Protolysis Kinetics of some Amino-acids Studied by the Nuclear Magnetic Resonance Spin Echo Technique

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Received 4th September, 1972

Proton magnetic relaxation times have been measured as a function of pH and temperature for aqueous solutions of amino-acids in the series $H_3\overset{+}{N}—[CH_2]_n—\overset{-}{C}O_2$ where n = 1, 2, 3, 5 and 7 and also for solutions of α-alanine, sarcosine, L-proline, picolinic acid and 2-pyrrolidone. The T_1 values were independent of pH but T_2 had a minimum value near to pH 6 at room temperature for most of the solutions studied. In many cases, a second minimum occurred close to pK_{a1} for the amino-acid. The data are analysed in terms of proton exchanges between amino groups and sites which are oxygen attached. Kinetic parameters are derived for some exchange reactions which have not previously been considered in connection with amino-acid solutions.

The importance of proton transfer steps in general acid-base catalysis of both chemical and enzymatic reactions makes the rates and mechanisms of these processes of particular interest. In the case of the α-amino-acids glycine and sarcosine the exchange rates for the amino group protons in aqueous solution have been measured by means of proton n.m.r.[1-3] The effect of the exchange is to collapse the multiplets due to the methyl or methylene group adjacent to the nitrogen atom, when from a comparison of the observed line shape with theoretical curves the mean interval between events leading to exchange of a proton (τ) can be inferred. In addition the frequency of exchanges of protons from solvent molecules to nitrogen can be measured from the broadening of the water line. By observing τ as a function of pH, the rate constants for several reactions contributing to the exchange of protons were obtained for both the cationic and zwitterionic forms of the acids.

An alternative method of measuring proton exchange rates using a Carr-Purcell spin-echo train was described by Luz and Meiboom.[4] In this method, the decay of a Carr-Purcell train is measured as a function of pulse repetition rate. The apparent relaxation rate for a system in which spins are exchanging between two sites differing in Larmor frequency by $\delta\omega$ is given by

$$\frac{1}{T_2} = \frac{1}{T_2^\circ} + \left[1 - \frac{2\tau}{(tcp)} \tanh \frac{(tcp)}{2\tau}\right] \tau p_a p_b \delta\omega^2 \qquad (1)$$

where T_2° is the transverse relaxation time in the absence of exchange, τ the average lifetime of a spin between exchanges, (tcp) the time between consecutive 180° pulses and p_a, p_b the fractional populations of the two sites. Allerhand and Gutowsky [5, 6] examined the conditions under which eqn (1) could be applied and found that it was valid as long as either $(tcp) \ll T_2$ or $\tau^{-1} \gg \omega$. In the present work, we report some measurements made on solutions of amino-acids in the series $H_3\overset{+}{N}—[CH_2]_n—\overset{-}{C}O_2$

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where $n = 1$, 2, 3, 5 and 7 using the spin-echo technique. Measurements were also made on other amino-acids not in the series. These compounds were α-alanine, sarcosine, L-proline, picolinic acid and 2-pyrrolidone.

EXPERIMENTAL

RELAXATION TIMES

The pulsed n.m.r. spectrometer, which operated at a frequency of 45 MHz, was built in this department by Mr. W. E. Porter and Mr. R. Parsons. Induced signals were displayed on an oscilloscope and photographed on Polaroid film. Measurements of T_1 were made by using 90°, τ, 90° pulse sequences and plotting "infinity" amplitude (obtained from single 90° pulses) minus signal amplitude against time on semilog graph paper. Carr-Purcell sequences with the r.f. phase of the 90° pulse offset by 90° from the phase of the following 180° pulses (Meiboom-Gill modification) were used. To obtain T_2, a graph of echo amplitude against time was plotted on semilog graph paper. In both cases, provided that a straight line graph was obtained, the time for the amplitude to fall from 2.72 units to 1 unit was recorded as T_1 or T_2. Experiment showed that in general T_2 values were reproducible on a single sample to better than 3 % and T_1 values to better than 6 %.

Temperature control of the sample was achieved by evaporating liquid nitrogen and passing the gas through a Dewar tube around the sample coil either directly for temperatures below room temperature or via a heat exchanger for higher temperatures. The heat exchanger used was a double spiral coil condenser fitted to a flask containing a suitable refluxing solvent. Fine temperature control could be obtained by altering the rate of nitrogen evaporation by means of a small electrical heater run from a Variac. Temperatures, which were measured by means of a copper-constantan thermocouple, were precise to within 0.5°C of the stated value.

PREPARATION OF SAMPLES

A.R. grade samples of the amino-acids were used without further purification. Trial multiple recrystallisations of glycine were found to be without effect upon relaxation times. Solutions of known pH were prepared by placing 25 ml of the neutral amino-acid at (for example) 1 molar concentration in a magnetically stirred beaker containing glass and calomel electrodes attached to an E.I.L. pH meter and adding a second solution, also 1 molar in amino-acid and 2 molar in HCl or NaOH until the desired pH was obtained. A 1 ml sample of the well-mixed solution was then de-oxygenated by bubbling moist, oxygen-free nitrogen through it for ten minutes. After de-oxygenation, sample liquid was sealed off in a sample tube which had been prepared from a short length of 6 mm diam. Pyrex tubing by sealing one end and blowing out a small spherical bulb (8 mm diam.). Experiments with distilled water showed that the procedure completely removed interfering quantities of dissolved oxygen.

pH values were measured at 25°C. Away from room temperature it was difficult to hold the electrodes at constant temperature while making additions to the solution. Because of this difficulty it was thought preferable to measure the pH of a specimen at 25°C and to calculate changes in pH with temperature using published values of dissociation constants (Appendix). pH values were recorded to 0.01 unit using potassium hydrogen phthalate as standard.

CONTINUOUS WAVE N.M.R. SPECTRA

Conventional n.m.r. spectra were obtained by means of an A.E.I. RS2 spectrometer operating at 60 MHz. No temperature controller was used and measurements refer to 25±2°C. Solutions were not degassed.

