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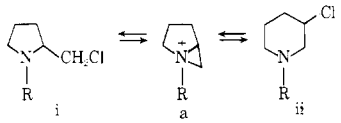

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Electron Spin Resonance Study of Liquids during Photolysis. XIX. Aliphatic Dipeptides[1,2]

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Abstract: Aqueous solutions of simple dipeptides containing about 1% hydrogen peroxide have been photolyzed at room temperature and the resulting radicals studied by electron spin resonance. The peroxide gives ȮH which abstracts hydrogen from the peptide. Abstraction of a hydrogen from the carbon adjacent to the peptide nitrogen to give a single radical occurs in the following cases: glycylglycine, β-alanylglycine, L-alanylglycine, β-alanyl-L-alanine, and glycyl-L-alanine. A mixture of radicals is obtained from glycyl-β-alanine and β-alanyl-β-alanine formed by hydrogen abstraction from each of the CH_2 groups at the carboxylate end of the dipeptide. Hyperfine couplings and g values have been measured and are discussed.

We have started a systematic study by electron spin resonance of short-lived radicals at room temperature formed by photolyzing aqueous solutions containing simple peptides and a small amount, ca. 1%, of hydrogen peroxide. Upon photolysis the peroxide gives ȮH which then attacks the peptide by abstracting a hydrogen to give the observed radical. Attack by ȮH is of basic importance in radiation biology. Its formation from H_2O_2 has frequently been used in our photolytic studies, and, with few exceptions, its action has been the abstraction of hydrogen from the substrate. The seven dipeptides studied here contain the simplest aliphatic amino acids: glycine, L-alanine, and β-alanine. All of the spectra exhibit abundant hyperfine splittings leading in most cases to a detailed analysis with identification of the radicals. The nature of the ȮH attack is similar in all of the cases presented, a feature, however, that should not be generalized to more complex dipeptides. The peptides were synthesized by one of the authors (D.G.D.) and thus far have been primarily studied in aqueous solution at or near the pH of the isoelectric point; they were present as zwitterions.

Experimental Section

The spectrometer operated at a nominal frequency of 9.5 GHz and used 100-kHz field modulation. Magnetic field strength was measured by proton magnetic resonance, and this resonance frequency and the microwave frequency were measured with a frequency counter. Further details of the sample handling operations, the equipment, and method of measuring hyperfine couplings and g values are described in earlier papers of this series.[2] Estimated error limits of hyperfine couplings and g values are ±0.03 G and ±0.00004, respectively, unless otherwise stated. The solutions, typically a few grams of the dipeptide in 25 ml of water, contained about 1% H_2O_2 (added as 98% H_2O_2). Adjustments in pH were

Table I. Physical Constants of Peptides and Derivatives

	Carbobenzoxy peptide methyl ester		Carbobenzoxy peptide		Peptide	
	Mp, °C[a]	$[\alpha]^{22}D$, deg	Mp, °C	$[\alpha]^{22}D$, deg	Mp, °C	$[\alpha]^{22}D$, deg
β-Ala-Gly	100–102[b]		148–150[c]		229–230[d]	
Gly-β-Ala	93–94[b]		140–141[e]		229–230[f]	
β-Ala-L-Ala	83–85[b]	−27.0 (*c* 2, EtOH)	132–133[b]	−12.0 (*c* 2, EtOH)	237–238[b]	−55.8 (*c* 2, 0.5 *N* HCl)
β-Ala-β-Ala	108–110[b]		144–146[g]		212[h]	
Gly-L-Ala	93–94.5[i]	−32.0 (*c* 2, EtOH), −8.2 (*c* 2, EtOAc)	138[j]	−10.1 (*c* 2, EtOH)	230–231[k]	−59.0 (*c* 2, 0.5 *N* HCl)
L-Ala-Gly	100–102[l]	−23.5 (*c* 5, MeOH)	132–133[m]	−17.0 (*c* 2, EtOH)	230–231[n]	+24.0 (*c* 1, 0.5 *N* HCl), +50.0 (*c* 2, H_2O)

[a] All melting points were taken in capillary tubes, uncorrected. [b] New compound. [c] H. T. Henson and E. L. Smith, *J. Biol. Chem.*, 175, 833–848 (1948), report mp 146–149°. [d] Footnote *c*, mp 226°. [e] Footnote *c*, mp 140°. [f] Footnote *c*, mp 228°. [g] Footnote *c*, mp 144–145°. [h] Footnote *c*, mp 212°. [i] J. H. Jones, B. Liberek, and G. T. Young, *J. Chem. Soc.*, 2371 (1967), report mp 62–64°, $[\alpha]^{20}D$ −8.1° (*c* 1, EtOH). [j] H. Goldschmidt and K. K. Gupta, *Chem. Ber.*, 98, 2831 (1965), report mp 130–131°, $[\alpha]^{24}D$ −9.1° (*c* 2, EtOH). [k] B. F. Erlanger and E. Brand, *J. Am. Chem. Soc.*, 73, 3508 (1951), report $[\alpha]^{24}D$ −59.3° (*c* 2, 0.5 *N* HCl). [l] L. Zervas, D. Borovas, and E. Gazis, *J. Am. Chem. Soc.*, 85, 3660 (1963), report mp 98–99°, $[\alpha]^{18}D$ −25.1° (*c* 5, MeOH). [m] F. C. Stewart, *Aust. J. Chem.*, 21, 2543 (1968), report mp 134–135°, $[\alpha]^{24}D$ −17° (*c* 2, EtOH). [n] Footnote *k*, $[\alpha]^{24}D$ +22.6° (*c* 2, 0.5 *N* HCl); F. H. C. Stewart, *Aust. J. Chem.*, 19, 1067 (1966), report mp 230–231.5°.

made with either HCl or most often LiOH. (LiOH was usually used in order to facilitate subsequent recovery and purification of the peptides.) All solutions were freed of dissolved oxygen by purging with helium and were photolyzed near room temperature as they slowly flowed (≃1 ml/min) through a flat silica cell positioned in the microwave cavity of the spectrometer. The uv source was a high-pressure mercury arc, Philips Type SP 500 W. The solutions were recirculated with a peristaltic pump and only contacted glass, silica, and Teflon. Rarely did spurious signals develop from prolonged photolysis; however, the spectra usually became weaker after a few hours of operation.

