parameters of 2-oxazolidone radical resemble closely those of radical IX″ and are not affected much by the substitution of an NH group for an oxygen atom. Moreover they differ significantly from those of radical II, reflecting more nonplanarity around the α-carbon atom due to the bonding with an adjacent oxygen atom.[10,20] Consequently structure IX seems to be favorable.



The spectral lines were too broadened to be observed around pH 11. However, addition of 0.3 M phosphate buffer increased the rate of interchange between the acid and basic forms[12,21] resulting in a narrow line spectrum which is a weighted average of the two forms. Above pH 11.5 the lines were narrow without phosphate buffer. The pH dependence of the spectrum thus obtained over the range of pH 6 to H_- = 13.46 ([KOH] = 0.30 M) gave the pK_a value of 10.90 ± 0.15 for the NH proton dissociation of radical IX. The hyperfine coupling constant of nitrogen did not change markedly upon the dissociation, supporting also the assignment to radical structure IX in preference to IX′. The remarkable increase in α-proton coupling constant with an almost unchanged β-proton coupling constant might be ascribed to the introduction of less nonplanarity and subsequent decrease in s character of the unpaired-electron orbital of the α-carbon atom upon the dissociation.

In order to obtain ESR parameters for reference to 2-oxazolidone radical, ESR spectra were observed while irradiating aqueous solutions of γ-butyrolactone. A triplet (1:2:1)-doublet (1:1)-triplet (1:2:1) pattern was observed. However, the ESR parameters depended upon the pH value: a_β^H = 32.03 G, a_α^H = 15.92 G, a_γ^H = 0.37 G, and g = 2.002 93 for pH 6.22 and 7.12, while a_β^H = 19.22 G, a_α^H = 11.47 G, a_γ^H = 0.70 G, and g = 2.003 44 for pH 12.70 and H_- = 13.72 ([KOH] = 0.48 M). The former can be attributed to structure X rather than



(X) (X') (X")

X′, because of its relatively low g value. Zeldes and Livingston[19] reported a similar radical X″ from propylene carbonate. The ESR parameters of radical X″ are a_β^H = 32.07 G, $a^H(CH_3)$ = 21.20 G, and g = 2.002 96, which are in good agreement with those of radical X. The radical from γ-butyrolactone observed in basic solution is probably $^-O\dot{C}HCH_2CH_2COO^-$ resulting from the alkaline hydrolysis of the parent compound and subsequent reaction with O^- radical. ESR parameters of this radical are comparable to those of $CH_3\dot{C}HO^-$ radical[12] ($a^H(CH_3)$ = 20.16 G, a_α^H = 11.11 G, and g = 2.003 59).

The α-proton coupling constant of radical X is larger than that of radical IX or IX″, while the β-proton coupling constant of X is smaller than that of IX or IX″. This might be explained by less rigid nonplanar structure for the ring atoms of radical X than that for IX or IX″.

Radical from 2,5-Piperazinedione (Glycine Anhydride). A somewhat complex ESR spectrum was obtained during the electron irradiation of an aqueous neutral solution of 10 mM 2,5-piperazinedione. It consists apparently of two nitrogen hyperfine splittings (coupling constant, 0.51 and 1.53 G), three proton hyperfine splittings (2.26, 2.48, and 17.16 G), and two equivalent proton splittings (8.58 G). This ESR spectrum is attributed to the radical XI formed by hydrogen abstraction from a methylene group by OH radical. Of the expected 216 lines for the acid form of this radical, about 130 lines could be detected and separated. Comparing the ESR parameters with those of a corresponding linear peptide radical from glycylglycine,[4] it is notable that a_δ^H increases from 3.30 to 8.58 G to reveal more delocalization of π-electron spin density in the cyclic peptide radical. The ESR spectrum observed above pH 10.8 lacked one proton hyperfine splitting compared with that of the acid form and can be ascribed to the basic form of radical XI with one proton dissociated from the NH group adjacent to the α-carbon atom. Between pH 9.2 and 9.6, the superimposed ESR spectra of the two forms were observed. The pK_a value for NH proton dissociation has been determined to be 9.62 ± 0.04 by the direct measurement of the relative intensity ratio of the two forms. Assignments of radical XI and its pK_a value are in agreement with those obtained in a previous work using optical pulse radiolysis.[6] The ESR spectra of 2,5-piperazinedione could not be observed above pH 12 probably because of hydrolysis of the substrate. Consequently the pK_a value for the second dissociation of an NH proton could not be determined.