RESULTS

The echo width and receiver recovery time limited the period between successive echoes to a minimum value of 0.758 ms. At this pulse repetition frequency there

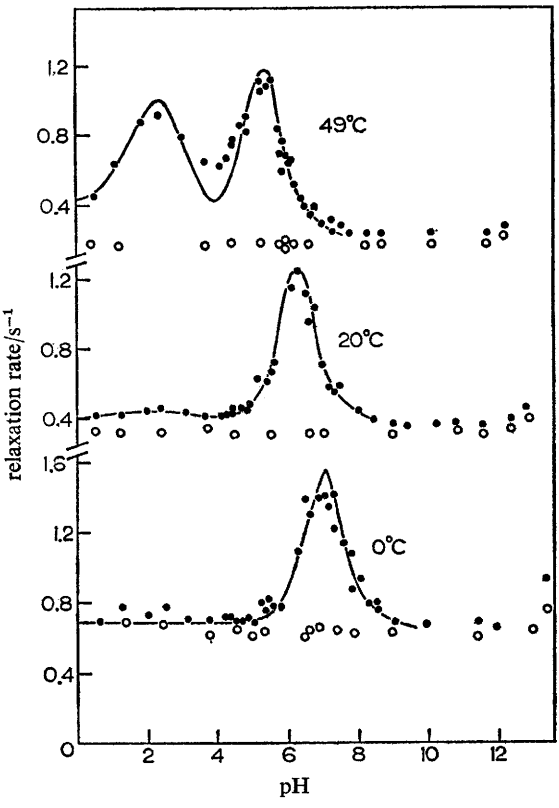

FIG. 1.—Relaxation rates for 0.4 M glycine solutions; ○, T_1^{-1}; ●, T_2^{-1}.

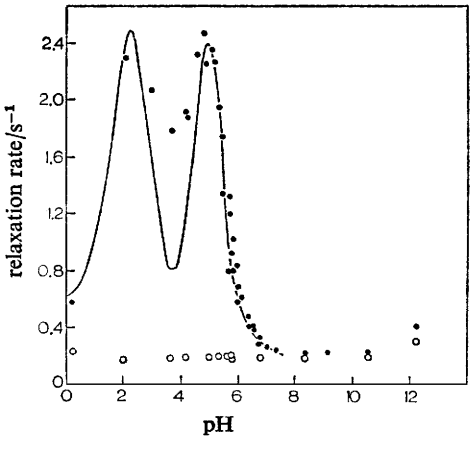

FIG. 2.—Relaxation rates for 1.0 M glycine solutions at 49°C; ○, T_1^{-1}; ●, T_2^{-1}.

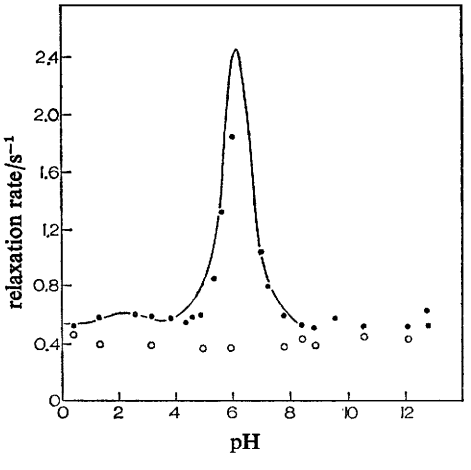

FIG. 3.—Relaxation rates for 1 M α-alanine solutions at 20°C; ○, T_1^{-1}; ●, T_2^{-1}.

was no change in the T_2 of pure water over a range of pH from 0 to 13, i.e., the exchange of protons to ^{17}O molecules was not observable. The pulse separation was, in general, maintained near its minimum usable value to reduce diffusion effects. Only in the experiments with ω-aminocaprylic acid and 2-pyrrolidone were longer pulse separations (2.13 ms) used in order to increase sensitivity.

Unless otherwise stated, solutions were left unbuffered in order to avoid introducing other proton accepting sites. 2-Pyrrolidone is not appreciably ionised at intermediate pH values; solutions were, therfore, prepared with sodium phosphate as buffer. Fig. 12 shows that there was no significant difference in relaxation times between solutions 0.2 M and 0.1 M in sodium phosphate. It was concluded that sodium phosphate had no measurable effect within the pH range shown. Only a

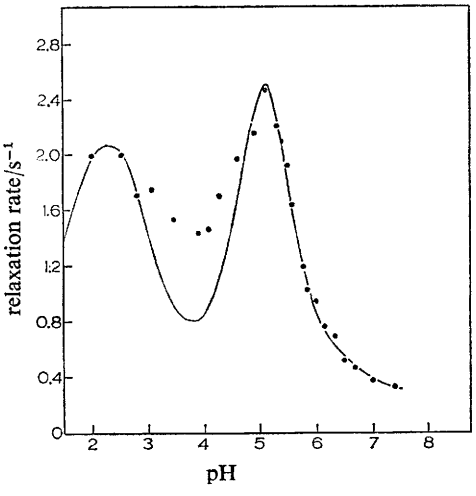

FIG. 4.—Relaxation rates (T_2^{-1}) for 1 M α-alanine solutions at 49°C.

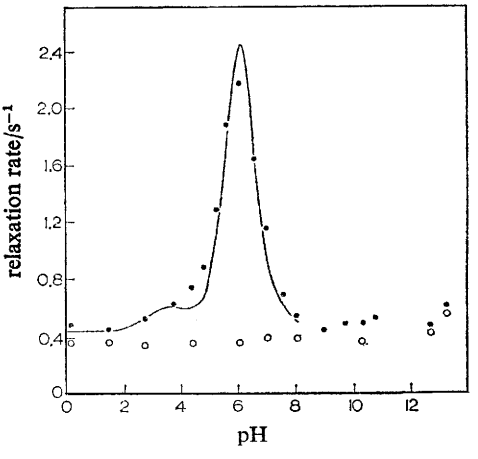

FIG. 5.—Relaxation rates for 1 M β-alanine solutions at 20°C; ○, T_1^{-1}; ●, T_2^{-1}.

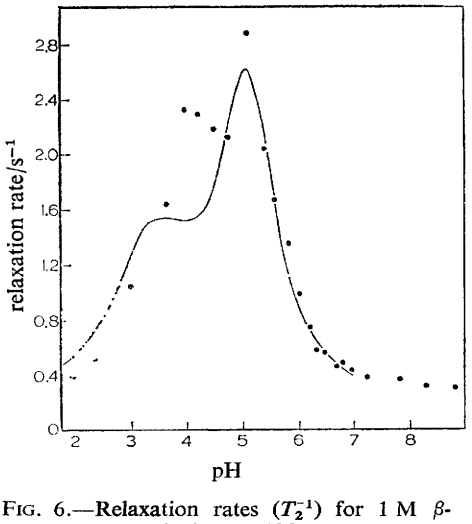

FIG. 6.—Relaxation rates (T_2^{-1}) for 1 M β-alanine at 49°C.

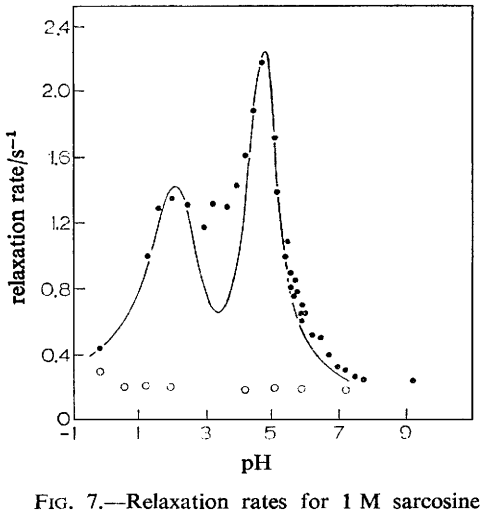

FIG. 7.—Relaxation rates for 1 M sarcosine solutions at 49°C; ○, T_1^{-1}; ●, T_2^{-1}.

very small quantity of ω-aminocaprylic acid was available and the concentration of 0.2 M again necessitated the use of sodium phosphate buffer to stabilize the pH.

