308. *Proton Resonance Spectra of some Amino-acids in Aqueous Solution.*

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The spectra of some amino-acids in aqueous solutions in the pH range 1—13 are discussed. The changes in the chemical shifts of the CH protons give some information about the ionisation sequences, and the coupling constants indicate the conformations of the ions produced.

As part of a study of the proton resonance spectra of diamagnetic metal complexes of the amino-acids, we have measured the spectra of some of the acids themselves; they give some information about the structures of these compounds in solution. Previously, Jardetsky and Jardetsky [1] have given some details of the spectra of amino-acids, but they did not study the effect of pH in detail; McDonald and Phillips [2] have recently done so in the case of histidine. The spectra of a few acids at certain pH values have been reported by Fujiwara and Arata,[3] and an analysis of the spectra of β-substituted α-amino-acids is being published by Pachler.[4] Bovey and Tiers have also measured the spectra in solution in trifluoroacetic acid.[5]

[1] Jardetsky and Jardetsky, *J. Biol. Chem.*, 1958, **233**, 383.
[2] McDonald and Phillips, *J. Amer. Chem. Soc.*, 1963, **85**, 3769.
[3] Fujiwara and Arata, *Bull. Chem. Soc. Japan*, 1963, **36**, 578.
[4] Pachler, *Spectrochim. Acta*, 1963, **19**, 2085.
[5] Bovey and Tiers, *J. Amer. Chem. Soc.*, 1959, **81**, 2870.

Experimental.—The spectra were recorded at a frequency of 56·45 mc./sec. and at a temperature of 22° with a Varian spectrometer. The amino-acids, which were obtained from B.D.H. Ltd. and from the Nutritional Biochemicals Corporation, were made up to solutions of known concentrations, about 0·2—0·5M, in water containing sufficient acid or alkali to produce the required pH value, which was measured at 20° with a glass electrode in a miniature cell (volume *ca.* 0·5 ml.). For each acid 15—30 samples of the same concentration but of different pH were measured. A trace of t-butyl alcohol was added to each sample, the methyl proton line being taken as the internal reference. When the strong line of the solvent protons obscured part of a spectrum, it was recorded again under equivalent conditions in solution in deuterium oxide. The error in the shifts is about ±0·01 p.p.m., and in the coupling constants, ±0·2 c./sec.

Results and Discussion.—The protons in the species $-COOH$, $-NH_2$, OH^-, H_3O^+, and H_2O exchange rapidly and appear as a single line whose position, a weighted average of the shifts in the several environments, is close to that of water itself at a given pH. In solutions of pH less than about 1, the protons in $-NH_3^+$ groups exchange slowly enough to give separate broad lines on the low-field side of the solvent. The spectra consist mainly of the lines of the aliphatic CH protons, which lie on the high-field side of the solvent. The pH dependence of the chemical shifts is given in Figs. 1 and 2, and some additional data are in Table 1.

TABLE 1.

Proton resonance spectra of valine, threonine, and allothreonine.

	pH 1			pH 6			pH 12		
	Val	Thr	Allothr	Val	Thr	Allothr	Val	Thr	Allothr
Shift of α-CH proton [a]	−2·74	−2·81	−2·90	−2·36	−2·34	−2·60	−1·83	−1·85	−2·10
Shift of β-CH proton [a]	−1·14	−3·21	−3·16	−1·03	−3·02	−3·12	−0·73	−2·69	−2·82
J(α-CH to β-CH) [b] ...	4·3	3·8	3·6	4·4	4·8	4·2	5·1	4·9	4·6
Shift of CH_3 protons [a]	+0·16 [c] +0·19	−0·12	−0·09	+0·21 +0·26	−0·08	+0·04	+0·32 +0·39	+0·04	+0·13
J(β-CH to CH_3) [b]	6·7 [d]	6·6	6·6	7·0	6·7	6·6	6·8	6·7	6·6

[a] Shifts are given in p.p.m., negative values indicating a line on the low-field side of the reference, $Me_3C{\cdot}OH$. [b] Coupling constants are given in c./sec. and are assumed to be positive. [c] The upper set of values refers to one of the methyl groups in valine and the lower set refers to the other. [d] The coupling to the β-CH proton is the same for the two methyl groups.

In an aliphatic group CH–X–H, where X–H is $-COOH$ or $-NH_3^+$, the α-CH proton lies at a lower field than it does in the ionised group CH–X^-, partly because X^- is less electronegative than X–H, and partly because the two groups have different magnetic anisotropies.[6] The fast exchange of hydrogen atoms in solutions containing both the acid group and its ionised form causes the line position of the α-CH proton to be a weighted average of those of the two species, and it moves upfield over the region of pH in which the acid ionises. Hence the ionisation is shown by a step in the graph of the shift of this proton against pH.

