The Activity Ratio of Zwitterions and Uncharged Molecules in Ampholyte Solutions.   The Dissociation Constants of Amino Acid Esters

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A simple amino acid, such as glycine, may exist in either of two electrically neutral forms: (1) the "zwitterion," $^+H_3NRCOO^-$, and (2) what we may call the "uncharged molecule," $H_2NRCOOH$. The zwitterion possesses an extremely high electric moment; it is very soluble in water, nearly insoluble in organic solvents, and its melting point is high. The uncharged molecule has a relatively small electric moment; it has, relatively to the corresponding zwitterion, a greater affinity for organic solvents than for water and melts at a lower temperature. The two forms also differ greatly in their heats of reaction with acids and bases, and in other properties. One form may pass into the other by molecular rearrangement: $H_2NRCOOH \rightleftharpoons {}^+H_3NRCOO^-$. The equilibrium constant of this reaction will be denoted as K_Z

$$\frac{(^+H_3NRCOO^-)}{(H_2NRCOOH)} = K_Z \tag{1}$$

In an infinitely dilute aqueous solution of the amino acid, which will be taken as the standard state in all that follows, K_Z is equal to the ratio of the concentrations (or mole fractions) of zwitterions and uncharged molecules in solution. In any other state, at the same temperature and pressure, K_Z is by definition the activity ratio of the two forms. The value of this constant is thus an important index of many physico-chemical properties of the system containing the amino acid.

It has now been abundantly demonstrated[1] that the zwitterion form is predominant ($K_Z \gg 1$) in aqueous solutions of the aliphatic amino acids, whereas in the aminobenzoic acids, the uncharged molecule appears to predominate ($K_Z \leqq 1$). Bjerrum and Ebert have estimated K_Z for a number of amino acids by various methods. Of these the most accurate appears to be one developed by Ebert, involving the dissociation constants of the amino acids and their esters. The data on which Ebert based his calculations were (as he pointed out) few and scattered, and not highly accurate. Since that time Emerson and Kirk[2] have determined accurately

(1) Bjerrum, Z. physik. Chem., **104**, 147 (1923); Michaelis and Mizutani, ibid., **116**, 135 (1925); Ebert, ibid., **121**, 385 (1926); Harris and Birch, Biochem. J., **24**, 1080, 1086 (1930); Weber, Biochem. Z., **218**, 1 (1930); Miyamoto and Schmidt, Univ. Cal. Pub. Physiol., **8**, 1 (1932); Cohn, Naturwissenschaften, **20**, 663 (1932). Concerning dielectric constants of amino acids, see Hedestrand, Z. physik. Chem., **135**, 36 (1928); Devoto, Gazz. chim. ital., **60**, 528 (1930); **61**, 906 (1931); **63**, 50 (1933); Atti. Accad. Lincei, **15**, 471 (1932); Wyman and McMeekin, THIS JOURNAL, **55**, 908, 915 (1933). Concerning solubilities, see Bjerrum and Cohn (above), and Cohn, McMeekin, Edsall and Weare, Proc. Am. Soc. Biol. Chem., **7**, No. 6, 44 (1931), and unpublished data.

(2) Emerson and Kirk, J. Biol. Chem., **87**, 597 (1930).

the apparent dissociation constant of glycine ethyl ester.   Other esters of amino acids, however, have not been studied; and we have attempted to remedy this deficiency, by the experiments reported below, from which conclusions can be drawn as to the relation between K_Z and chemical structure.   We have also investigated the effect of temperature upon K_Z; and the relation of the dielectric constant of the medium to the relative number of zwitterions and uncharged molecules present.   For the latter purpose we have extended the existing data on the dissociation constants of amino acids and their esters in alcohol–water mixtures, following the procedure of Michaelis and Mizutani.

The basis of the method may be briefly stated.[3]   The cation of the amino acid ($^+H_3NRCOOH$) may give off a hydrogen ion from either the carboxyl or the charged amino group; $i.$ $e.$, two dissociation constants are involved

$$\frac{(H^+)(^+H_3NRCOO^-)}{(^+H_3NRCOOH)} = K_A \qquad (2) \qquad \frac{(H^+)(H_2NRCOOH)}{(^+H_3NRCOOH)} = K_B \qquad (3)$$
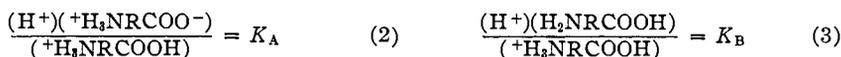
Likewise each of the two isoelectric forms may give off a hydrogen ion to form the amino acid anion

$$\frac{(H^+)(H_2NRCOO^-)}{(^+H_3NRCOO^-)} = K_C \qquad (4) \qquad \frac{(H^+)(H_2NRCOO^-)}{(H_2NRCOOH)} = K_D \qquad (5)$$

The activity ratio of zwitterions to uncharged molecules is, in terms of these constants

$$K_Z = \frac{(^+H_3NRCOO^-)}{(H_2NRCOOH)} = \frac{K_A}{K_B} = \frac{K_D}{K_C} \qquad (6)$$

The dissociation constants, as determined directly by titration, are related as follows to the constants given above

$$K_1 = \frac{(H^+)[(^+H_3NRCOO^-) + (H_2NRCOOH)]}{(^+H_3NRCOOH)} = K_A + K_B \qquad (7)$$

$$K_2 = \frac{(H^+)(NH_2RCOO^-)}{[(^+H_3NRCOO^-) + (H_2NRCOOH)]}; \quad \frac{1}{K_2} = \frac{1}{K_C} + \frac{1}{K_D} \qquad (8)$$

K_1 and K_2 can be determined by direct experiment.   If we know in addition any one of the four constants K_A, K_B, K_C and K_D, the values of the other three are uniquely determined by equations (6), (7) and (8).   Of these constants, K_B can be determined as the dissociation constant of the ester of the amino acid in question, if we assume that the —COOH group is identical with the —COOCH$_3$ or —COOC$_2$H$_5$ group in its effect on the dissociation of a neighboring group.   That this assumption is a close approximation to the truth has been shown by Wegscheider.[4,5]

(3) E. Q. Adams, THIS JOURNAL, **38**, 1503 (1916); Ebert, Z. physik. Chem., **121**, 385 (1926).