The peptides were prepared by coupling the carbobenzoxyamino acid with the amino acid methyl ester in methylene chloride or chloroform using either cyclohexylcarbodiimide or carbonyldiimidazole as carboxyl-activating agents. After conventional work-up the methyl esters were hydrolyzed at room temperature with an equivalent of base and acidified and the carbobenzoxy group was removed by hydrogenation with Pd black catalyst. The free peptides were recrystallized from aqueous ethanol. The process is illustrated by the synthesis of the previously unreported β-alanyl-L-alanine. The physical properties of the other peptides are shown in Table I.

***N*-Carbobenzoxy-β-alanyl-L-alanine Methyl Ester.** Triethylamine (14 ml) was added with stirring to an ice-cold mixture of 14 g of L-alanine methyl ester hydrochloride in 150 ml of $CHCl_3$ followed by 22.4 g of *N*-carbobenzoxy-β-alanine.[3] Cyclohexylcarbodiimide (21.6 g in 50 ml of $CHCl_3$) was added to the resultant solution and the mixture stirred 2 hr at 0° and 5 hr at room temperature. After the addition of 1 ml of acetic acid and filtration of the dicyclohexylurea, the solution was washed successively with cold dilute HCl, saturated bicarbonate, and water, dried briefly over Na_2SO_4, and evaporated in vacuo at 40° to dryness. The solid was taken up in 75 ml of hot ethyl acetate, a small amount of dicyclohexylurea filtered off, petroleum ether (bp 30–60°) added to incipient crystallization, and the product filtered after remaining overnight at 5°. The yield was 26.3 g (85%) having mp 81–83° and $[\alpha]^{22}D$ −26.5° (*c* 2, EtOH). Recrystallization from the same solvents raised the melting point to 83–85° and the $[\alpha]^{22}D$ to −27.0°.

Anal. Calcd for $C_{15}H_{20}ON_2O_5$ (308.33): C, 58.43; H, 6.54; N, 9.09. Found: C, 58.53; H, 6.57; N, 9.15.

Alternatively, 3.4 g of carbonyldiimidazole was added to a stirred mixture of 4.5 g of carbobenzoxy-β-alanine in 50 ml of CH_2Cl_2 followed after 0.75 hr by 2.8 g of L-alanine methyl ester hydrochloride. After 18 hr the clear solution was worked up in the above fashion, and 5.2 g (84%) of product was obtained with mp 83–85° and $[\alpha]^{22}D$ −26.5° (*c* 2, EtOH).

Carbobenzoxy-β-alanyl-L-alanine. The above ester (3.1 g) was dissolved in 20 ml of MeOH, 10 ml of 1 *N* NaOH added, and after 45 min the solution acidified with 11 ml of 1 *N* HCl. Concentration in vacuo to 0.5 vol and crystallization at 5° gave 2.6 g (88%) of product having mp 129–130° and $[\alpha]^{22}D$ −12.0° (*c* 2, EtOH).

Anal. Calcd for $C_{14}H_{18}N_2O_5$ (294.30): C, 57.13; H, 6.17; N, 9.52. Found: C, 56.85; H, 6.06; N, 9.25.

β-Alanyl-L-alanine. Hydrogenation of 6 g of the carbobenzoxy dipeptide in 75 ml of EtOH–25 ml of H_2O plus 1.5 ml of HOAc with Pd black followed by neutralization to pH 6.8, evaporation in vacuo, and crystallization from 60 ml of 80% ethanol yielded 3 g of the pure dipeptide having mp 237–238°. When dried over $CaCl_2$ in vacuo the peptide contained 0.5 mol of water which could be removed by drying at 120° in vacuo.

Anal. Calcd for $C_6H_{12}N_2O_3$ (160.18): C, 44.99; H, 7.55; N, 17.49. Found: C, 45.10; H, 7.75; N, 17.58.

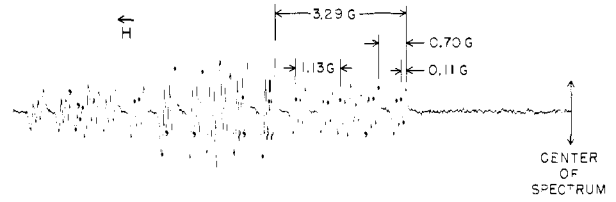

Figure 1. The higher field group of lines from 5 g of glycylglycine in 40 ml of water containing 1.8% H_2O_2. A similar group of lines occurs at 17.5 G lower field.

Results and Discussion

Glycylglycine. The spectrum from glycylglycine contains two similarly appearing groups of lines that are spaced by 17.5 G. The higher field group obtained from a solution of 5 g of the peptide in 40 ml of water and containing 1.8% H_2O_2 is shown in Figure 1. The lower field group was of identical appearance except that the intensity was noticeably weaker. This effect is due to a deviation from thermal equilibrium in the populations of the spin states and is very frequently observed with short-lived radicals.[4] It is usually the lower field lines that are weaker. The multiplicity of lines arises from five hyperfine couplings: both nitrogens, a pair of equivalent hydrogens, a weakly coupled hydrogen, and a strongly coupled hydrogen. From the nature of the couplings it is clear that a hydrogen is abstracted from one of the CH_2 groups of the peptide, but there is ambiguity as to which one. The assignment has been made by comparison with other dipeptides of this series and is given along with the *g* value and measured couplings in Figure 2.