An example is γ-aminobutyric acid, which has the structure $^+H_3N{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}COOH$ in acid solution. As the pH is increased, the α-CH_2 protons first show a shift to higher field, since the cation loses a proton from the carboxyl group to give the zwitterion as the main neutral species. The γ-CH_2 group, which is identified by the additional fine structure it shows in acid solution from spin coupling with the NH_3^+ protons, shifts upfield when the NH_3^+ group ionises at a higher pH.

The shift of the CH proton appears to be related linearly to the degree of dissociation of the group attached to it, as Grunwald *et al.* found for aliphatic amines.[7] The pH at the midpoint of a step in the graph is nearly equal to the pK value of the group. In the above example, the midpoints occur at the pH values 3·9 and 10·7, corresponding to pK

[6] Spiesecke and Schneider, *J. Chem. Phys.*, 1961, **35**, 722.
[7] Grunwald, Loewenstein, and Meiboom, *J. Chem. Phys.*, 1957, **27**, 641.

values of 4·0 and 10·6, estimated from the pH data. (Accurate values of the apparent dissociation constants at 20° are given as $pK_1 = 4{\cdot}038$ and $pK_2 = 10{\cdot}70$ by King.[8])

The shifts of protons which are further away from the group X–H also show steps when ionisation occurs These result from changes in inductive effects, which are probably small,[6] and in the long-range shielding, which depends not only on the magnetic anisotropies of the groups X–H and X^- but also on their spatial arrangement, and can be different in each of the rotational isomers of the molecule.[9] The rate of interconversion of these isomers is usually fast enough to make the measured shift appear as a weighted average of those in the isomers. Hence the shift depends on their relative populations, which are expected to alter when ionisation occurs, for X–H and X^- differ in charge and size and therefore in their electrostatic, steric, and hydrogen-bonding interactions. At the second dissociation of γ-aminobutyric acid, both the α- and the β-CH_2 protons show

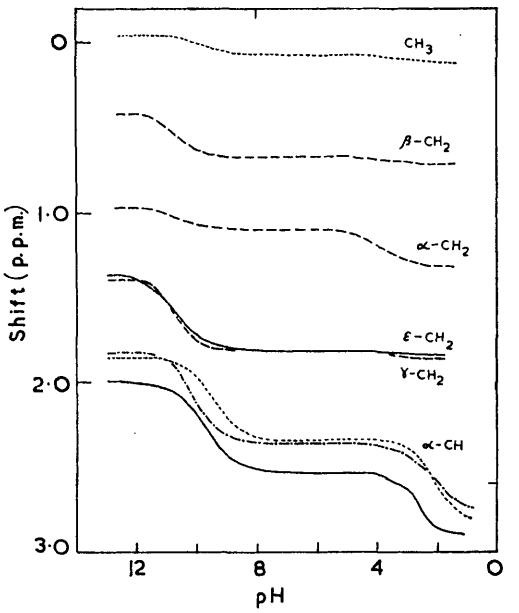


FIG. 1. Chemical shifts, in p.p.m. on the low-field side of t-butyl alcohol, for the α-, β-, and γ-CH_2 protons of γ-aminobutyric acid (– – – –), the α-CH proton of valine (— · — · —), the α-CH and CH_3 protons of threonine (· · · ·), and the α-CH and ε-CH_2 protons of lysine (full lines).

steps, indicating that the average configuration of the anion is different from that of the zwitterion.

The long-range shielding from groups on other carbon atoms can also be a third contribution to the shift of the CH proton in the group CH–XH; consequently the heights of the steps for the ionisation of a given group X–H in a molecule $R^1R^2CH{\cdot}X{-}H$ are not necessarily the same when R^1 or R^2 is changed. However, in the present example, the heights of the first step (0·22 p.p.m.) and the second (0·41 p.p.m.) are similar to those found in several aliphatic carboxylic acids and primary amines, respectively.

DL-*Valine.* As is found with several α-amino-acids, the first step in the shift of the α-CH proton is smaller than the second (Fig. 1), the first dissociation involving the loss of the proton from the carboxyl group of the cation $(CH_3)_2CH{\cdot}CH(COOH){\cdot}NH_3^+$ to give the zwitterion. The α-CH proton is split into a doublet by spin coupling to the β-CH proton. At a given value of pH, the coupling constant J is a weighted average over all the ionic species present and their rotational isomers. By comparison with other aliphatic compounds, in which the values of the vicinal coupling constants are in the ranges 1—3 and

[8] King, *J. Amer. Chem. Soc.*, 1954, **76**, 1006.
[9] Gutowsky, *J. Chem. Phys.*, 1962, **37**, 2196.