(4) Wegscheider, Monatsh., **16**, 153 (1895); **23**, 287 (1902).

(5) The effect of a substituent on acid and basic dissociation is closely related to its dipole moment. It is therefore worthy of note that the dipole moments of fatty acids and their esters (excepting formic acid and the formates) are almost identical.   Smyth ("Dielectric Constant and Molecular Structure," New York, 1931, Appendix) gives for acetic acid $\mu = 1.73 \times 10^{-18}$ e.s.u., for propionic acid 1.74, and for a variety of fatty acid esters values ranging from 1.75 (methyl acetate) to 1.87×10^{-18} (isobutyl acetate).   These results appear to be strong independent evidence for the approximate correctness of Wegscheider's conclusions.

We assume, then

$$K_B = \frac{\left(R\begin{smallmatrix}NH_2\\COOH\end{smallmatrix}\right)(H^+)}{\left(R\begin{smallmatrix}NH_3{}^+\\COOH\end{smallmatrix}\right)} = \frac{\left(R\begin{smallmatrix}NH_2\\COOC_2H_5\end{smallmatrix}\right)(H^+)}{\left(R\begin{smallmatrix}NH_3{}^+\\COOC_2H_5\end{smallmatrix}\right)} = K_E \qquad (9)$$

It should be remembered in all that follows that equation (9) is only[5] approximate, in contrast to the earlier equations, which are exact. Assuming $K_B = K_E$, however, we may derive from (6), (7) and (8) the following relations

$$K_A = K_1 - K_E \qquad (10) \qquad\qquad K_D = \frac{K_1 K_2}{K_E} \qquad\qquad (12)$$

$$K_C = \frac{K_1 K_2}{K_1 - K_E} \qquad (11) \qquad\qquad K_1 K_2 = K_A K_C = K_B K_D = K_E K_D \qquad (13)$$

and for K_Z

$$K_Z = \frac{K_A}{K_B} = \frac{K_A}{K_E} = \frac{K_1}{K_E} - 1 \qquad (14)$$

Hence, if K_1 is very much larger than K_E (which proves to be the case for all the aliphatic amino acids) we have, as a close approximation

$$K_Z = K_1/K_E \qquad (15) \qquad\qquad \text{and } \log K_Z = pK_E - pK_1 \qquad (15a)$$

and likewise

$$K_D = K_2 K_Z \qquad (16) \qquad\qquad \text{and } pK_D = pK_2 - \log K_Z \qquad (16a^6)$$

Experimental

The hydrochlorides of the amino acid esters are in general more stable than the esters themselves. We have therefore prepared the hydrochlorides, and determined the dissociation constants by titrating them electrometrically with sodium hydroxide.

Preparation of Materials.—The ester hydrochlorides were prepared in the usual manner[7] by the action of dry hydrogen chloride gas on a suspension of the amino acid in absolute alcohol. They were purified, after the alcohol was evaporated off *in vacuo*, by being dissolved in absolute alcohol and crystallized by addition of ether. Chloride content was determined by the method of Wilson and Ball.[8] Melting points were determined in several cases, but not in all. The accompanying table gives the substances prepared.

ESTER HYDROCHLORIDES OF AMINO ACIDS

Hydrochloride of esters	Cl calcd., %	Cl found, % (1)	(2)	M. p. (uncorr.), °C.
Glycine methyl	28.25	28.25	28.18	.....
α-Alanine ethyl[a]	23.09	22.84	22.94	87–88
β-Alanine ethyl[b]	23.09	22.92	22.84	69–70

(6) In accordance with the definition of K_Z, it is clear that K_1, K_2 and K_E should all be taken as the true dissociation constants at infinite dilution. In general, however, the known values at present are the apparent dissociation constants at a finite concentration (generally 0.04 molal). In using these apparent values instead of the true dissociation constants, an error is introduced into the evaluation of K_Z. The uncertainty should not be greater than about 0.2 in log K_Z, but it cannot yet be evaluated exactly.

(7) Fischer, *Sitzber. Berliner Akad.*, 1062 (1900).

(8) Wilson and Ball, *J. Biol. Chem.*, **79**, 221 (1928).

ESTER HYDROCHLORIDES OF AMINO ACIDS   (*Concluded*)

Hydrochloride of esters	Cl calcd., %	Cl found, % (1)	Cl found, % (2)	M. p., (uncorr.), °C.
α-Amino-*n*-butyric acid ethyl[c]	21.16	21.36	21.23	139–140
dl-Leucine ethyl[d]	18.13	18.16	18.21	115
Glycylglycine ethyl[e]	17.78	18.04	18.04	.....
ε-Aminocaproic acid ethyl[f]	18.13	18.10	...	.....
Aspartic acid diethyl[g]	15.72	...	...	.....

[a] Melting point agrees with value of Johnson and Ticknor, THIS JOURNAL, **40**, 642 (1918).   [b] Prepared from succinimide. See Hoogewerf and van Dorp, *Rec. trav. chim. Pays-Bas*, 10, 5 (1891), and Holm, *Arch. Pharm.*, 242, 590 (1904).   Melting point agrees with Weidel and Roithner, *Monatsh.*, 17, 172 (1896).   [c] Curtius and Müller, *Ber.*, 37, 1273 (1904), give 130.5° as the melting point.   [d] Röhmann, *Ber.*, **30**, 1980 (1897), gives melting point as 134°, but it is not clear whether he refers to the methyl or the ethyl ester.   [e] Prepared by method of Fischer and Fourneau, *Ber.*, 34, 2868 (1901).   [f] See Ruzicka, *Helv. Chim. Acta*, 4, 472 (1921).   [g] Chloride content was about 5% too low, and further purification was not undertaken.   Hence reported dissociation constant is less accurate than other values reported.