2-Oxohexamethylenimine (ε-caprolactam) and 2-piperidone (δ-valerolactam) gave only poorly resolved ESR spectra of the intermediate radicals formed in the reaction with hydroxyl radical.

Discussion of the Changes in ESR Parameters upon Dissociation of the NH Proton. In cyclic peptide radicals, dissociation of the NH proton occurs from two different sites relative to the α-carbon atom: >Ċ-NH-CO- and >Ċ-CO-NH-.[22]

As the skeletal structure of radicals in the first group (>Ċ-NH-CO-) is the same as that of linear peptide radicals,[4] similar changes in ESR parameters have been expected and, in fact, observed upon the NH proton dissociation. Actually, in this group, g values and hyperfine coupling constants of protons beyond the peptide bond from the α-carbon atom increase while α-proton and β-proton coupling constants decrease upon the dissociation of the NH proton. (Nitrogen hyperfine coupling constants also increase to some extent upon the dissociation. This is contrary to the decreasing tendency in the cases of linear peptide radicals.[4]) This fact suggests much more spin density on the carbon atom of the carbonyl group adjacent to the nitrogen of concern in the dissociated form. Spin density which increases in the peptide group should come from the α-carbon atom. However, the remarkable decrease in the α-proton coupling constant observed in linear peptide radicals[4] was not generally observed here upon the dissociation of NH proton.

For example, in radicals I, II, and IV from three pyrrolidinones, the hyperfine coupling constant of γ protons increases by 2.5 to 3.4 G, that of nitrogen increases slightly, and g values increase also by 0.000 07 to 0.000 17 upon the NH proton dissociation. Moreover, β-proton coupling constant decreases by 3.5 to 4.7 G, which means the decrease of spin density on the α-carbon atom $\rho_{\alpha\text{-c}}$. According to the ESR data of five-membered alicyclic free radicals,[10] $\rho_{\alpha\text{-c}}$ could be estimated from β-proton coupling constant a_β^H and eq 4, assuming the planarity of radical structure and the angle of 30° between the unpaired electron orbital of the α-carbon atom and the C_β–H bond ($\cos^2 30° = 3/4$).

$$\rho_{\alpha\text{-c}} = a_\beta^H/(B_0 + 3B/4) \quad (4)$$

Parameters B_0 and B are assigned as 3.0 and 46.7 G, respectively, so that $\rho_{\alpha\text{-c}}$ obtained for an acid form of radical I is equal to that obtained from the value of $a^H(CH_3)$ and the well-known relation,[23] $\rho_{\alpha\text{-c}} = a^H(CH_3)/29.3$. These parameters are in the reasonable range obtained previously.[10] The decrease of $\rho_{\alpha\text{-c}}$ upon the NH proton dissociation is thus estimated as follows: from 0.692 to 0.601 for radical I, 0.753 to 0.652 for II, and 0.637 to 0.514 for IV. Applying eq 4 with observed a_γ^H value, the increase of the spin density on a peptide carbon atom might be estimated as follows: 0.095 to 0.184 for I, 0.103 to 0.192 for II, and 0.075 to 0.141 for IV. Apparently 0.07 to 0.09 of spin density flows from an α-carbon atom to a carbon atom in a peptide group. However, the α-proton coupling constant which might be proportional to spin density on the α-carbon atom does not decrease so remarkably in radical II. If the decrease in β-proton coupling constant of these radicals is mainly based on the decrease in the π electron spin density on the α-carbon atom, then σ character might be taken into account to explain the almost unchanged value of the α proton coupling constant of radical II. It is probable that after the dissociation of the NH proton, a somewhat nonplanar configuration of bonding orbitals around the α-carbon atom, bending of the ring, and a consequent s character of the orbital containing the unpaired electron are introduced. However, these effects seem to be small judging from the observed equivalence of the two β- and γ-proton coupling constants of radical II in the basic form.