Fig. 1-14 show a selection of the relaxation time measurements obtained. The continuous lines were calculated as explained in the discussion below.

The separation of the N-proton doublet in [^{15}N]glycine was found to be 75 Hz at pH zero and the shift of the doublet centre from the water peak 173 Hz.

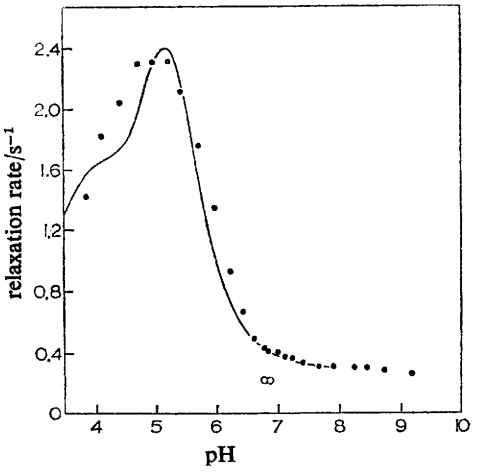

FIG. 8.—Relaxation rates for 1 M γ-aminobutyric acid solutions at 49°C; ○, T_1^{-1}; ●, T_2^{-1}.

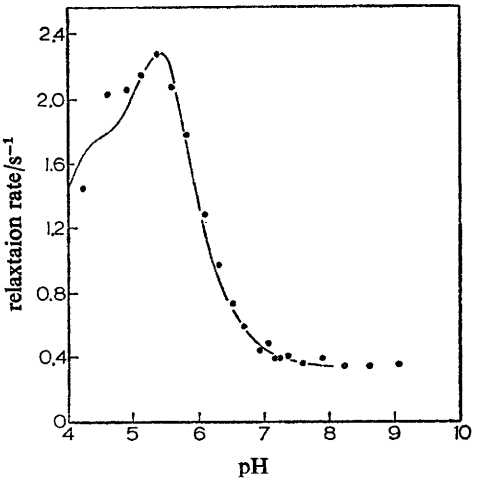

FIG. 9.—Relaxation rates (T_2^{-1}) for 1 M ε-aminocaproic acid solutions at 49°C.

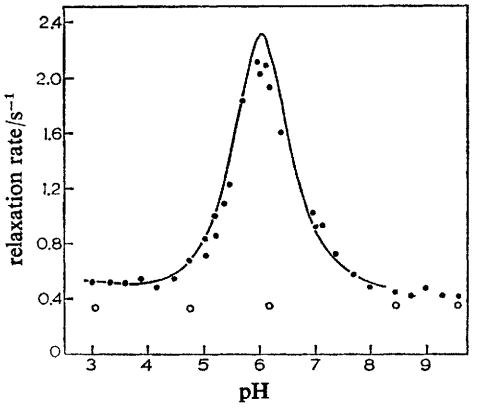

FIG. 10.—Relaxation rates for 1 M L-proline solutions at 25°C; ○, T_1^{-1}; ●, T_2^{-1}.

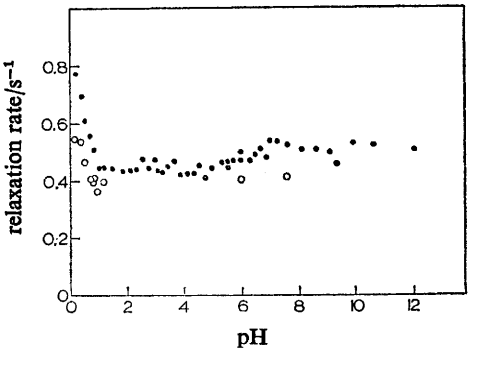

FIG. 11.—Relaxation rates for 1 M picolinic acid solutions at 22°C; ○, T_1^{-1}; ●, T_2^{-1}.

DISCUSSION

The results show that, within experimental error, T_1 for the amino-acids studied does not change appreciably with pH except in very alkaline solution. It is therefore reasonable to assume that over the same pH range, T_2° (absence of exchange) if it were measurable, would also not vary. For reasons given later, it is almost certain that at pH values above about 9, all exchange processes involving water molecules are extremely fast (i.e., $1/\tau \gg \omega$). In this case, $T_2 \rightarrow T_2^\circ$ and the latter can be estimated

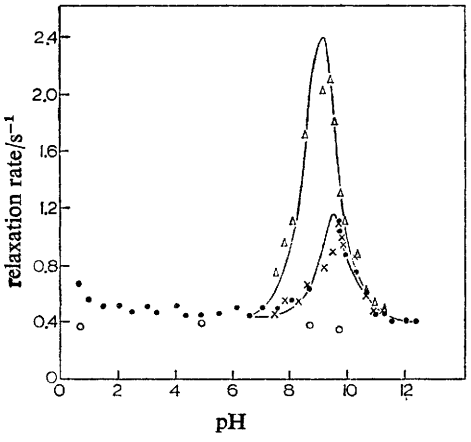

FIG. 12.—Relaxation rates for 1 M 2-pyrrolidone solutions at 25°C; ○, T_1^{-1} (0.2 M phosphate buffer); ●, T_2^{-1} (0.2 M phosphate buffer); ×, T_2^{-1} (0.1 M phosphate buffer, (tcp) = 0.758 ms); △, T_2^{-1} (0.1 M phosphate buffer, (tcp) = 2.13 ms).

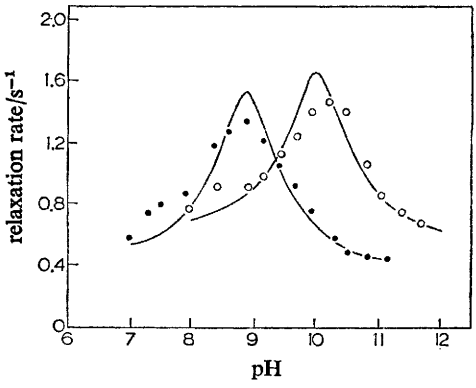

FIG. 13.—Relaxation rates (T_2^{-1}) for 0.5 M 2-pyrrolidone solutions in 0.1 M phosphate buffer ((tcp) = 2.13 ms); ○, 10°; ●, 25°C.