ε-Aminocaproic acid was prepared from cyclohexanone oxime by the method of Wallach.[9]   A pure sample of β-aminobutyric acid was supplied to us by Dr. T. L. Mc-Meekin, to whom we are indebted for much valuable advice in the preparation of the esters.

The Determination of the Dissociation Constants.—The titrations were carried out on a water-jacketed hydrogen electrode of the bubbling type, in conjunction with a 0.1 N potassium chloride calomel half cell, with a saturated potassium chloride bridge. The electrodes were standardized with 0.1 N hydrochloric acid, whose P_H is taken as 1.076.[10]   The e. m. f. of the calomel half-cell is taken as 0.3353 volt at 25°, the liquid junction potential being neglected.   All measurements were made at 25° except a few taken at 20 and 30° to study the effect of temperature.   A 2-cc. sample of the ester hydrochloride solution (generally 0.04 molal) was titrated with sodium hydroxide (of twice the equivalent concentration) which was run in from a 1-cc. buret, graduated in 0.01 cc. divisions, which could be read to 0.002 cc.   The logarithmic values of the dissociation constants (pK') were calculated by the equation

$$pK' = P_H + \log \frac{\text{(ester hydrochloride)}}{\text{(free ester)}} = P_H + \log R$$

the amount of free ester present being taken as equivalent to that of the alkali added.

The free esters of the amino acids all tend to hydrolyze rapidly in aqueous solution. Up to the point of half neutralization or beyond, however, the e. m. f. readings were very steady over a period of a half hour or more, and the calculated values of pK from the different points on the titration curve were in good accord.   With increasing alkalinity, the calculated apparent values of pK' shifted more and more toward the acid side, presumably because the free ester hydrolyzes more rapidly than the hydrochloride.

In Tables I and II are given titration curves of glycylglycine ethyl ester hydrochloride, and of β-alanine ethyl ester hydrochloride, which are typical examples of the titration curves obtained.   Table III gives a complete summary of the dissociation constants obtained in aqueous solution.

(9) Wallach, *Ann. Chem.*, **312**, 171 (1900).
(10) Scatchard, THIS JOURNAL, **47**, 698 (1925).

TABLE I

GLYCYLGLYCINE ETHYL ESTER HYDROCHLORIDE, 0.0406 MOLAL, 2 Cc.   TITRATED WITH
0.08 M NaOH.   TEMPERATURE, 25.2°

Time	NaOH added, cc.	P_H	log R	pK'
3.25	0.100	6.777	0.962	7.739
3.30	.100	6.784	.962	7.746
3.35	.200	7.141	.610	7.751
3.40	.300	7.376	.377	7.753
3.45	.400	7.575	.187	7.762
3.50	.500	7.718	.013	7.731
3.55	.500	7.708	.013	7.721
4.00	.600	7.858	−.160	7.698
4.05	.700	8.020	−.347	7.673
4.10	.800	8.213	−.570	7.643
4.15	.900	8.460	−.893	7.567
4.20	.900	8.444	−.893	7.551

There is obviously a steady fall in the apparent value of pK' after more than 0.4 cc. of sodium hydroxide has been added.   The subsequent points, therefore, are given no weight in estimating the value of pK', which is taken as 7.75.

TABLE II

β-ALANINE ETHYL ESTER HYDROCHLORIDE, 0.0420 MOLAL.   TITRATED WITH NaOH,
0.08 M.   TEMPERATURE, 25°

Time	NaOH added, cc.	P_H	log R	pK'
3.20	0.100	8.134	0.978	9.112
3.25	.100	8.136	.978	9.114
3.30	.200	8.525	.628	9.153
3.35	.300	8.758	.398	9.156
3.40	.400	8.941	.211	9.152
3.45	.400	8.949	.211	9.160
3.50	.500	9.109	.041	9.150
3.55	.600	9.265	−.125	9.140
4.00	.700	9.399	−.301	9.098
4.05	.700	9.406	−.301	9.105
4.10	.800	9.578	−.507	9.071
4.15	.900	9.768	−.778	8.990
4.20	.900	9.746	−.778	8.968

Here again there is a similar fall in the apparent pK' values after 0.6 cc. of sodium hydroxide has been added.   The value of pK' is taken as 9.15.   (Two other titrations gave values of 9.12 and 9.13 for pK', from which the final value is taken as 9.13.)

Titrations in Alcohol–Water Mixtures.

The method employed was essentially that of Michaelis and Mizutani.[1]   A given buffer mixture of an amino acid and its hydrochloride was made up to a fixed volume concentration in water and in various alcohol–water mixtures, and the e. m. f. measured on the cell

$$H_2 \mid \text{amino acid solution} \mid\mid \text{saturated KCl} \mid\mid 0.1 \ N \ \text{KCl} \mid \text{HgCl} \mid \text{Hg}$$
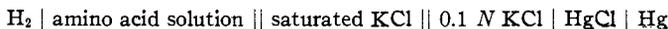
As in water, P_H values were calculated from the formula