Glycylglycine was also studied in D_2O solution. On the time scale of the experiment (many minutes to hours) the hydrogen on the peptide nitrogen is exchangeable, and it was confirmed that the coupling of 1.13 G (Figure 2) is properly assigned. The hydrogens on the other nitrogen also probably exchange, but resolved splittings are not seen for those hydrogens. There are frequently small shifts in values of couplings in going to D_2O as a solvent. The value of the α coupling constant increased from 17.53 (Figure 2) to 17.58 G. The *g* value and $a_{CH_2}{}^{H}$ were unchanged. The peptide nitrogen coupling increased from 0.70 to 0.75 G. The sum of the remaining two constants was directly measurable, but

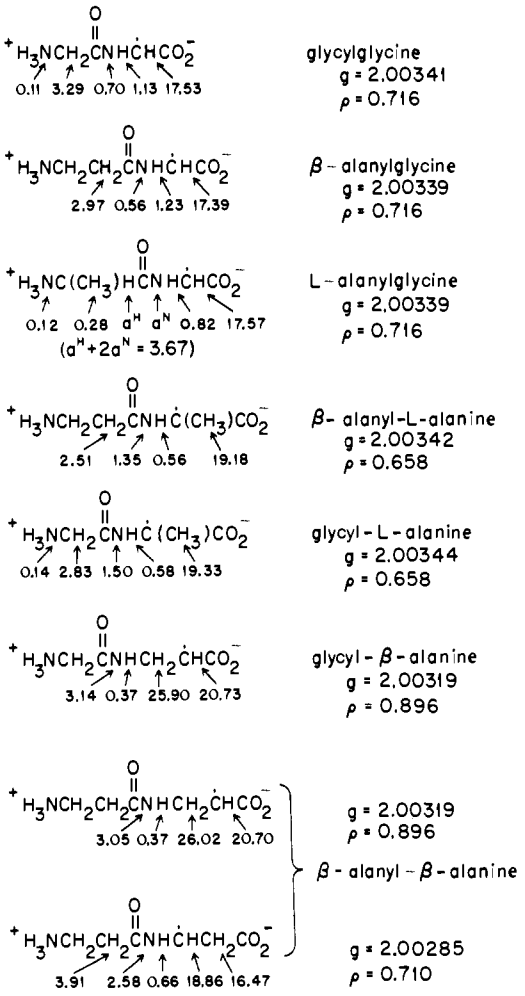

Figure 2. Formulas of radicals studied in photolyzed aqueous solutions containing the indicated dipeptide and hydrogen peroxide. Numbers below the formulas are hyperfine coupling constants in gauss for the indicated nuclei. g values and spin densities (described in the text) are also listed.

not the individual values because of lack of resolution. A computer simulation of the spectrum was made, and an excellent fit was obtained by leaving the smaller nitrogen coupling unchanged and by taking the value of 0.186 G for the deuteron constant. This value translates to 1.21 G in terms of the proton to be compared with 1.13 G (Figure 2) obtained in H_2O.

A somewhat similar radical, $CH_3CONHCHCO_2^-$, has been reported[5,6] from acetylglycine. Parameters reported[6] for a_α^H, $a_{NH}{}^H$, and a^N are 17.33, 1.32, and 0.51 G, respectively, which are similar to the corresponding couplings for the radical for glycylglycine (Figure 2). Garrison[7] and coworkers have studied the chemical products resulting from γ-radiolysis of aqueous solutions of simple peptides. Their products are accounted for by an initial reaction in which ȮH forms a radical from the peptide by abstraction of hydrogen from the carbon adjacent to the peptide nitrogen, the same radical reaction reported here.

Seeing only one radical was of especial interest. First, it meant that the reaction of ȮH was highly specific for one place in the molecule. This feature is repeated for all of the peptides reported here except those containing β-alanine at the carboxylate end of the dipeptide. Second, the peptide bond is a partial double bond which leads to cis and trans isomers.[8] The trans isomer is the more stable one for the peptides reported here. Only one isomer is observed for the free radical, and this is likely the same isomeric form as the

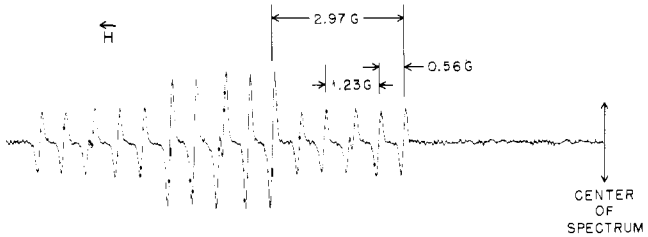

Figure 3. The higher field group of lines from 2 g of β-alanylglycine in 25 ml of water containing 1% H_2O_2 (pH 6.5). A similar group of lines occurs at 17.4 G lower field.

parent peptide. The carbon atom of glycylglycine which lost a hydrogen is expected to form sp^2 orbitals which are occupied by the bonding electrons leaving the unpaired electron in a π p orbital. The radical should be essentially planar about this carbon. Because of double bond character the peptide unit, -CCONHC-, has considerable rigidity and is also planar.[8] All of these atoms along with those of the carboxylate group probably lie in the same plane with the unpaired electron conjugating through much of the molecule. This places all atoms of the radical except the terminal NH_3 and the hydrogens on the adjacent CH_2 in essentially the same plane. The great degree of conjugation that results is consistent with seeing resolved hyperfine couplings from most of the magnetic nuclei throughout the radical.