9—12 c./sec. between CH protons in the *gauche* and *trans* arrangements, respectively,[10-12] the present values (Table 1) suggest that in valine one or both of the *gauche* rotational isomers is more populated than the *trans*. This is unexpected if steric repulsions determine the relative stabilities, since the *trans*-isomer should be the more stable one. The corresponding coupling constant in α-alanine, $CH_3 \cdot CH(COOH) \cdot NH_3^+$, in which the isomers are equally populated, is 7·2 c./sec. over the pH range 1—12; however, there are no bulky groups on the β-carbon atom of α-alanine, and the bond lengths and angles, upon which J partly depends,[12] may differ from those in valine. In both acids, the β-CH proton shifts upfield mostly at the second dissociation.

The methyl protons in valine are split into a doublet by the β-CH proton, but appear as a double doublet since the two methyl groups are not equivalent, due to the low

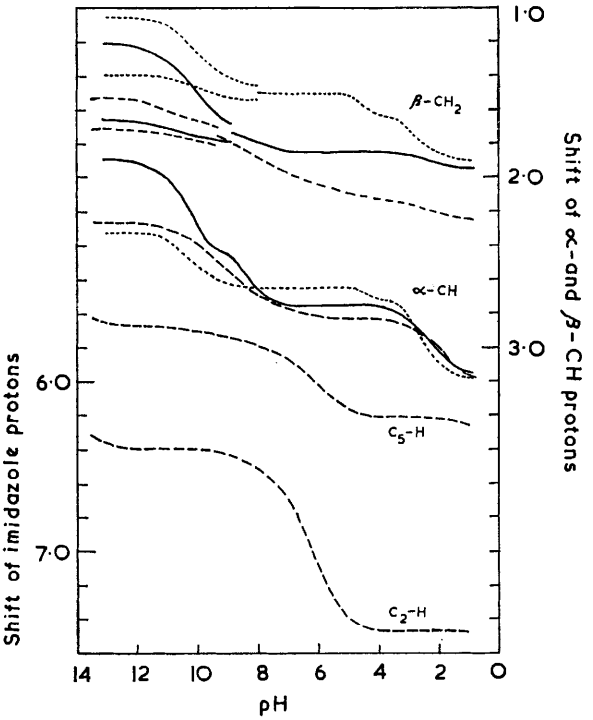


FIG. 2. Chemical shifts, in p.p.m. on the low-field side of t-butyl alcohol, for aspartic acid (· · · ·), cysteine (full lines), and histidine (– – – –). The two scales are displaced by 2·80 p.p.m. Above pH 8—9, the shifts of the two CH_2 protons are shown separately, in each case the upper line (at higher field) represents the proton (H_A) with the greater spin coupling to the α proton (H_C).

symmetry of valine together with the differences in population of the isomers.[9] The pH dependence of the chemical shifts, produced by long-range shielding, is slightly different for the two methyl groups.

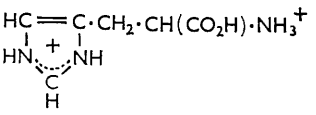
L-*Threonine* $CH_3 \cdot CH(OH) \cdot CH(COOH) \cdot NH_3^+$. The pH dependence of the shifts of the α-CH, the β-CH, and the methyl protons, and the values of the coupling constants, are similar to those in valine and may be explained in the same way, although in threonine a *gauche* configuration can be stabilised by intramolecular hydrogen bonding. The hydroxyl proton exchanges with the solvent and is not seen separately.

[10] Gutowsky and Juan, *J. Chem. Phys.*, 1962, **37**, 120.
[11] Bothner-By and Naar-Colin, *J. Amer. Chem. Soc.*, 1962, **84**, 743; Anet, *ibid.*, p. 747.
[12] Banwell and Sheppard, *Discuss. Faraday Soc.*, 1962, **34**, 115.