In the second group with a structure >Ċ-CO-NH-, g values and nitrogen hyperfine coupling constant (except in the case of succinimide radical VIII) decrease upon the NH proton dissociation. There are no marked changes in α-proton coupling constant. These features differ from those of the first group suggesting that in the second group there is no drastic change in π-electron spin distribution but a local flow of π electrons, such as the flow from peptide nitrogen and/or oxygen atom to the peptide carbon atom, occurs upon the NH proton dissociation. The spin density on the α-carbon atom does

not seem to be much influenced by the dissociation of a proton in the NH group rather distant from the α-carbon atom itself.

Surprisingly, the β-proton coupling constant in radical VIII from succinimide decreases on dissociation of the NH proton suggesting that the spin density on the α-carbon atom decreases from 0.775 to 0.702 using eq 4. However, the observed α-proton coupling constant does not decrease much. In order to explain this discrepancy, nonplanarity around the α-carbon atom and subsequent σ character in the unpaired electron orbital might be introduced again after the NH proton dissociation.

Conclusion

Using the in situ radiolysis–steady state ESR method, the pK_a values for the peptide proton dissociation have been determined to be 7.6 to 13.6 for eight cyclic peptide and one related cyclic radicals. The more delocalized the π-electron system was, the lower the pK_a value became. Significant changes in the ESR parameters of cyclic peptide radicals upon the dissociation were discussed and nonplanarity of the radical structure was introduced to explain the α-proton coupling constant unexpected from simple π-electron spin distribution. To estimate the line-broadening effect in ESR spectra of 2-pyrrolidone-5-carboxylic acid radical, a forward rate constant of 1×10^9 M^{-1} s^{-1} was necessary for $-CONH\dot{C}(COO^-)- + OH^- \rightarrow -CON^-\dot{C}(COO^-)- + H_2O$. The deviation of the rate from the value expected under diffusion control might come from the negative charge on the carboxyl group adjacent to the radical center. Structurally related cyclic radicals from 1-methyl-2-pyrrolidinone and γ-butyrolactone were identified and discussed.

Acknowledgment. The authors wish to thank Drs. R. H. Schuler, R. W. Fessenden, and P. Neta for critical reading of this manuscript.

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Peptide Strain. Conformation Dependence of the Carbon-13 Nuclear Magnetic Resonance Chemical Shifts in the Ferrichromes[1a]

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Abstract: The flexible, nonrandom conformation of the metal-free ferrichromes in $(CD_3)_2SO$ is reflected in their carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra. The resonances are readily assigned by reference to literature data, previous studies in D_2O, and the comparative spectra of various serine- and alanine-containing analogues. Single-residue substitutions cause resonance shifts in the carbonyl spectral region as a consequence of concomitant conformational drifts. On metal binding the carbonyl and aliphatic ^{13}C resonances spread over wider chemical shift ranges, consistent with a tight chelate structure. Comparison of the spectra of the Al^{3+} analogues of ferrichrome (alumichrome), ferrichrome C (alumichrome C), sake colorant A (alumisake), ferricrocin (alumicrocin), ferrichrysin (alumichrysin), and ferrichrome A (alumichrome A) enables assignment of the peptide aliphatic resonances and identification of those arising from the side chains. The unusual chemical shift span exhibited by the ^{13}C NMR spectra of the alumichromes cannot be accounted for on the basis of theories based solely on electric field and anisotropy effects. Given the strained conformation revealed by the crystallographic model, proved valid for solution conditions by ^1H- and ^{15}N-NMR studies, it is proposed that the aliphatic chemical shifts are likely to be highly dependent on distorted bond geometries causing local orbital rehybridizations which deviate the carbon atoms from a pure sp^3 valence configuration. On going from one analogue to the other the carbonyl resonances shift in good agreement with literature data on model peptides which show the effect of varying the residue at position i on the chemical shifts of carbonyls at locations $i - 1$ and $i + 1$. These observations and the isomorphic nature of the alumichrome suite of analogues lead to a unique solution of the difficult problem of assigning the peptide carbonyl ^{13}C resonances.

The sensitivity of carbon-13 nuclear magnetic resonance (^{13}C NMR)[2] spectroscopy to polypeptide conformations has been amply demonstrated.[3] For ferrichrome and its serine-containing analogues ferricrocin and ferrichrysin (Figure 1) we have shown in a previous report[4] that the conformational change induced by metal binding is revealed dramatically by the aliphatic and carbonyl resonances. These experiments, performed on aqueous solutions at 15 MHz, were consistent