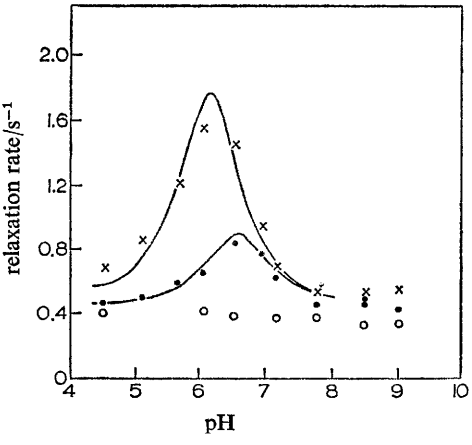

FIG. 14.—Relaxation rates for 0.2 M ω-aminocaprylic acid solutions in 0.2 M phosphate buffer, ○, T_1^{-1}; ●, T_2^{-1} ((tcp) = 0.758 ms); ×, T_2^{-1} ((tcp) = 2.13 ms).

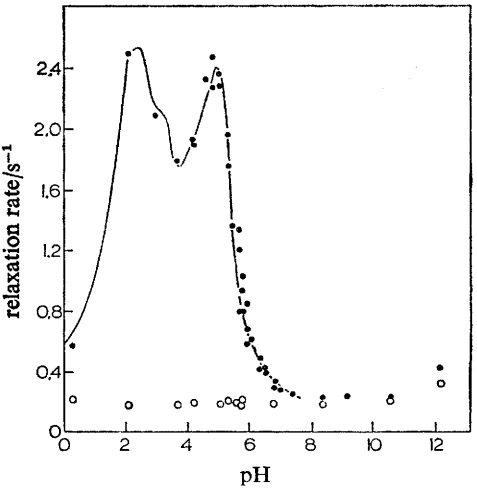

FIG. 15.—Relaxation rates for 1.0 M glycine solutions at 49°C, ○, T_1^{-1}; ●, T_2^{-1}.

for the whole pH range. In very alkaline solution, increased viscosity contributes towards a lowering of T_1 and T_2. For the series of simple amino-acids investigated, a plot of $1/T_2$-$1/T_2^{\circ}$ against pH shows the following three major features.

(*a*) There is always a peak within the pH range 4.5 to 7.5. The maximum occurs near to pH 6 at room temperature for all simple acids. The height of the peak increases slightly as temperature increases. The position of the peak moves to lower pH as temperature increases indicating that the rate of exchange increases with reduction of acidity.

(*b*) In many cases, a second peak is clearly present on the acid side of the primary peak. The pH value at this peak maximum is close to pK_{a1} for the amino acid. The second peak differs from the first in that its height is extremely temperature dependent and that its position does not change greatly with temperature.

(*c*) On the acid side of pK_{a1}, the quantity $1/T_2 - 1/T_2^\circ$ increases with temperature.

Assuming for the moment that the equation for simple exchange between two sites of equal T_2° is applicable, eqn (1) simplifies to an expression of the form

$$y = q\left(1 - q \tanh \frac{1}{q}\right) \tag{2}$$

where

$$q = \frac{2\tau}{(tcp)}, \quad y = A\left(\frac{1}{T_2} - \frac{1}{T_2^\circ}\right) \quad \text{and} \quad A = \frac{2}{p_a p_b (\delta\omega)^2 (tcp)}.$$

y (or the peak in $1/T_2$) has a maximum when

$$2\tau = 0.623(tcp). \tag{3}$$

Substitution shows that

$$\left(\frac{1}{T_2} - \frac{1}{T_2^\circ}\right)_{max} = 0.1325 p_a p_b (\delta\omega)^2 (tcp). \tag{4}$$

An important property of the maximum which allows the "fast" and "slow" sides to be recognised is that an increase in temperature shifts its position in the direction of increasing τ. The exchange mechanisms determine the relationship between pH and τ and, by comparing experimental points on a ($1/T_2$, pH) plot with a theoretical curve based on eqn (2), they can be identified.

A monoacidic monobasic amino-acid may be present in four forms, conveniently written RH_2^+, $RH^\pm$, RH and R^-. Exchange of protons between these species may occur without the intervention of water. In addition, exchange of protons with water also takes place. The simplest exchanges that can be written down are as follows:

[1] $$H_3O^+ + RH^\pm \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} H_2O + RH_2^+$$

[2] $$H_3O^+ + RH \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} H_2O + RH_2^+$$

[3] $$H_3O^+ + R^- \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} H_2O + RH^\pm$$

[4] $$H_3O^+ + R^- \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} H_2O + RH$$

[5] $$H_2O + R^- \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} OH^- + RH^\pm$$

[6] $$H_2O + R^- \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} OH^- + RH$$

[7] $$H_2O + RH^\pm \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} OH^- + RH_2^+$$

[8] $$H_2O + RH \underset{k_{-8}}{\overset{k_8}{\rightleftharpoons}} OH^- + RH_2^+.$$

The exchange of protons between water, oxonium and hydroxyl oxygen is so rapid that all these protons contribute to a single n.m.r. line. Furthermore, except in extremely acid conditions, the exchange with water of the carboxyl proton of both RH_2^+ and RH is very rapid and only a single sharp water line is seen. Reactions [1], [4], [6] and [7] which are all oxygen-oxygen proton exchanges are therefore unobservable. Reactions [2], [3], [5] and [8] are proton exchanges between oxygen (of water or oxonium ion) and nitrogen ($-\overset{+}{N}H-$). These would be simple two site exchanges were it not for the fact that the magnetic environment of nitrogen protons depends upon the spin states of the nitrogen nucleus and adjacent carbon attached, non exchanging, protons. The most drastic assumption in this work is that these short lived, different, magnetic environments are of no great importance. An average site is described which includes all protons attached to nitrogen. It is admitted that individual states within this site are largely, but not entirely, averaged out by nitrogen spin fluctuation and by intermolecular exchange as the results of Sheinblatt and Gutowsky [3] indicate. In other words, the simplifying assumption is that a proton interchanges between site A (oxygen attached) and site B (nitrogen attached) but that its movements while on site A or B are unimportant to this particular experiment. The protons on site A are present as OH^-, H_2O, H_3O^+ and —COOH. The protons on site B are present as $-\overset{+}{N}-H$ and $\diagdown N-H$. The following reactions must also be considered since they too consist of proton exchanges between sites A and B.