At a given pH, the spectrum of threonine is slightly different from that of the diastereoisomer allothreonine (Table 1) and the proportions of the two compounds in a mixed sample are easily determined by measuring the relative intensities of the lines. Several other examples of the different spectra of diastereoisomers have been reported.[13]

L-*Lysine.* The first dissociation of the doubly charged cation is mainly that of the α-carboxyl group, giving the cation $^{+}H_3N{\cdot}[CH_2]_4{\cdot}CH(COO^{-}){\cdot}NH_3^{+}$ (= A). The ionisations of the two ammonium groups in this cation overlap. As the pH is increased (Fig. 1) the second step for the α-CH proton reaches a given degree of completion at a lower pH than the step for the ε-CH_2 protons, showing that the α-ammonium group has the greater tendency to lose a proton. The zwitterion $^{+}H_3N{\cdot}[CH_2]_4{\cdot}CH(COO^{-}){\cdot}NH_2$ (= B) is the main neutral species, $H_2N[CH_2]_4{\cdot}CH(COO^{-}){\cdot}NH_3^{+}$ (= C) is a smaller proportion of the zwitterion being formed concurrently by ionisation of the ε-ammonium group of the cation. The anion (= D) is formed mainly by loss of the ε-ammonium proton from the zwitterion B. Since the two ammonium groups are separated by five aliphatic carbon atoms, we assume that the shift of the α-CH proton is related linearly to the degree of ionisation of the α-ammonium group, the fractional height of the step giving the ratio of concentrations ([A] + [C])/([B] + [D]). Estimating the values $pK_2 \simeq 9{\cdot}6$ and $pK_3 \simeq 10{\cdot}9$ from the pH data (in 0·5M solution) we derive the microscopic dissociation constants $k_{A \rightarrow B} \simeq 2 \times 10^{-10}$ and $k_{A \rightarrow C} \simeq 4 \times 10^{-11}$, whose ratio gives the relative proportions of the zwitterions as [B] : [C] = 5 : 1. A similar value is found by using the shift of the ε-CH_2 protons. The result agrees with the ratio 5·6 : 1 calculated by Edsall and Blanchard [14] from comparisons of pK values; the closeness of the agreement is fortuitous since our present data are not very accurate.

Aspartic acid, cysteine, and histidine. These acids have a third ionisable group on the β-carbon atom; in acid solutions their cations have the structures

$HOOC{\cdot}CH_2{\cdot}CH(CO_2H){\cdot}NH_3^{+}$, $HS{\cdot}CH_2{\cdot}CH(CO_2H){\cdot}NH_3^{+}$, and 

respectively. Each shows three steps in the graph of shifts against pH (Fig. 2), corresponding to the three macroscopic dissociation constants, but these steps can usually give no more than a qualitative indication of which group is ionising, since the ionisation of any one group affects the shifts of the protons on the other carbon atom.

In each acid, the first ionisation (pK_1 = 1·88, 1·71, and 1·78, respectively, at 25° [15]) is mainly that of the α-carboxyl group. The accompanying shift of the β-CH_2 protons is largest for aspartic acid, in which the ionisation of the β-carboxyl group overlaps that of the α, and the loss of the first proton occurs partly from the β-carboxyl. Edsall and Blanchard [14] calculated that the neutral species of aspartic acid is a mixture of the zwitterions $HOOC{\cdot}CH_2{\cdot}CH(COO^{-}){\cdot}NH_3^{+}$ and $^{-}OOC{\cdot}CH_2{\cdot}CH(COOH){\cdot}NH_3^{+}$ in the ratio 17·5 : 1. The successive large and small steps of the shift of the α-CH proton agree with this qualitatively; for the β-protons, the first step is again larger than the second, although the first step will include the major part of the change due to the ionisation of the α-carboxyl group. The third dissociation is that of the α-NH_3^{+} group.

In cysteine, for which pK_2 = 8·33 and pK_3 = 10·78 at 25°,[15] there is good evidence from quantitative measurements of ultraviolet absorption [16] and Raman [17] spectra that the acid strength of the thiol group in the neutral zwitterion is about twice that of the $-NH_3^{+}$ group, the loss of one proton giving the anions $^{-}S{\cdot}CH_2{\cdot}CH(COO^{-}){\cdot}NH_3^{+}$ and

[13] Hutton and Schaeffer, *Canad. J. Chem.*, 1963, **41**, 1857; Jardetsky, *J. Biol. Chem.*, 1963, **238**, 2498.
[14] Edsall and Blanchard, *J. Amer. Chem. Soc.*, 1933, **55**, 2337.
[15] Greenstein and Winitz, "Chemistry of the Amino Acids," John Wiley and Sons, Inc., New York, 1961, p. 486.
[16] Benesch and Benesch, *J. Amer. Chem. Soc.*, 1955, **77**, 5877.
[17] Elson and Edsall, *Biochemistry*, 1962, **1**, 1.