$$P_H = \frac{\text{E. m. f. observed} - 0.3353}{0.00019837 \ T}$$

<div align="center">TABLE III</div>

VALUES OF pK' FOR AMINO ACID ESTERS AND FOR ε-AMINOCAPROIC ACID

Substance	Concentration	Temp., °C.	pK'
Glycine methyl ester	0.0400	20.1	7.80
		25	7.66
		30	7.51
α-Alanine ethyl ester	.0240	25	7.80
β-Alanine ethyl ester	.0400	25	9.13
α-Aminobutyric acid ethyl ester	.0180	25	7.53
	.0405	25	7.71
	.0759	25	7.80
Leucine ethyl ester	.0217	25	7.63
Aspartic acid diethyl ester	.04	25	6.5
ε-Aminocaproic acid ethyl ester	.0400	25.1	10.37
Glycylglycine ethyl ester	.0400	25.2	7.75
ε-Aminocaproic acid pK_1	.0401	25.1	4.43
ε-Aminocaproic acid pK_2	.0401	25.1	10.75

where T is 298.1°. The negative logarithm of the apparent dissociation constant (pK^*) was calculated by the formula

$$pK^* = P\text{H} + \log \frac{\text{(amino acid hydrochloride)}}{\text{(amino acid)}}$$

(concerning the interpretation of pK^*, see the discussion below).

The second term on the right of this equation should be corrected for the concentration of hydrogen ions dissociated from the hydrochloride. Nothing is at present known with certainty, however, as to the relation between PH and the absolute value of hydrogen-ion concentration in al-cohol–water mixtures. Hence we have not attempted to make this correction, but simply wish to point out that it should be made when further knowledge concerning these systems is attained. In water the correction factor can be fairly accurately estimated, and the corrected pK or (pK^*) values in water are given in brackets in Table IV. As the PH values rise rapidly with increasing alcohol concentration, it appears cer-tain (on any probable assumption regarding the relation between PH and C_H) that this correction term diminishes, and in 90% alcohol should not (on the most unfavorable assumptions) alter the calculated value of pK^* by more than 0.04. In β-aminobutyric acid and ε-aminocaproic acid, the correction term is very small, even in water.

The PH of equimolal buffer mixtures of glycine and sodium glycinate in alcohol–water mixtures was also measured. In agreement with Michaelis and Mizutani, the PH of these systems was found not to alter significantly between water and 90% alcohol. The measurements are not reported here. Similar measurements in alkaline solutions on other amino acids gave unstable e. m. f. readings, and are, therefore, likewise not reported. All alcohol percentages are given by volume. All PH values are averages of at least three or four closely agreeing readings.

TABLE IV
TITRATIONS IN ALCOHOL–WATER MIXTURES AT 25°

C₂H₅OH by volume, %	Glycine		Alanine		Leucine		β-Aminobutyric acid	ε-Aminocaproic acid	
	P_H	pK_1*	P_H	pK_1*	P_H	pK_1*	$P_H = pK_1*$	P_H	pK_1*
0	2.937	2.57 (2.42)	2.760	(2.43) 2.59	2.695	2.60 (2.34)	3.559 (3.533)	4.266	4.442 (4.436)
10	2.975	2.61	2.781	2.61	...	..	...	...	..
20	3.080	2.71	2.902	2.73	2.827	2.74	3.724	4.498	4.67
30	3.130	2.76	2.983	2.81	2.942	2.85	3.824	4.783	4.96
40	3.287	2.92	3.141	2.97	...	..	4.007	5.005	5.18
50	3.389	3.02	3.278	3.10	3.325	3.24	4.117	5.288	5.46
60	3.571	3.20	3.459	3.28	...	..	4.367	5.537	5.71
70	3.775	3.41	3.665	3.49	3.66	3.57	4.548	5.870	6.05
80	4.02	3.65	3.929	3.75	3.92	3.83	...	6.127	6.30
90	...	3.79	4.234	4.06	4.20	4.11	5.133	6.486	6.66

Glycine 0.014 M, glycine hydrochloride 0.006 M; alanine 0.012 M, alanine hydrochloride 0.008 M; leucine 0.0086 M, leucine hydrochloride 0.007M; β-aminobutyric acid 0.01 M, β-aminobutyric hydrochloride 0.01 M; ε-aminocaproic acid 0.008 M, ε-aminocaproic hydrochloride 0.012 M. The values in brackets are the corrected values of pK_1* in water (see text).

The values of pK_1' for glycine are in good agreement with those of Michaelis and Mizutani (see footnote 1). The value for glycine in 90% alcohol is taken from their paper.

The ethyl ester hydrochlorides of alanine and leucine were also titrated with base in 90 per cent. alcohol, and e. m. f. readings taken. The instability of the esters increased the uncertainty of the readings considerably, but in each case the apparent value of pK' shifted from its value in water (7.7) to approximately 6.8. The magnitude of this shift is in good agreement with that found by Mizutani[11] for the aliphatic amines, and appears to be characteristic for the amino group (see discussion below).

Discussion

The Relation of K_Z to Chemical Structure.—The logarithmic dissociation constants of the esters studied in this paper, together with those of the corresponding amino acids, are presented in Table V. With them are tabulated the values of log K_Z and pK_D, as calculated from equations (15a) and (16a).

TABLE V

LOGARITHMIC DISSOCIATION CONSTANTS OF AMINO ACIDS AND THEIR ESTERS[a] AT 25°

Substance	pK_1	pK_2	pK_{Ester}	log $K_Z =$ $pK_E - pK_1$	pK_D
Glycine	2.31	9.72	7.73 (ethyl)	5.42	4.30
			7.66 (methyl)		
α-Alanine	2.39	9.72	7.80	5.41	4.31
α-Aminobutyric acid	2.55	9.60	7.71	5.16	4.44
Leucine	2.34	9.64	7.63	5.29	4.35
β-Alanine	3.60	10.19	9.13	5.53	4.66
ε-Aminocaproic acid	4.43	10.75	10.37	5.94	4.81
Glycylglycine	3.14	8.07	7.75	4.61	3.46
Aspartic acid	2.08	3.87	6.5	..	..
		(pK_3) 9.85			

[a] The values for pK_1 and pK_2 are taken from the review by Cohn, *Ergebnisse Physiol.*, **33**, 822, 842 (1931), except those for α-aminobutyric acid, which are from Czarnetsky and Schmidt, *Z. physiol. Chem.*, **204**, 129 (1932), and those for ε-aminocaproic acid, from the present investigation. The value of pK_E for glycine ethyl ester is from Emerson and Kirk, *J. Biol. Chem.*, **87**, 597 (1930); the other ester values are from the present investigation.