[9] $$RH_2^+ + RH \underset{k_{-9}}{\overset{k_9}{\rightleftharpoons}} RH_2^+ + RH^{\pm}$$

[10] $$RH \underset{k_{-10}}{\overset{k_{10}}{\rightleftharpoons}} RH^{\pm}$$

[11] $$RH + RH \underset{k_{-11}}{\overset{k_{11}}{\rightleftharpoons}} RH_2^+ + R^-$$

[12] $$RH + R^- \underset{k_{-12}}{\overset{k_{12}}{\rightleftharpoons}} RH^{\pm} + R^-$$

[13] $$RH_2^+ + R^- \underset{k_{-13}}{\overset{k_{13}}{\rightleftharpoons}} RH^{\pm} + RH^{\pm}.$$

The rate constants, k_{-l}, refer to the rate of transfer of protons from nitrogen to oxygen in each case. If τ_B is the average lifetime of any one proton on a B site,

$$\begin{aligned}\frac{3\Sigma B}{\tau_B} &= k_{-2}[H_2O][RH_2^+] + k_{-3}[H_2O][RH^{\pm}] + k_{-5}[OH^-][RH^{\pm}] \\ &\quad + k_{-8}[OH^-][RH_2^+] + k_{-9}[RH^{\pm}][RH_2^+] + k_{-10}[RH^{\pm}] \\ &\quad + k_{-11}[R^-][RH_2^+] + k_{-12}[R^-][RH^{\pm}] + k_{-13}[RH^{\pm}]^2. \qquad (5)\end{aligned}$$

ΣB is the total concentration of B sites, i.e., amino-acid concentration. The factor 3 arises because in most of the compounds studied there are three exchangeable nitrogen attached protons per molecule. In others (proline, sarcosine, 2-pyrrolidone) the appropriate factor is used. Each term in the expression for $3/\tau_B$ contains a fraction $x/\Sigma B$ to account for the fact that only this fraction of the B sites are occupied by species in the form of reactant x.

Now

$$\frac{[RH_2^+]}{\Sigma B} = \frac{[H_3O^+]^2}{[H_3O^+]^2+K_{a1}[H_3O^+]+K_{a1}K_{a2}} \tag{6}$$

and

$$\frac{[RH^\pm]}{\Sigma B} = \frac{K_{a1}[H_3O^+]}{[H_3O^+]^2+K_{a1}[H_3O^+]+K_{a1}K_{a2}}. \tag{7}$$

Only pH values considerably more acid than pK_{a2} are of interest here and this simplification leads to

$$\begin{aligned}\frac{3}{\tau_B} = \frac{1}{[H_3O^+]+K_{a1}}(&k_{-2}[H_2O][H_3O^+]+K_wk_{-8}+k_{-9}[H_3O^+][RH^\pm]\\ &+k_{-11}[R^-][H_3O^+]+K_{a1}k_{-3}[H_2O]+k_{-5}K_{a1}[OH^-]\\ &+K_{a1}k_{-10}+k_{-12}K_{a1}[R^-]+K_{a1}k_{-13}[RH^\pm]).\end{aligned} \tag{8}$$

The terms including k_{-8}, k_{-11}, k_{-3}, k_{-10} and k_{-13} are all constant at pH values between pK_{a1} and pK_{a2}. The experimental results indicate that certainly no large constant contribution to $1/T_2-1/T_2^\circ$ exists in this range and therefore that the contribution of these terms to $3/\tau_B$ must be very small at all pH values below pK_{a2}.

The expression reduces to

$$\begin{aligned}\frac{3}{\tau_B} = \frac{1}{[H_3O^+]+K_{a1}}(&k_{-2}[H_2O][H_3O^+]+k_{-9}[H_3O^+][RH^\pm]\\ &+k_{-5}K_{a1}[OH^-]+k_{-12}K_{a1}[R^-]).\end{aligned} \tag{9}$$

Only the terms involving k_{-5} and k_{-12} can account for the observed peak centred near to pH 6, since only these terms lead to an increase of exchange rate with pH. Insertion of approximate values for τ_B, $[R^-]$ and $[OH^-]$ lead to the alternative solutions $k_{-12}\simeq 10^8\ M^{-1}\ s^{-1}$ or $k_{-5}\simeq 10^{12}\ M^{-1}\ s^{-1}$. Sheinblatt and Gutowsky did not consider reaction [12] but for the reverse reaction [13], which is closely related to [12], they obtained $k_{-13}<10^3\ M^{-1}\ s^{-1}$. It is therefore most improbable that k_{-12} is as high as $10^8\ M^{-1}\ s^{-1}$ which would rule out reaction [12] as the cause of the central peak. A rate constant as high as $10^{12}\ M^{-1}\ s^{-1}$ on the other hand is also unexpected. Even with the large diffusion coefficients of oxonium and hydroxyl ions, the specific recombination rate of these ions in water at room temperature is less than $2\times 10^{11}\ M^{-1}\ s^{-1}$.[26] For glycine, the upper limit to the rate constant k_{-5} was found to be $2\times 10^{11}\ M^{-1}\ s^{-1}$.[3] It seems that the explanation for the central peak lies elsewhere. A possibility is

[14] $$RH^\pm+H_2O+R^-\underset{k_{-14}}{\overset{k_{14}}{\rightleftharpoons}}R^-+H_2O+RH^\pm.$$

This corresponds with the reaction shown to be of great importance in the amines.[4] Insertion of experimental values in the rate equation (below) leads to k_{14} of order $10^7\ M^{-2}\ s^{-1}$, which is most reasonable. Exchange reaction [14] is second order in amino-acid concentration; the pH of the maximum should show a first order shift in the slow direction with increase of concentration. Exchange reaction [5] would not result in a change of peak position with change of concentration. In fact, the the results for glycine show a small but definite shift of peak position with concentration in the predicted direction. To summarise, the most reasonable explanation of the central peak is the exchange reaction [14] which leads to the equation

$$\frac{3}{\tau_{B1}} = \frac{k_{14}[H_2O][R^-]K_{a1}}{[H_3O^+]+K_{a1}} \tag{10}$$

where τ_B is related to the τ of eqn (2) by

$$\tau = p_A\tau_B. \tag{11}$$

Labelling the total amino-acid concentration by M and noting that [RH] and [R^-] are only very small fractions of [$RH^\pm$] in the solutions of interest here, M = [RH_2^+]+[$RH^\pm$] near to pK_{a1}

$$[R^-] = [RH^\pm]K_{a2}/[H_3O^+]$$

and eqn (10) in convenient form becomes

$$\frac{3}{\tau_{B1}} = \frac{k_{14}[H_2O]K_{a1}^2K_{a2}M}{[H_3O^+](K_{a1}+[H_3O^+])^2}. \tag{12}$$

The second peak has a very temperature sensitive amplitude and is centred close to pK_{a1}. The explanation for this is evidently that an exchange process exists which is slow both above and below pK_{a1} and which leads to τ values of order (*tcp*) only in the vicinity of pK_{a1}. Inspection of the general eqn (8) shows that only the part referring to exchange reaction [9] can satisfy this requirement. In convenient form, this part becomes

$$\frac{3}{\tau_{B2}} = \frac{K_{a1}k_{-9}[H_3O^+]M}{(K_{a1}+[H_3O^+])^2}. \tag{13}$$

The reaction [15], analogous to [14] must also be considered

[15] $$RH_2^+ + H_2O + RH \underset{k_{-15}}{\overset{k_{15}}{\rightleftharpoons}} RH + H_2O + RH_2^+.$$

The relevant expression for this reaction is

$$\frac{3}{\tau_{B2}} = \frac{K_xk_{15}[H_2O][H_3O^+]M}{(K_{a1}+[H_3O^+])^2} \tag{14}$$

where K_x = [RH][H_3O^+]/[RH_2^+].