Radicals could not be observed from highly acidic solutions where the hydrochloride was used instead of the isoelectric peptide.

β-Alanylglycine. Part of the spectrum obtained from 2 g of β-alanylglycine in 25 ml of water and containing 1% H_2O_2 (pH 6.5) is shown in Figure 3. A similar group of lines occurs at 17.4 G lower field. The only reasonable choice for the radical along with measured parameters is given in Figure 2. If the hydrogen had been abstracted from one of the CH_2 groups of the β-alanine residue there would have been a very strong β coupling constant from a pair of equivalent hydrogens instead of the rather small value from the pair of hydrogens. Resolved couplings are not observed for atoms beyond the indicated CH_2 of the β-alanine residue; the terminal nitrogen and the adjacent CH_2 do not give noticeable splittings. For this reason spectra from radicals with β-alanine at the amine end of the peptide tend to be simpler than those with glycine or L-alanine.

A similar experiment was carried out with a solution of the above composition but with the pH adjusted to 8.5. All of the parameters (Figure 2) remained unchanged, and the line width did not appear to change, but the intensity of the spectrum was only about one-half that observed at a pH of 6.5.

L-Alanylglycine. Aqueous solutions of L-alanylglycine had a pH of 5.7, and this was adjusted upward slightly, to 6.3, in the experiments described here. Initially it was felt that this gave some improvement in line width, but the effect may have been instrumental. Eventually we intend to make a detailed study of the pH dependence of some of the spectra.

The spectrum of Figure 4 was obtained with 3 g of L-alanylglycine in 25 ml of water containing 1% H_2O_2 (pH adjusted to 6.3). The top portion of Figure 4 is the entire spectrum at low resolution from which the coupling constant for a strongly coupled single hydrogen (17.57 G) and the g value can be obtained. The only reasonable choice for the identity of the radical is given in Figure 2. Note that the α coupling constant of 17.57 G matches quite closely the value for glycylglycine and for β-alanylglycine. This coupling constant increases to 17.64 G in D_2O solution, an increase very similar to that observed for glycylglycine.

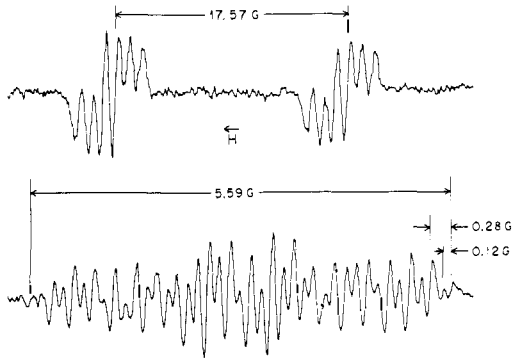

Figure 4. The spectrum from 3 g of L-alanylglycine in 25 ml of water containing 1% H_2O_2 and with the pH adjusted to 6.3. Top: the entire spectrum at low resolution. Bottom: the higher field group of lines at high resolution. The lower field group has a similar appearance.

Not all of the remaining coupling constants of L-alanylglycine could be evaluated because of lack of resolution. The bottom part of Figure 4 shows the appearance of each group of lines (the higher field group is illustrated) under high resolution. There is a great deal of overlap of lines; only 41 lines with varying degrees of resolution can be made out whereas 144 components are expected. However, the outer-most components are resolved, and they give the smallest coupling value, 0.12 G in the present case. The second smallest coupling constant of 0.28 G is also readily identified with the lines occurring with a 1:3 intensity ratio. This constant must be for the hydrogens of the CH_3, whereas the value of 0.12 G can most reasonably be assigned to the nitrogen of the L-alanine residue even though the third component of the expected triplet is not resolved. An approximate value of the coupling for the hydrogen on the peptide nitrogen can be obtained by observing the spectrum in D_2O. This is the only coupling that changes due to isotopic replacement of hydrogen. The appearance of each group of lines changes completely, but the coupling may be deduced from the contraction of the overall width of the group. The value found in this way was 0.84 G. It must be regarded as approximate because of small shifts that may occur in going to D_2O as a solvent. After subsequent computer simulations, a spacing of lines attributed to this coupling constant was identified and a more appropriate value of 0.82 G was taken from the spacing. This constant is known with somewhat less accuracy than our usually stated ±0.03 G. Couplings that remain unevaluated are for the hydrogen on the carbon of the L-alanine residue, a^H, and for the peptide nitrogen, a^N, but the following relation obtains: $a^H + 2a^N = 3.67$ G. This relation was deduced from a measurement of the total span of the group of lines (bottom of Figure 4) and from the known values of all couplings other than a^H and a^N. It appears then that only one parameter need be varied in attempting a fit of the spectrum by computer simulation. Unfortunately, there is so much overlap of components that an extraordinarily small change in value of any coupling makes a very large change in the appearance of the simulation. A satisfactory fit has not been found, and the individual values for a^H and a^N remain unknown. However, the spacings and intensities of the components at each end of the group of lines could be excellently simulated which gives confidence that those couplings with small values that were measured are known quite reliably.

β-Alanyl-L-alanine. The spectrum of β-alanyl-L-alanine consists of four groups of lines with nominal intensities of 1-3-3-1 and with spacings of the groups of about 19 G. The appearance of typical groups of lines is shown in Figure 5 for a solution of 2.9 g of the peptide in 25 ml of water containing 1% H_2O_2 (pH 6.3). The major splitting into four

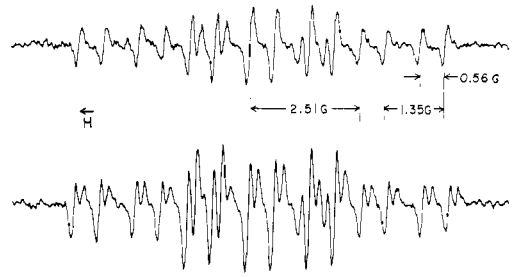

Figure 5. The spectrum from 2.9 g of β-alanyl-L-alanine in 25 ml of water containing 1% H_2O_2 (pH 6.3). There are four groups of lines with nominal intensities of 1-3-3-1. Shown are the higher field 1-strength group (top) and higher field 3-strength group (bottom).