It is clear that the values of K_Z are very great for all these substances, ranging from about 40,000 for glycylglycine to nearly 1,000,000 for ε-aminocaproic acid. These values are even higher than those estimated by Bjerrum and Ebert; and in spite of some uncertainty as to the accuracy of the assumptions involved in this evaluation of K_Z, they can leave no doubt that the zwitterion is overwhelmingly predominant in all these solutions.

The values of pK_D (about 4.3 for the α-amino acids as compared to 4.8 for the unsubstituted fatty acids) represent the effect of an *uncharged* amino group on the dissociation of a neighboring carboxyl. Evidently the amino group produces a moderate increase in the acidity of the carboxyl (at least in aliphatic amino acids); a result in accord with the views of Vorländer[12]

(11) Mizutani, *Z. physik. Chem.*, **116**, 350 (1925).
(12) Vorländer, *Ann.*, **320**, 99 (1902).

and Adams.[13] The calculated values of pK_D are somewhat uncertain, owing to the nature of the theory by which they are derived; but the uncertainty is hardly great enough to invalidate this conclusion.

Several other systematic relationships emerge from the table. In the first place it is clear that the esters of all the alpha amino acids possess essentially identical dissociation constants ($pK_E = 7.7 \pm 0.1$), regardless of the length of the attached carbon chain, a result entirely in accord with the behavior of other substituents. The enormous effect of the —$COOC_2H_5$ group (and presumably therefore of the —COOH group) in weakening the basicity of the amino group is clearly revealed. A primary amine is approximately a thousand times as strong a base as the ester of an α-amino acid. This effect is so great that even the charged $C\diagup^{O}_{\diagdown O}$ group in the amino acids reduces ten-fold the basicity of a neighboring amino group, although the electrostatic effect of the negative charge on the group would in itself work powerfully in the opposite direction.

As the distance between the amino group and the —$COOC_2H_5$ group increases, the effect of the substituent diminishes. The relation between the dissociation constant and the distance of the substituent group may be approximately represented by an equation first developed by MacInnes,[14] which is (in our notation)

$$pK' = pK_\infty - (S/d) \qquad (17)$$

in which pK_∞ is the dissociation constant, when the substituent is at an infinite distance along the carbon chain,[15] S is a constant characteristic of the substituent, and d (the "distance") is taken as the number of carbon atoms separating the dissociating group from the substituent. For the amino acid esters, equation (17) becomes

$$pK' = 10.8 - (3.0/d) \qquad (18)$$

MacInnes has shown this equation to hold for a variety of substituents, and Schmidt[16] has shown that it applies approximately to both dissociating groups in the amino acids. In Fig. 1 we have plotted pK_1, pK_2 and pK_E as a function of $1/d$ for the amino acids and their esters, utilizing Schmidt's data for the amino acids (to which we have added our own determinations for ϵ-aminocaproic acid). The figure shows also the values of the P_H of the isoelectric point, pI (calculated from the equation $pI = (pK_1 + pK_2)/2$) and the values of $\log K_Z$ and pK_D, as a function of the reciprocal of the distance between the groups.

It is evident that the linear relationship is valid only as a first approxima-

(13) Adams, THIS JOURNAL, **38**, 1503 (1916).

(14) MacInnes, *ibid.*, **50**, 2587 (1928).

(15) pK_∞ is close to, but not in general identical with, the pK value (pK_0) for the corresponding unsubstituted acid or base.   In general $pK_\infty > pK_0$ or $K_\infty < K_0$.   For the amino group, pK_∞ lies between 10.7 and 10.8; for the carboxyl group, between 4.8 and 4.9.

(16) Schmidt, Kirk and Appleman, *J. Biol. Chem.*, **81**, 723 (1929).   See also Greenstein, *ibid.*, **96**, 499 (1932).

tion.   Particularly the values for pK_2 and pK_E, when $1/d$ is small, deviate considerably from the straight line.   As MacInnes has pointed out, the dissociation constants, to be strictly comparable, should be determined for a series of compounds in which the length of the carbon chain remains con‧ stant, the influence of the substituent being examined for all possible positions in this chain.   This condition has not been fulfilled here, which may be the cause of some of the observed deviations.
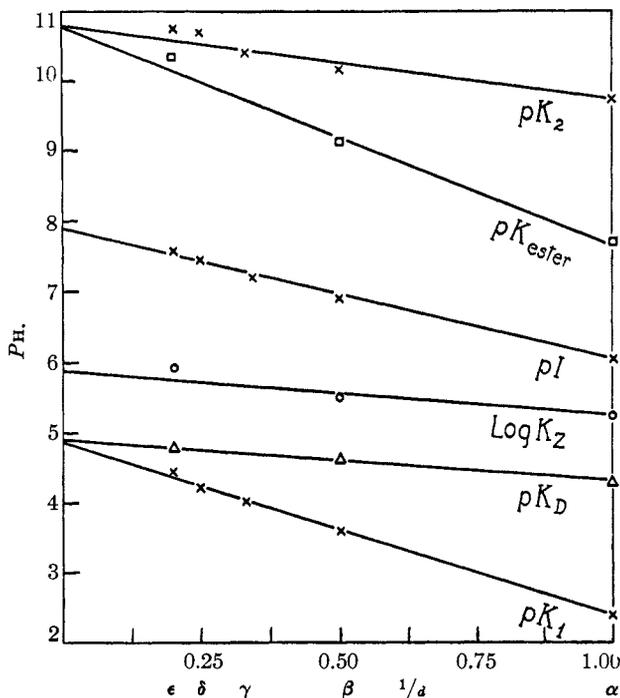
Fig. 1.—Dissociation constants and related constants for amino acids and their esters, as a function of the reciprocal distance between the polar groups.