There is no *a priori* reason for favouring either reaction [9] or [15]; the observed peak must be regarded as resulting from contributions by both exchanges. Expressions [13] and [14] reach a maximum value when [H_3O^+] = K_{a1}

$$\left(\frac{3}{\tau_{B2}}\right)_{max} = \frac{k_{-9}M}{4} + \frac{k_{15}M}{4}\cdot\frac{K_x[H_2O]}{[H_3O^+]}. \tag{15}$$

The third observation, that there is a contribution to $1/T_2 - 1/T_2^\circ$ on the acid side of pK_{a1}, suggests an exchange process which involves the positive ion RH_2^+. The only appropriate reaction so far written down is reaction [2] for which

$$\frac{3}{\tau_{B3}} = \frac{k_{-2}[H_2O][H_3O^+]}{[H_3O^+]+K_{a1}}. \tag{16}$$

When $[H_3O^+] \gg K_{a1}$, $3/\tau_{B3} = k_{-2}[H_2O]$, which is constant at a given temperature. This value falls as pH increases towards pK_{a1} and is vanishingly small at higher pH values.

The theoretical curves shown in fig. 1 to 14 have been calculated from τ_B obtained from

$$\frac{1}{\tau_B} = \frac{1}{\tau_{B1}} + \frac{1}{\tau_{B2}} + \frac{1}{\tau_{B3}}$$

derived from eqn (12), (13), (14) and (16). The rate constants used are presented in table 2. These were obtained by a trial and error best fit of calculated curves to the experimental points in the following manner. A set of "standard" curves based on eqn (2) was drawn on tracing paper for a sequence of peak amplitudes. In these standard curves, y was plotted against $-\log q$ in order that they could be compared with $1/T_2$ values plotted against pH. By this means, for each graph, the pH value of the central peak maximum and the peak ampltiude were obtained. From these figures, k_{14} and $\delta\omega$ (eqn (4)) were calculated. The equilibrium constants are tabulated in the Appendix. The theory indicates that the central peak height is only dependent upon concentration and chemical shift, $\delta\omega$. As the temperature is raised the central peak moves to lower pH values and also increases in height, indicating an increase of chemical shift. In order to proceed further it was necessary

TABLE 1.—CHEMICAL SHIFTS (Hz) BETWEEN NITROGEN PROTONS AND WATER PROTONS

	pH							
	1	2	3	4	5	6	7	9
0.4 M glycine	172	167	162	158	153	148	144	
1.0 M glycine	174	167	159	152	145	138	130	
1.5 M glycine	170	163	156	149	143	136	129	
1.0 M α-alanine	171	164	158	151	144	138	131	
1.0 M β-alanine	174	166	159	152	145	138	131	
1.0 M sarcosine	190	184	179	174	169	163	158	
1.0 M γ-aminobutyric acid	159	155	151	147	143	139	135	
1.0 M ε-aminocaproic acid	145	143	141	139	137	135	133	
1.0 M L-proline	169	168	167	166	164	163	162	
0.2 M ω-aminocaprylic acid	—	—	—	—	—	144	—	
1.0 M 2-pyrrolidone	—	—	—	—	—	—	—	143

to make an assumption about the way the chemical shift varies with pH and temperature. The course adopted was to make the approximation that the chemical shift varies linearly with pH over the range pH 1 to 7 and that it is independent of temperature in the range considered. These approximations are only justified by the fact that they lead to a predicted value for the chemical shift in [^{15}N]glycine at pH zero in good agreement with that observed, that they are consistent with the observations and that in the past it has been the practice to assume that a chemical shift measured in the absence of exchange at low temperatures, remains invariant with increase of temperature. The approximations, if not truly valid, do not affect the calculated values of k_{14} but would have a small effect upon the curve shape and on the values of the other rate constants. It is very unlikely that there is a gross error from this source. Table 1 shows these chemical shifts, calculated from the observed variation in height of the central peak. The next step was to obtain the contribution to $1/T_2$ from reaction [14] at pH = pK_{a1} from eqn (12). This value of $(1/T_1)'$ was subtracted from the observed figure to give the contribution due to all other processes, $(1/T_2)''$. Strictly $1/T_2$ values are only additive if they are directly proportional to $1/\tau$ values. This is true on the slow side, well away from the central peak, at pH = pK_{a1}. The corrected value of $(1/T_2)''$ at pK_{a1} was then expressed as a fraction of the theoretical maximum value $(1/T_2)''$ could achieve at that pH for the calculated chemical shift and particular (*tcp*) interval. From this fraction and the "standard" curves described above, the value of $1/\tau_2$ resulting from the combined exchanges [9] and [15] was obtained and hence the value of $K_{a1}k_{-9}+K_x[H_2O]k_{15}$. k_{-2} was obtained in a similar manner from the observed $1/T_2$ value at pH zero, well below pK_{a1}.

The composite curve was then calculated from these three sets of parameters by computing the overall mean exchange frequency, $1/\tau$, at 0.3 unit intervals of pH over the measured range and hence $1/T_2 - 1/T_2^\circ$.

TABLE 2.—AMINO-ACIDS

system	temp./°C	k_{14}/M^{-2} s^{-1}	$k_{-9} + \frac{k_{15}K_x[H_2O]}{K_{a1}}$ / M^{-1} s^{-1}	k_{-2}/ M^{-1}s^{-1}	$k_{-10}+k_{-13}$M/ s^{-1}
0.4 M glycine	0	2.19×10^6	$<8\times10^2$	<1	—
	20	3.39	22	3.6	—
	49	5.83	430	18	1500
1.0 M glycine	0	1.51	9	<1	—
	20	2.34	32	2.1	270
	37	4.30	96	5.5	1100
	49	4.53	220	12	1400
1.5 M glycine	0	1.04	4	2.0	—
	20	2.02	22	3.0	360
	49	3.90	160	11	3000
1 M α-alanine	0	0.92	<3	<1	—
	20	1.79	7	(1)	—
	25	2.08	23	(3)	120
	49	3.39	167	(10)	1500
1 M β-alanine	0	2.25	4	—	—
	20	4.25	15	—	330
	25	3.86	19	—	333
	49	8.84	(114)	<1	(3000)
1 M sarcosine	0	1.59	<2	<1	—
	25	3.64	14	3.8	130
	49	9.01	84	5.3	1250
1 M L-proline	1	2.64	—	—	—
	25	5.84	(20)	—	—
	49	7.33	(100)	—	780
1 M γ-amino-n-butyric acid	10	4.76	7	—	—
	25	7.21	(24)	—	(220)
	49	13.1	(142)	—	(1400)
1 M ε-aminocaproic acid	1	3.29	<3	—	—
	25	9.04	(21)	—	—
	49	10.9	(173)	—	(1700)
0.2 M ω-aminocaprylic acid	25	26	—	—	—