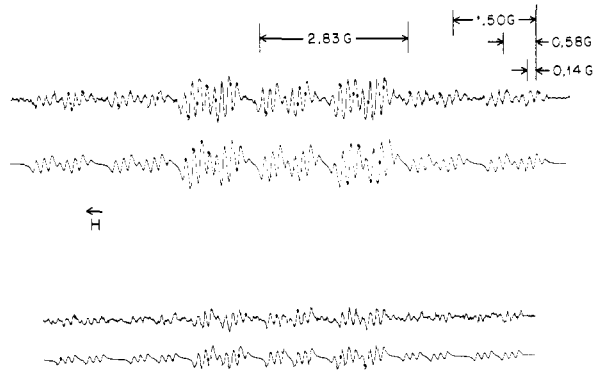

Figure 6. The spectrum from 4.8 g of glycyl-L-alanine in 42 ml of water containing about 1% H_2O_2 and 1 ml of 1 *N* NaOH. Top: the higher field 3-strength group of lines with a computer simulation beneath using parameters described in the text. Bottom: the higher field 1-strength group of lines with a computer simulation beneath.

groups of lines arises from the three equivalent hydrogens of the CH_3, and the identification of the radical and assignment of couplings as given in Figure 2 are without ambiguity. The greater complexity of the 3-strength group of lines as compared to the 1-strength (Figure 5) results from a second-order splitting[9] which is almost completely resolved.

Glycyl-L-alanine. Photolysis of aqueous solutions of glycyl-L-alanine containing H_2O_2 gives rise to a spectrum of four widely spaced groups of lines. It is apparent that the radical formed is analogous to that formed in β-alanyl-L-alanine (Figure 2) and that the fourfold splitting arises from the hydrogens of the CH_3 group. The nominal intensities of the groups are 1-3-3-1; however, there is a pronounced intensity anomaly due to spin polarization, and those lines at low field are quite weak. Moreover, the multiplicity of components within a group of lines is greater than in β-alanyl-L-alanine because of extra splittings due to coupling with the terminal nitrogen. As a consequence, the individual components were quite weak. Most detailed measurements were made on the 3-strength groups even though these lines had the extra complexity of second-order splittings.[9] Some measurements were also made on the higher field 1-strength group.

Glycyl-L-alanine showed another complexity. The appearance of the spectrum changed upon prolonged photolysis. There were small shifts in some of the coupling values, and some but not all of the lines showed changes in width. At the start of photolysis some of the clusters of lines were much more poorly resolved than shown in Figure 6. This effect appears to be associated with a small change in pH during the course of photolysis. The parameters given in Figure 2 were measured with a solution of 1.6 g of glycyl-L-alanine in 25 ml of water containing 1.4% H_2O_2. The measurements were made after a few hours of photolysis

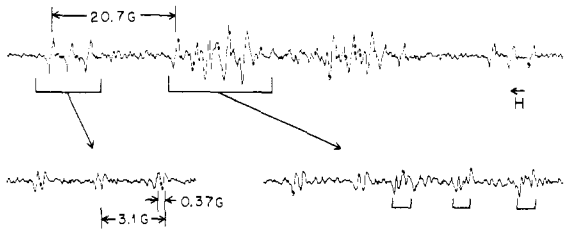

Figure 7. The spectrum from 2 g of glycyl-β-alanine in 25 ml of water containing 1.2% H_2O_2. Top: the entire spectrum with low resolution. Bottom: indicated portions of the spectrum with higher resolution. The brackets at lower right indicate the positions of 2-strength lines that are split by second-order effects.

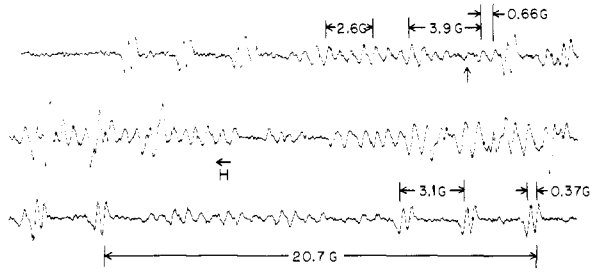

Figure 8. The spectrum formed by photolysis of 3.2 g of β-alanyl-β-alanine in 25 ml of water containing 1% H_2O_2 (pH 6.8) displayed in three sections with the low-field end at lower right. The line spacings given on the bottom third of the spectrum correspond to some of the couplings for one radical while those given in the top third of the spectrum are for a second radical described in the text. The vertical arrow marks an extraneous line that only appears after prolonged photolysis.

when the spectrum stopped changing. The spectrum of Figure 6 was obtained with 4.8 g of glycyl-L-alanine in 42 ml of water containing about 1% H_2O_2. The spectrum changed with time, and finally 1 ml of 1 *N* NaOH was added which immediately sharpened those clusters of lines that were broad and stabilized the spectrum to the form seen after prolonged photolysis. The spectrum was simulated (Figure 6) using the measured parameters, and an excellent fit was obtained. It appears that in order to obtain a sharp lined spectrum the pH must be increased somewhat above the isoelectric point. A few measurements were also made of sharp lines present in the spectrum early in the photolysis before the spectrum had changed to its final form. The couplings for the terminal nitrogen (0.13 G) and the CH_2 hydrogens (2.81 G) were not significantly different than the values of Figure 2. The values for the peptide nitrogen (1.45 G) and attached hydrogen (0.52 G) probably are significantly different. A better understanding will have to await more detailed measurements as a function of pH. Because of the line broadening it appears that a dynamic process is involved such as a change in conformation and/or exchange of hydrogen which is acid catalyzed and that the region of the peptide nitrogen is the likely region of involvement.