MacInnes suggested that the term S/d in equation (17) might represent the effect upon the potential energy of the molecule produced by the approach of charged groups to the distance d, and that this potential energy should be closely related to the change in the free energy of ionization ($RT \ln K$) of the molecule.   We may picture the dissociating group as a charged sphere, at whose surface the electric potential is V_0 in the absence of an external field.   Then charges $e_1, e_2, \ldots$ at distances $d_1, d_2, \ldots$ will alter the value of the potential to

$$V = V_0 + \frac{e_1}{d_1} + \frac{e_2}{d_2} + \ldots = V_0 + \sum \frac{e}{d} \qquad (19)$$

if we neglect the distortion of the relative positions of the charges due to

their mutual induction. Similarly, for a dissociating compound containing several substituent groups (which we treat as point charges) we may write

$$pK' = pK_\infty - \frac{S_1}{d_1} - \frac{S_2}{d_2} - \ldots \tag{20}$$

Thus for aspartic acid diethyl ester we should calculate, from equations (17), (18) and (20)

$$pK' = 10.8 - \frac{3.0}{1} - \frac{3.0}{2} = 6.3 \tag{21}$$

whereas the observed pK' value is 6.5. The agreement is as good as could be expected, considering the uncertainty of the assumptions made and the neglect of the distortion effect. Equation (20) is at any rate empirically useful in making approximate estimates of dissociation constants, when exact values are unknown. For instance, we may make use of it in estimating the relative numbers of the three isoelectric forms of aspartic acid in aqueous solution.

$$
\begin{array}{ccc}
\begin{array}{c} COOH \\ | \\ CH_2 \\ | \\ CHNH_3^+ \\ | \\ COO^- \end{array} \quad (A) &
\begin{array}{c} COO^- \\ | \\ CH_2 \\ | \\ CHNH_3^+ \\ | \\ COOH \end{array} \quad (B) &
\begin{array}{c} COOH \\ | \\ CH_2 \\ | \\ CHNH_2 \\ | \\ COOH \end{array} \quad (C)
\end{array}
$$
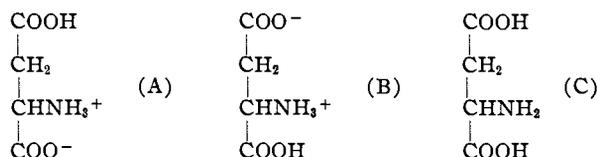
A and B are zwitterions, B having a much higher dipole moment than A; C is an uncharged molecule. Any one of the three forms may arise when a hydrogen ion is given off from the cation $HOOC—CH_2—\overset{NH_3^+}{\underset{}{CH}}—COOH$ (D). Three dissociation constants are therefore involved

$$\frac{(H^+)(A)}{(D)} = K_A \quad (22) \qquad \frac{(H^+)(B)}{(D)} = K_B \quad (23) \qquad \frac{(H^+)(C)}{(D)} = K_C \quad (24)$$

Hence

$$K_A/(A) = K_B/(B) = K_C/(C) \tag{25}$$

pK_C may be taken as equal to the pK value of aspartic acid diethyl ester (6.5). For the effect of the NH_3^+ group on the dissociation of the carboxyl in (A) or (B) we may use Greenstein's[17] equation $pK_{COOH} = 4.83 - (2.50/d)$; for the effect of the other carboxyl we may use the equation (which holds for the mono esters of the dicarboxylic acids beyond succinic acid)

$$pK_{COOH} = 4.8 - (0.5/d)$$

Hence, assuming a summation of these effects according to equation (20)

$$pK_A = 4.8 - \frac{2.50}{1} - \frac{0.5}{2} = 2.05$$

$$pK_B = 4.8 - \frac{2.50}{2} - \frac{0.5}{2} = 3.3$$

$$pK_C = pK_E = 6.5$$

(17) Greenstein, *J. Biol. Chem.*, **96**, 499 (1932).

pK_A is, as it should be, approximately equal to the measured value of pK_1 (2.08). Hence A, B and C are present in solution approximately in the proportion: 28,000 to 1600 to 1. The concentration of the uncharged molecule, C, is negligible; that of the highly polar form B is appreciable; but the form A, corresponding to the zwitterion of the simple amino acids, predominates.

A similar calculation may be applied to a dibasic amino acid such as lysine which may exist in the three isoelectric forms

$$
\begin{array}{lll}
\begin{array}{l}
NH_2 \\
| \\
(CH_2)_4 \\
| \\
CHNH_3{}^+ \\
| \\
COO^-
\end{array} \quad (A) &
\begin{array}{l}
NH_3{}^+ \\
| \\
(CH_2)_4 \\
| \\
CHNH_2 \\
| \\
COO^-
\end{array} \quad (B) &
\begin{array}{l}
NH_2 \\
| \\
(CH_2)_4 \\
| \\
CHNH_2 \\
| \\
COOH
\end{array} \quad (C)
\end{array}
$$
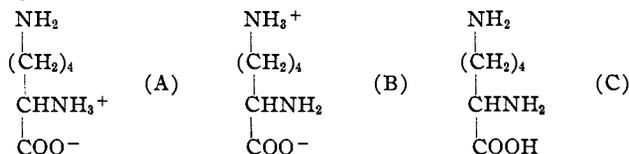
Any one of these may lose a hydrogen ion to form the anion (D) $NH_2-(CH_2)_4-CHNH_2-COO^-$.