Inspection of the graphs indicates that, in general terms, the shape of each theoretical line is correct. In addition, there is, very satisfactory agreement on the fast (basic) side of the central peak and agreement, within experimental error, on the acid side of pK_{a1}. There is, however, a region of pH lying between pK_{a1} and the central peak where the observed deviations are not accountable for by instrumental errors. In this region, further processes significantly reduce the mean exchange lifetime. The deviations are very small at 0°C but increase strongly with increase of temperature. For example, 1.5 M glycine at 49°C shows an unexplained difference in $1/T_2$ of 1.5 s^{-1}, equivalent to a process with a mean exchange frequency, $1/\tau$, of approximately 800 s^{-1}. Furthermore, it is apparent that this exchange frequency reaches a constant value in the pH region where the zwitterion concentration is constant and falls to near zero below pK_{a1}. If this were not so, other unexplained phenomena would be observed. Reactions [3], [8], [10], [11] and [13] are of the correct kinetic form. In order that reaction [3] should contribute measurably,

k_{-3} would have to exceed 2 M^{-1} s^{-1}. Sheinblatt and Gutowsky found k_{-3} to be <0.1 M^{-1} s^{-1} for glycine and sarcosine at 25°C and therefore reaction [3] can be neglected. k_{-8} would have to exceed 100 K_{a1}/K_w if reaction [8] were to contribute measurably. This is very improbable, since for all the amino acids under study, $pK_{a1} \leqslant 4.55$. At temperatures above 50°C, a small contribution to the exchange of the longer chain amino-acids might be observable if reaction [8] is diffusion-controlled. This reaction has previously been ignored. Similarly k_{-11} would have to exceed 100 K_{a1}/K_{a2} in 1 M solution if reaction [11] were to be of importance. This is 5×10^7 M^{-1} s^{-1} for the most favourable amino-acid. Now reactions [9], [11], [12] and [13] are all intermolecular proton exchanges between positively charged nitrogen and negative carboxylate ions and there is no reason to expect gross differences between respective rate constants. This present work indicates that k_{-9} is no greater than 500 M^{-1} s^{-1}. Elsewhere,[3] k_{-13} was measured as 10^3 M^{-1} s^{-1} or less. It is highly unlikely that k_{-11} contributes measurably here. Reactions [10] and [13] on the other hand could well account for the observed graph deviations. In the region of interest

$$\frac{3}{\tau_{B4}} = \frac{k_{-10}K_{a1}}{K_{a1}+[H_3O^+]} + \frac{k_{-13}K_{a1}M}{(K_{a1}+[H_3O^+])^2}. \qquad (17)$$

As the two terms on the right hand side of (17) have a different functional dependence on pH and concentration, they can in principle be separated. Unfortunately the relaxation times could not be measured with sufficient precision by means of the present apparatus. For this reason it has not been possible to include the contribution from $1/\tau_{B4}$ in drawing the theoretical curves. It is however possible to make a rough estimate of $k_{-10}+k_{-13}M$, the value of $3/\tau_{B4}$ well on the basic side of pH = pK_{a1}. These values are included in table 2 but they have an estimated possible error of 50 %. Only the glycine results permit a separation of k_{10} and k_{13} from the values at three different concentrations. These two constants were found to be equal in magnitude (in appropriate units) at 49°C including k_{-10} and k_{-13}. This showed very satisfactory agreement with the experimental points throughout the complete pH range (fig. 15).

The two major assumptions used in deriving the relation between exchange rate and measured relaxation time can now be examined. These are the existence of only two sites and the equality of T_2 on each site. The nitrogen protons give rise to a single n.m.r. line if the mean exchange frequency from any particular nitrogen atom is rather greater than the highest spin-spin coupling constant between the relevant proton and any other nucleus. The coupling with nitrogen is strongest. This coupling constant is unknown for the amino-acids. For [^{15}N]glycine, the observed separation of the N proton doublet at pH zero was 75 Hz. It is unlikely that the ^{14}N proton spin-spin coupling constant exceeds 100 Hz. If $1/\tau_B$ is greater than, say 600 s^{-1}, the assumption is valid. Calculations showed that this is certainly true in basic solution at all pH values down to and below the pH of the central maximum. At pH values below pK_{a1}, the assumption is not truly valid and this of course is the reason that the ^{15}N proton doublet can be seen in acid solution. The values of k_{-2} are therefore subject to an unassessable possible error. The assumption of equal T_2 cannot be examined in detail but is certain to be invalid. The effect of this is uncertain but, subject to the condition below, is probably small since, in the absence of exchange, the spin echo apparatus would only " see " the relaxation behaviour of the water protons which are present in many times the concentration of the protons of other species. Rather more serious is that if the N proton transverse relaxation time approaches the mean residence time of a proton on nitrogen, the exchanging

proton transfers additional phase on jumping from nitrogen to water. This could well be expected to make eqn (1) inapplicable. It is possible that this difficulty may arise here, in fairly acid solutions only, again perhaps affecting k_{-2}.

The values of k_{14} are of a very similar magnitude to the values of the rate constants for the same reaction of ammonia and the methylamines. These are expressed as pseudo second order constants in the original literature and they should be compared with $k_{14}[H_2O]$ in this work. k_{14}, at room temperature, ranges from 1.4×10^6 M^{-2} s^{-1} for ammonia to 1.4×10^7 M^{-2} s^{-1} for dimethylamine. Into this same range fall most of the values for the amino acids. For all these compounds there is a significant correlation between the acid dissociation constant of the substituted ammonium ion and k_{14}. The exchange is apparently favoured by reduced acidity of the ion. This would support the view of Grunwald *et al.*[8] that the rate determining step is the transfer of a proton from a water molecule in the solvation shell of the ion to the base molecule and not from the positive ion to a water molecule. Activation energies calculated from k_{14} are shown in table 3. With the exception of sarcosine they lie between 16 and 21 kJ mol^{-1}. Again these are very similar to the activation energies observed by Grunwald [9] and Loewenstein [10] for the corresponding reactions of di- and tri-methylamines.