Glycyl-β-alanine. Unlike the peptides thus far described, more than one radical contributes to the spectrum from glycyl-β-alanine. The spectrum is illustrated in Figure 7 for 2 g of the peptide in 25 ml of water containing 1.2% H_2O_2. The pH was about 6.1. One of the radicals, the one that gives all of the strong lines of Figure 7, was readily identified, and the assignment and measured parameters are given in Figure 2. There is a relatively large α coupling constant of 20.73 G suggesting that the spin density on this carbon is somewhat greater than for the α carbons of the other peptides. This appears reasonable because of a lack of conjugation with the peptide link due to the intermediate CH_2. This is largely the reason for assigning the radical as shown in Figure 2 rather than to the radical where the hydrogen is abstracted from the other CH_2. In the latter case coupling to nuclei across the peptide link would be expected. This is also borne out by the results to be described on β-alanyl-β-alanine. The relatively large coupling constant for the hydrogens of the CH_2 is reasonable.

There are lines from at least one more radical in Figure 7. These are weak and very numerous. Most or all of these lines likely arise from the radical formed by abstracting a hydrogen from the CH_2 adjacent to the peptide nitrogen.

β-Alanyl-β-alanine. The multiplicity of lines with glycyl-β-alanine was too great to deduce parameters for the second radical formed during photolysis. This was the reason for choosing β-alanyl-β-alanine. Less multiplicity would be expected, and this has proven to be the situation. The spectrum obtained from 3.2 g of β-alanyl-β-alanine in 25 ml of water containing 1% H_2O_2 (pH of 6.8) is shown in Figure 8. One of the radicals has a spectrum and parameters virtually identical with that measured for glycyl-β-alanine (Figure 2). Parameters for the second radical with their assignments are also given in Figure 2. A few very weak extraneous lines have also been seen in the spectrum from β-alanyl-β-alanine, but they are only present after prolonged photolysis.

Discussion of Coupling Constants. The α and β coupling constants and spin densities may be treated in a manner described by Fischer.[10] The treatment is intended only for uncharged radicals, and although there is no net charge on the peptides studied here, they were present as zwitterions. The charges are probably highly localized and do not cause trouble. High spin density occurs at the carboxylate end of the peptide. Comparisons with other aliphatic radicals indicate that the charge of the carboxylate group remains localized. For example, a radical made from citric acid,[11] $HO_2CCH_2C(OH)(CO_2H)\dot{C}HCO_2H$, has an α coupling constant of 20.9 G which changes relatively little to 20.3 G in the triply charged anion. Similarly, the values for $\dot{C}H_2COOH$ and the anion, 21.4 and 21.1 G, respectively, show relatively little change.[12]

Radicals from the L-Alanine Residue. The β coupling constant, a_β^H, may be estimated from the relation[13]

$$a_\beta^H = B_\beta^H (\cos^2 \theta)\rho \qquad (1)$$

where ρ is the electron spin density for the unpaired electron in the $2p_z$ orbital of the α carbon, and θ is the azimuthal angle between the axis of the p_z orbital and the bond with which the β hydrogen is attached to the radical. In the presence of motion an appropriate average of $\cos^2 \theta$ must be taken. The value[10] used for B_β^H is 58.6 G which stems from Fessenden and Schuler's measurements[14] on β coupling constants for hydrogens in freely rotating CH_3. Like essentially all free radicals that have been studied, we regard the CH_3 of β-alanyl-L-alanine and glycyl-L-alanine to be freely rotating which gives a value of ½ for $(\cos^2 \theta)$ in eq 1. From values of a_β^H (Figure 2) the spin densities on the α carbons are 0.655 and 0.660, respectively. These values are virtually identical, and we use the average of 0.658 in the following treatment.

Radicals from the Glycine Residue. Fischer[10] has shown that the spin density on the α carbon can be found from the relation

$$\rho = \prod_{i=1}^{3}[1 - \Delta(X_i)] \qquad (2)$$

where $\Delta(X_i)$ is a constant for each substituent on the trigonal α carbon and has the value zero for hydrogen, $\Delta(H) = 0$. The value[10] for CH_3 is $\Delta(CH_3) = 0.081$. The essential change in going from the above radicals with freely rotating CH_3 to the radicals from glycylglycine, β-alanylglycine,

and L-alanylglycine (Figure 2) is the replacement of a CH_3 by H. With the above spin density of 0.658 and eq 2 we deduce a spin density on the α carbon of 0.716 for each of these three radicals. The α coupling constants of the three radicals are essentially the same (Figure 2, average of 17.50 G) which leads to a value of McConnell's constant of $Q = -24.4$ G. An interesting parameter that may be deduced is Δ(RCONH). In applying eq 2 we use the spin density of 0.716 and take the value $\Delta(CO_2^-) = 0.072$. This stems from Fischer's[10,15] value of $\Delta(COOR') = 0.072$ where R′ is H or an alkyl group. The justification for taking the same value for the carboxylate ion is the above observation that couplings do not shift greatly in going from the electrically neutral acid to the anion. With these values we deduce Δ(RCONH) = 0.228. Fischer[15] has stated that $\Delta(X_i)$ increases with increasing mesomeric effect of the substituent. The value for RCONH is larger than any reported by Fischer;[15] the nearest contenders are OH (0.160), $COCH_2CH_3$ (0.162), and OCH_2CH_3 (0.172). This is a reflection of the high degree of conjugation of the peptide unit.