The three dissociation constants involved are then

$$\frac{(H^+)(D)}{(A)} = K_A \quad (26) \qquad \frac{(H^+)(D)}{(B)} = K_B \quad (27) \qquad \frac{(H^+)(D)}{(C)} = K_C \quad (28)$$

Hence

$$K_A(A) = K_B(B) = K_C(C) \qquad (29)$$

In estimating pK_A and pK_B it is probably best to ignore the effect of the distant $-NH_2$ group on the dissociation of the charged $-NH_3{}^+$ group, which is probably very small and will be about the same for both the forms A and B. To obtain the value of pK_A we may then employ Greenstein's[17] equation $pK_{NH_3{}^+} = 10.72 - (0.9/d)$. Hence

$$pK_A = 10.72 - \frac{0.9}{1} = 9.8$$

$$pK_B = 10.72 - \frac{0.9}{5} = 10.54$$

while pK_C may be taken as equal to pK_D as deduced from equation (16a) for the mono-amino mono-carboxylic acids: $pK_C = 4.3$ (pK_B, it may be noted should be approximately equal to the measured value of pK_3 for lysine (10.53) and this is indeed the case). The ratio of A to B to C in solution is then roughly, from (29): 320,000 to 1,800,000 to 1. Here then, the very highly polar form (B) is predominant. The dielectric constants of lysine solutions should be very high, approaching those of ε-aminocaproic acid (DeVoto, Wyman[1]) and lysine may thereby exert an important "medium effect" on solutions in which it is dissolved. For arginine the same conclusion is obtained by similar reasoning; indeed, the extremely polar form (in which a positive charge resides on the guanidine radical and a negative charge on the carboxyl) predominates even more than the corresponding form in lysine. Histidine, on the other hand, is a less polar zwitterion, similar to the mono-amino mono-carboxylic acids (compare Miyamoto and Schmidt[1]).

The Effect of Temperature upon K_Z.—From equation (15a) log K_Z = $pK_E - pK_1$. For glycine the change of pK_1 and pK_E with temperature is known over a limited range. pK_1 is virtually independent of temperature between 0 and 25°,[18] while the values of pK_E at 20, 25 and 30° are given in Table III.

$$\log K_Z(20°) = 7.80 - 2.31 = 5.49$$
$$\log K_Z(25°) = 7.66 - 2.31 = 5.35$$
$$\log K_Z(30°) = 7.51 - 2.31 = 5.20$$

It is clear that K_Z decreases with rising temperature, being approximately halved for a rise of 10°. The formation of a zwitterion from a neutral molecule is hence an exothermic process, the heat of reaction as calculated by the equation

$$-\Delta H = \frac{\Delta(R \ln K)}{\Delta(1/T)}$$

being 11,500 ± 1000 calories.

$$H_2NCH_2COOH \rightleftharpoons {}^+H_3NCH_2COO^-$$
$$\Delta H_{298} = -11,500 \text{ cal.} \tag{30}$$
$$\Delta F^\circ_{298} = -7300 \text{ cal.} = -RT \ln K_Z$$
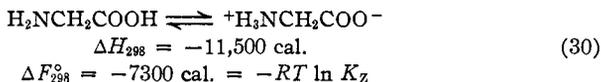
The heat of the reaction (30) is primarily that of the process RNH_2 + $H^+ \longrightarrow RNH_3{}^+$, a reaction known to involve the evolution of 10,000 to 13,000 calories per mole in the aliphatic amines and amino acids.[19] It is worthy of note that the total energy involved in this reaction is almost independent of the nature of adjacent groups in the molecule, being essentially the same for the primary amines, the amino acids and the amino acid esters; whereas the free energy of the reaction is profoundly altered by the introduction of substituents into the molecule.

Rise of temperature thus increases the concentration of uncharged molecules at the expense of zwitterions. This effect may be of considerable importance in the aminobenzoic acids, in which K_Z is not greatly different from unity at room temperature, according to the calculations of Bjerrum and Ebert. Variations of temperature might produce important alterations in the dielectric and other properties of these substances.

The Relative Concentrations of Zwitterions and Uncharged Molecules in Relation to the Dielectric Constant of the Solvent.—Highly polar molecules are most soluble in highly polar solvents; slightly polar or non-polar molecules in less or non-polar solvents.[20] This general principle is strikingly illustrated by a comparison of the solubilities of the aliphatic and aromatic amino acids in water and in 90% alcohol (Table VI). The highly polar zwitterions are all far more soluble in water than in 90% alcohol; the aminobenzoic acids (which are known on other grounds[21] to

(18) Cohn, *Ergebnisse Physiol.*, **33**, 838 (1931).

(19) See Weber, *Biochem. Z.*, **218**, 1 (1930), and Schmidt's data for the amino acids conveniently tabulated by Cohn, Ref. 18. The removal of a hydrogen ion from a carboxyl group in aqueous solution is accompanied by a very small heat change.

(20) Hildebrand, "Solubility," Chemical Catalog Co., New York, 1923.

(21) Compare Bjerrum, Ebert, Michaelis and Mizutani, Harris and Birch (see footnote 1).

exist largely as uncharged molecules, even in water) are far more soluble in the organic solvent.