TABLE 3.—ACTIVATION ENERGIES FOR REACTION [14]

	activation energy/ kJ mol^{-1}
0.4 M glycine	(10.7)
1.0 M glycine	19.9
1.5 M glycine	20.0
1.0 M α-alanine	19.9
1.0 M β-alanine	20.3
1.0 M sarcosine	25.8
1.0 M L-proline	16.5
1.0 M γ-aminobutyric acid	19.8
1.0 M ε-amniocaproic acid	19.3

Sheinblatt and Gutowsky obtained a value for k_{-9} at 25°C for glycine of 1.2×10^3 M^{-1} s^{-1}. When this is taken from the value obtained for the term which includes k_{-9} and k_{15} in this work, k_{15} is found to be 1.0×10^7 M^{-2} s^{-1}. This again is very similar in order of magnitude to k_{14} as expected from the reactions involved. More precise work would be needed to enable the temperature dependance of k_{-9} and k_{15} to be separated. The ratio K_x/K_{a1} is the ratio of the concentrations of uncharged amino-acid, RH, and zwitterion, $RH^{\pm}$. Its temperature dependence has not been measured but since the heat absorbed in transforming $RH^{\pm}$ into RH is of the order of 50 kJ mol^{-1},[11] K_x/K_{a1} increases quite strongly with temperature.

Reaction (2) is the exchange between cation and water which has been studied in some detail by conventional n.m.r. The results here are necessarily imprecise for the reasons given but are of the same order of magnitude as those obtained previously. They do not provide any information on the proposed mechanism.

Rate constant k_{-10} for glycine at 25°C has been determined elsewhere and agrees closely at 25°C with the calculated value here. Reaction (13) has not been considered previously.

It is seen that the proposed kinetic scheme is consistent with the experimental observations and with the results of other workers. The spin echo measurements have provided some information on those exchange reactions [13], [14] and [15] that have not been considered in connection with amino acid solutions elsewhere. It

should be noted that while two of these reactions are formally termolecular, they almost certainly result from a bimolecular collision of an ion or molecule with a solvated ion.

K_{a1} and K_{a2} have not been measured for ω-aminocaprylic acid. The trend of these dissociation constants is very clear from the lower members of the series; extrapolated values are shown in the Appendix. The pH range studied affords an estimation of k_{14} only. In order to increase sensitivity to the relatively low concentration, a second set of measurements was made at a larger pulse interval, (*tcp*). Both solid lines in fig. 14 were calculated from $k_{14} = 2.6 \times 10^7$ M^{-2} s^{-1}. The agreement is very satisfactory although, as expected, there is some contribution on the slow side from other exchanges.

Picolinic acid is an α-amino-acid which contains the nitrogen atom within an aromatic ring. The results show that T_2 does not vary outside the limits of experimental error over the pH range 1 to 12. Both T_1 and T_2 fall sharply in solutions more acid than pH 1. The acid-base behaviour of the pyridinemonocarboxylic acids has been studied by several workers whose results have been reviewed and extended by Green and Tong.[12] They used the well established method of Ebert [13] to determine the proportion of uncharged molecules to zwitterions present at any concentration. This employs the assumption that the acid dissociation constant of the methyl ester, $\overset{+}{H}NRCOOMe$, is the same as that of the N proton of the acid, $\overset{+}{H}NRCOOH$. Both pK_{a1} and pK_{a2} are very much lower than those of the simple aliphatic amino-acids and furthermore, at 22°C, the concentration of uncharged molecules is as high as 6.3 % of the zwitterions. This is in strong contrast to the other compounds so far considered. The result is that exchange reaction [15] is strongly favoured and provided that k_{15} exceeds about 10^4 M^{-2} s^{-1} and that the other rate constants are not too different from those of the simple amino-acids, the exchange rate is very rapid in solutions less acid than pH 1. It is because $1/\tau \gg 1/(tcp)$ for all pH $\geqslant$ 1 that no significant change in T_2 with pH can be seen. Below pH 1 it is expected that the exchange rate falls but there is evidence of complex formation in very acid solution; this may account for the observed decrease of both T_1 and T_2 in this region.

2-Pyrrolidone is a lactam and so contains the peptide grouping as part of a ring. The lactam is a very weak acid [14] ($pK_{a2} = 15.3$) and the cation is a strong acid [15] ($pK_{a1} = -0.8$). Solutions of 2-pyrrolidone are not self buffering and sodium phosphate buffer was used. No difference was found between relaxation times of solutions at two different buffer concentrations. Within the pH range 1 to 12 a single peak in $1/T_2$ was found. The experiments were repeated at a different pulse repetition frequency and temperature in order to locate the fast and slow sides. The fast side clearly lies in the direction of increasing pH. Of the exchanges described above, only reactions [5] and [14] are of the correct form to account for the observed behaviour. The alternative rate constants would be approximately $k_{-5} = 10^8$ M^{-1} s^{-1} or $k_{14} = 10^7$ M^{-2} s^{-1}. Neither value is impossible. However, the peak

TABLE 4.—2-PYRROLIDONE

temp./°C	conc./M	(*tcp*)/ms	k_{-5}/M^{-1} s^{-1}
25	1.0	0.758	1.32×10^8
25	1.0	2.13	1.48×10^8
25	0.5	2.13	1.88×10^8
10	0.5	2.13	5.15×10^8

pH in both 0.5 M and 1.0 M solution lies within 0.05 pH unit of 8.95 at 25°C (tcp = 2.13 ms). If reaction [14] were responsible, a shift of 0.30 pH unit would be expected. Therefore, most probably, reaction [5] produces the fast exchange rate. The results in table 4 have been calculated on this basis. The value of k_{-5} at 25°C lies between that for *N*-methylacetamide and that for glycylglycine. This is expected if k_{-5} follows the predicted order of acidity of the exchanging proton in these compounds.

APPENDIX

DISSOCIATION CONSTANTS

compound	temp./°C	pK_{a1}	pK_{a2}	pK_a	pK_x (25°C)	ref.
glycine	0	2.454	10.528			16, 17
	20	2.364	9.918		7.70	
	37	2.323	9.482			
	49	2.306	9.204			
α-alanine	0	2.430	10.614			18, 17
	20	2.360	10.011		7.80	
	25	2.348	9.866			
	49	2.331	9.278			
β-alanine	0	3.66	11.00			19, 17
	20	3.57	10.38		9.13	
	25	3.55	10.23			
	49	3.50	9.62			
sarcosine	0	2.25	10.44			20
	25	2.23	10.01			
	49	2.05	9.52			
γ-aminobutyric acid	10	4.057	11.026			21, 22
	25	4.031	10.556		9.71	
	49	4.031	9.905			
ε-aminocaproic acid	1	4.420	11.666			23, 17
	25	4.373	10.804		10.37	
	49	4.407	10.060			
ω-aminocaprylic acid	25	4.55	10.90			extrapolated value
L-proline	1	2.011	11.296			24
	25	1.952	10.640			
	49	1.957	10.089			
2-pyrrolidone	25	(−0.8)	15.3			14, 15 (estimated)
ammonium	25			9.25		25
methylammonium	25			10.62		
dimethylammonium	25			10.78		
trimethylammonium	25			9.80		
picolinic acid	22	1.01	5.32		2.21	12

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