Radicals from the β-Alanine Residue. We first treat the radical from β-alanyl-β-alanine where the hydrogen has been abstracted from the carbon adjacent to the peptide nitrogen (bottom, Figure 2). Fischer has reported[10] $\Delta(CH_2COOH) = 0.080$ and $\Delta(CH_2COOCH_3) = 0.080$. We use this value for $\Delta(CH_2COO^-)$, the above value Δ(RCONH) = 0.228, and eq 2 to find $\rho = 0.710$. From the coupling of 18.86 G this gives a value of McConnell's constant of $Q = -26.6$ G. This radical is a little unusual in that the β coupling constant, 16.47 G, is smaller than the α coupling constant. If there were free rotation about the C-C bond of the residue $-\dot{C}HCH_2-$ we would predict from eq 1 a coupling constant of 20.8 G in contrast to the observed value of 16.47 G. This suggests that rotation is hindered. We calculate[10] the angles for the most stable conformations which are indicated schematically in Figure 9. These are energetically equivalent conformations which interchange the values of θ of the two β hydrogens. Attached to C_β are the two β hydrogens, $X_\beta = CO_2^-$, and C_α with the C_α-C_β bond perpendicular to the page. The z axis of the $2p_z$ orbital on C_α is in the plane of the page. Interconversion between the two conformations is sufficiently rapid that the pair of β hydrogens appear spectroscopically equivalent.[10] The average, experimentally observed, coupling is given by

$$a_\beta^H = {}^1/_2 B_\beta^H(\cos^2\theta_1 + \cos^2\theta_2)\rho$$
$$a_\beta^H = {}^1/_4 B_\beta^H(3 - 2\cos^2\phi)\rho \qquad (3)$$

The observed coupling of 16.47 G and spin density of 0.710 leads to a value of ϕ of 33°. The values for θ_1 and θ_2 are given by 60° ± ϕ or 93° and 27°.

The radical from glycyl-β-alanine and the similar radical from β-alanyl-β-alanine (Figure 2) have virtually identical coupling constants, and we use the average values in the following treatment. The β coupling constant is very much larger than the α constant which suggests that the situation is quite different than the case treated above. Unfortunately, a value of $\Delta(X_i)$ for $RCONHCH_2$ is not available, but the error is probably not large in using[15] $\Delta(NH_2CH_2) = 0.034$. With eq 2 this leads to $\rho = 0.896$. Combined with the coupling of 20.72 G this gives a value of −23.1 G for McConnell's constant which appears reasonable. If there were free rotation about the C_α-C_β bond the value of the β coupling constant predicted from eq 1 is 26.3 G which is strikingly close to the observed (average) value of 26.0 G. Although free rotation accounts excellently for the observed β coupling constant, the possibility of restricted rotation is not completely eliminated. For restricted rotation, eq 3 leads to $\phi = 44°$ which in turn leads to $\theta_1 = 104°$ and $\theta_2 = 16°$.

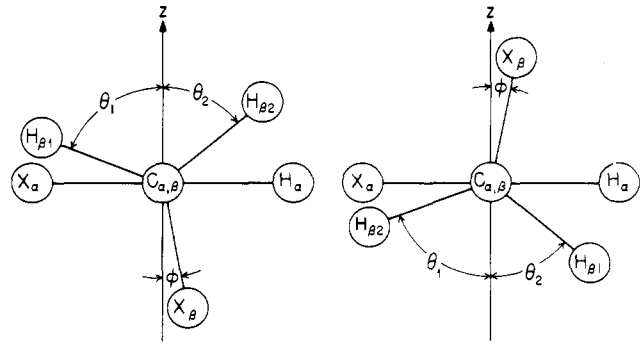

Figure 9. Rotationally equivalent conformations of $X_\alpha\dot{C}HCH_2X_\beta$ with the angles used in the text. The C_α-C_β bond is perpendicular to the page while the axis of the $2p_z$ orbital on C_α is in the plane of the page.

Summary

The seven peptides described show very similar behavior when attacked by ȮH. The reaction is highly selective for the abstraction of a hydrogen on a carbon located between the peptide nitrogen and the carboxylate group. In all cases only a single radical is observed except where β-alanine occurs at the carboxylate end of the dipeptide. With β-alanine in this position a mixture of radicals is obtained which is accounted for by hydrogen abstraction from each of the CH_2 groups. This systematic behavior should not be generalized to more complex peptides. These studies are continuing, and preliminary results show the nature of the radicals to be quite different when L-valine appears at the carboxylate end of the peptide. The experiments also suggest that there will be interesting effects upon changing the pH, and it is intended to make more detailed studies of selected cases.

Not only is the reaction for forming the radicals highly selective, but the values of the hyperfine couplings appear to be reasonable with systematic trends. The couplings for the peptide nitrogen and attached hydrogen (Figure 2) are always fairly small, but they do vary substantially. It is noted that generally a high value for one of these couplings is associated with a low value for the other one. Glycyl-β-alanine is at one extreme with couplings of 3.14 and 0.37 G for the nitrogen and hydrogen, respectively, while β-alanylglycine is at the other extreme with 0.56 and 1.23 G for the nitrogen and hydrogen, respectively.

All of the spectra are very rich in hyperfine structure, and it is apparent that nonnegligible spin density is distributed over a large portion of the radicals. This is probably going to make the study of longer peptides, one of the main goals of this program, very difficult. A simplifying feature, however, of which use will be made is to place a β-alanine residue at the amine end of the peptide. This will eliminate splittings from the terminal nitrogen.