$HS{\cdot}CH_2{\cdot}CH(COO^-){\cdot}NH_2$ in the ratio 2·1 : 1. The step of the α-CH proton does in fact show a smaller step at the second dissociation than at the third, but this is not reliable evidence in support of the ionisation sequence suggested, since the overall shift of the α-proton (1·29 p.p.m. between pH 1 and pH 13) is markedly larger than that in other α-amino-acids discussed here (0·9—1·0 p.p.m.), suggesting that in cysteine there is a greater long-range shielding contribution to the shift of the α-proton.

In histidine, the imidazole group itself contains two olefinic CH protons, whose lines lie on the low-field side of the solvent. For both of these protons, in particular the one between the nitrogen atoms, the shifts change only in the region of the second dissociation, showing clearly that the proton is lost mainly from the imidazole cation, as pointed out by McDonald and Phillips.[2] Our results are in good agreement with theirs. The pH at the midpoint of the step for the central CH proton is 6·3, the value of pK_2 being 5·97 (at 25°).[15] The third ionisation involves the α-ammonium group again, and the slight upfield shifts of the imidazole CH protons, when the pH is greater than about 12·8, indicate that the remaining NH group of the imidazole is beginning to ionise.

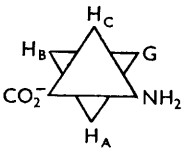
The two CH_2 protons are expected to be non-equivalent,[9] but in acid solutions the spectra appear superficially to be of the AB_2 type. The additional lines predicted for an ABC spectrum were not detected, and neither the coupling constants between the CH_2 protons (H_A and H_B) and the α-CH proton (H_C), nor the shift between H_A and H_B, could be determined. The shift is probably small, and only the average shift position is plotted (Fig. 2).

As the pH is increased, the shift between the protons H_A and H_B increases and the spectra are found to be of the ABC type. Approximate values for the separate shifts, and for the coupling constants, some of which are given below, were calculated on the assumption that the spectra were of the ABX type.

	Aspartic acid				Cysteine		Histidine		
pH	3·8	9·6	10·2	13·0	10·3	12·7 [b]	8·4	10·2	12·7
J_{AB} [a]	17·8	17·0	16·3	15·4	13·1	12·2	15·1	14·6	14·8
J_{AC}	9·3	8·3	8·8	9·9	8·0	8·7	8·8	8·7	8·6
J_{BC}	2·8	3·5	3·7	3·8	3·9	3·6	3·9	3·7	4·3

[a] Although the calculations do not give the sign of the geminal coupling constant J_{AB}, it is very likely to be negative relative to those of J_{AC} and J_{BC}, as in many other substituted ethanes.[12]
[b] Calculated for ABC type.

In the fully ionised species, the values of the vicinal coupling constants J_{AC} and J_{BC} suggest that the preferred conformation in each compound is one of the two rotational isomers in which the proton H_C is *gauche* to H_B and *trans* to H_A. This conformation is probably the most stable one throughout the third dissociation, since the coupling constants do not change greatly. The ammonium group, which is mainly involved in the dissociation, will therefore stay in roughly the same average position relative to the CH_2 protons, and is probably closer to H_A, for the shift of this proton moves upfield during the dissociation more than the shift of H_B does. Hence the most populated of the two *gauche* isomers is the one with the structure shown, in which the electrostatic and spatial repulsions between the α-carboxy group and the group G on the β-carbon atom should be at a minimum.



One difficulty in observing weak lines in these spectra is that in the pH region 8—12 there is a broadening of the lines, particularly those of the α-CH protons. A similar broadening occurs with the simple amino-acids, α-alanine and glycine, and seems to be associated with the ionisation of the ammonium groups, since it is also found with ethylamine and methylamine. For example, in solutions containing the glycine zwitterion (pH 7) the width of the CH_2 proton line is no more than 0·4 c./sec., but as the ionisation proceeds the line becomes broader, having a maximum width of about 1·7 c./sec. when

the ionisation is about half complete, and then narrows to a width of about 0·7 c./sec. in solutions which contain only the anion (pH 13). The effect cannot be due to a residual spin coupling with the NH protons, for it is still present in equivalent solutions in deuterium oxide, nor is is likely to arise from an incomplete averaging of the shifts of $R{\cdot}NH_3^+$ and $R{\cdot}NH_2$, since the proton exchanges which are responsible for this averaging are rapid in alkaline solutions.[18] In fact, in the pH range mentioned above, the rates of these exchanges are comparable with the proton resonance frequency, and it is possible that they provide an effective mechanism for the spin lattice relaxation, thereby broadening the proton energy levels. The line widths and relaxation times of the α-CH protons are being studied in solutions of different concentrations and temperatures.

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[18] Loewenstein and Connor, *Z. Electrochem.*, 1963, **67**, 280.