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Reactions of Metals with Vitamins. I. Crystal and Molecular Structure of Thiaminium Tetrachlorocadmate Monohydrate

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Abstract: The X-ray crystal structure of (protonated thiamine) tetrachlorocadmate monohydrate, $CdCl_4C_{12}H_{20}N_4O_2S$, has been determined. The unit cell parameters are $a = 16.874$ (2), $b = 15.553$ (2), $c = 7.906$ (2) Å; $\beta = 97.61$ (6)°; space group $P2_1/n$; $Z = 4$. A total of 2498 reflections having $F^2 > 2\sigma(F^2)$, $2\theta < 50°$ were collected on a four-circle diffractometer with Mo Kα radiation by the θ–2θ scan technique. The structure was solved by conventional heavy atom methods. All atoms heavier than hydrogen were refined anisotropically; positional parameters only were refined for the hydrogen atoms. Successive block-diagonal least-squares cycles yielded a conventional R factor of 0.027. The structure consists of protonated thiamine cations, tetrachlorocadmate anions, and a water molecule of hydration. The protonation site is on the pyrimidine nitrogen opposite the amino group. The observed thiamine conformation differs grossly from that usually found for thiamine derivatives but is one of the conformations theoretically predicted to be stable. The large number of hydrogen bonds, together with the short dipolar contacts involving the thiazolium sulfur and nitrogen atoms, may help to stabilize the present conformation. The tetrachlorocadmate anion is an almost regular tetrahedron with an average Cd–Cl distance of 2.453 Å. The coordination of thiamine to a metal appears to be an unlikely event since the electron pairs which would be used to bond a metal are generally involved in the π systems of the thiazolium and pyrimidine rings.

Various studies have indicated that metal complexes might be formed with thiamine and its derivatives.[2-7] There are several areas where complexing might be an important facet of the chemistry of thiamine. For example, complex formation is a possible mechanism for the prevention of cadmium[8,9] or manganese[10,11] poisoning by thiamine and would be involved if the oxidation of thiamine by metal ions[12,13] proceeded by an inner-sphere mechanism. However, definitive proof of metal–thiamine bonding is lacking, although some recent research[14-19] has been directed toward just this point.

We have been examining the reactions of thiamine with metal ions and report herein the structure of (protonated thiamine) tetrachlorocadmate monohydrate, $HThiCdCl_4 \cdot H_2O$.

Experimental Section

Crystals of $HThiCdCl_4 \cdot H_2O$, $CdCl_4C_{12}H_{20}SO_2N_4$, were prepared by mixing aqueous solutions of thiamine hydrochloride and cadmium nitrate (2:1 mole ratio) and allowing the solution to evaporate. Well-formed, colorless monoclinic crystals were obtained. A plate-like crystal, 0.20 × 0.31 × 1.1 mm, was mounted on a glass fiber with epoxy cement and aligned about the c* axis on a Picker four-circle diffractometer. The long dimension of the crystal was approximately parallel to the ϕ axis of the diffractometer. Cell dimensions were determined from scans of the axial and $h0l$ reflections out to $2\theta = 60°$ with Mo Kα radiation at a take-off angle of 1.5° ($\lambda = 0.70926$ Å for Mo Kα_1): $a = 16.874$ (2), $b = 15.553$ (2), $c = 7.906$ (2) Å; $\beta = 97.61$ (6)°; space group $P2_1/n$; $Z = 4$; $d_{obsd} = 1.78$ g/cc, $d_{calcd} = 1.74$ g/cc.

Previously described procedures were used for the data collection and reduction to the structure factors.[20] Of the 3816 reflections with $2\theta < 50°$ in the independent quandrant, 2948 had $F_o^2 > 2\sigma(F_o^2)$ and were classified as observed. Although the crystal turned brown during the data collection, it remained transparent and there was no significant decrease in the intensities of the standard reflections, which were measured approximately every 2 hr. No absorption correction was made in view of the low absorbance of the crystal (μ for Mo Kα radiation = 1.72 mm^{-1}).

Scattering factors for neutral Cl, S, O, N, C, and H atoms were taken from the International Tables for X-Ray Crystallography,[21] as were the real and imaginary parts of the anomalous scattering of cadmium. The neutral cadmium scattering factors were obtained from Cromer et al.[22] Major computer programs employed included Zalkin's FORDAP, Busing and Levy's ORFLS, and Busing, Martin, and Levy's ORFFE. In the least-squares refinements, the function minimized was $\Sigma w\Delta^2$, where $w = 1/\sigma^2(F)$ and $\Delta = ||F_o| - |F_c||$. Unweighted and weighted residuals, R_1 and R_2, respectively, were calculated after each refinement.

$$R_1 = \Sigma\Delta/\Sigma|F_o|$$

$$R_2 = \Sigma w\Delta^2/\Sigma wF_o^2$$

The structure was solved by the usual heavy atom methods. All atoms heavier than hydrogen were located and their positions and anisotropic thermal parameters were refined in the block diagonal approximation (position and thermal parameters for the Cd, 4(Cl), and thiazolium atoms were refined in one cycle, whereas the corresponding vairables for the pyrimidine atoms and the water molecule were refined in the next). At this stage, $R_1 = 0.055$, $R_2 = 0.074$. A difference map yielded the positions of the hydrogen atoms. Their positional parameters were refined but the isotropic temperature factors were kept fixed at 4.0 Å^2. Subsequent refinements of the nonhydrogen atoms yielded final values of 0.027 and 0.037 for R_1 and R_2, respectively. The average parameter shift on the last cycle was 0.4 σ. The error in an observation of unit weight was 1.0.

A weighting analysis showed that the strong reflections and those with low $\sin\theta/\lambda$ were somewhat overweighted, but as the number of affected reflections was small, no correction was made to the weighting scheme. A final difference Fourier map revealed no peaks higher than about 0.5 e/Å^3 and no holes deeper than