TABLE VI

SOLUBILITIES OF AMINO ACIDS IN WATER AND 90% ALCOHOL[a]

Temperature, 25° unless otherwise noted

Substance	Soly. in water, mole fraction (N_W)	Soly. in 90% alcohol, (N_A)	N_W/N_A (zwitterion)	N_W/N_A (uncharged molecule)
Glycine	. . .	. . .	250	1
α-Alanine	. . .	. . .	87	0.2
β-Alanine	. . .	. . .	80	.3
ε-Aminocaproic acid	. . .	. . .	26	.02
Leucine	. . .	. . .	9	.02
Betaine	.203	.036 (absolute alcohol)	5.64	. .
o-Aminobenzoic acid	.00046 (13.8°)	.0351 (9.6°)	. . .	.013
m-Aminobenzoic acid	.00078 (14.9°)	.0073 (9.6°)	. . .	.107
p-Aminobenzoic acid	.00045 (12.8°)	.0374 (9.6°)	. . .	.012

[a] Values of N_W/N_A (zwitterion) for aliphatic amino acids are calculated from solubility data (to be published later) of Cohn, McMeekin, Edsall and Weare; see discussion by Cohn, Naturwiss., 20, 663 (1932). Data on aminobenzoic acids from Oechsner de Coninck, Ann. chim. phys., 4, 528 (1895); on betaine interpolated from Stoltzenberg, Z. physiol. Chem., 92, 445 (1914). The low solubilities of the aminobenzoic acids in water may be partly due to the presence in the molecule of the large non-polar benzene ring; but this is not the whole explanation, for the benz-betaines [Willstätter and Kahn, Ber., 37, 412 (1904)], which are unquestionably zwitterions, are very soluble in water, moderately soluble in alcohol, and insoluble in non-polar solvents. Values in the last column for the aminobenzoic acids are derived directly from solubility data; for the uncharged molecules of the aliphatic amino acids they are calculated from titration data as explained in the discussion.

It may be inferred from these considerations that for any given amino acid, the *concentration* ratio of zwitterions to uncharged molecules should decrease[22] as the dielectric constant of the solvent is lowered. Ebert has calculated for the aliphatic amino acids that in pure ethyl alcohol the ratio is not far from unity. Further estimates of this ratio for various alcohol-water mixtures may be made on the basis of the titration data reported in Table IV.

In interpreting these data, we shall assume that, in any given solvent, at a constant ionic strength, the activity coefficient of the hydrogen ion is constant. Hence the P_H values measured in two different solutions under such circumstances, are determined only by the *relative* hydrogen-ion concentrations (C_H), or mole fraction (N_H) in these solutions.

$$(P_H)_2 - (P_H)_1 = \log \frac{[C_H]_1}{[C_H]_2} = \log \frac{[N_H]_1}{[N_H]_2} \tag{31}$$

This relation holds very accurately in aqueous solutions, which are maintained at a constant ionic strength by a neutral salt present in large con-

(22) The *activity* ratio (K_Z) of course remains constant by definition.

centration.[23]   That it holds approximately in alcohol–water mixtures at a low ionic strength is indicated by the fact that titration data on the hydrogen electrode in such solvents fit the Henderson–Hasselbalch equation[24] within 0.05 (or less) in P_H.

For brevity, let a zwitterion be denoted by the symbol $R^{\pm}$, the corresponding cation by R^+, and the uncharged molecule by R. Let K^* denote the apparent dissociation constant of a substance in any solvent, expressed in terms of the measured P_H and the concentrations of the components present,[25] then

$$pK_A{}^* = P_H + \log (N_{R^+}/N_{R^{\pm}}) \tag{32}$$
$$pK_B{}^* = P_H + \log (N_{R^+}/N_R) \tag{33}$$

As in aqueous solution, we assume that $pK_B{}^* = pK_E{}^*$. This implies that the ratio of the activity coefficients of the free ester (E) and the ester cation (E^+) is approximately the same in any solvent as the ratio for the corresponding uncharged molecule and its cation in the same solvent ($f_{E^+}/f_E = f_{R^+}/f_R$).

This is a special case of a more general principle. Let x and y be two compounds of an homologous series containing the same dissociating group. Then the ratio (K_x/K_y) of the two dissociation constants will be (as a first approximation) independent of the solvent. For water and alcohol–water mixtures the principle appears to hold within 0.2 to 0.3 in P_H for a wide variety of compounds.[26]   In the case here considered, the dissociating group is an amino group, to which is attached a hydrocarbon chain and either a —COOH or a —COOC$_2$H$_5$ group.   Then, setting $pK_E{}^* = pK_B{}^*$ in (33), and subtracting (32) from (33), we have

$$pK_E{}^* - pK_A{}^* = pK_E{}^* - pK_1{}^* = \log (N_{R^{\pm}}/N_R) \tag{34}$$

which is identical with (15a) if mole fractions be substituted for activities. Equation (34), however, applies to any alcohol–water mixture, but is only a rough approximation, on account of the assumptions made in its derivation.

As an illustration of the use of equation (34) we may select leucine in 90% alcohol.   From Table IV, $pK_E{}^* = 6.8$ and $pK_1{}^* = 4.1$; hence log $(N_{R^{\pm}}/N_R) = 2.7$;   therefore, in 90% alcohol the zwitterions outnumber the uncharged molecules by 500 to 1, instead of 200,000 to 1 as in water.

(23) Brönsted, *Trans. Faraday Soc.*, **23**, 430 (1927); Kilpatrick and Chase, THIS JOURNAL, **53**, 1732 (1931).

(24) Sano, *Biochem. Z.*, **171**, 277 (1926). Michaelis and Mizutani, *Z. physik. Chem.*, **116**, 135 (1925).

(25) If we assume that $P_H = \log (1/a_H)$ in any solvent, then K^* is the "acidity constant" of Brönsted [*Chem. Rev.*, **5**, 284–312 (1928)]. The treatment adopted here, however, requires no assumption regarding the activities of individual ions, or regarding the *absolute* values of hydrogen ion concentration in alcohol–water mixtures. In evaluating ($N_{R^{\pm}}/N_R$) comparisons are always made between P_H measurements on different substances *in the same solvent*. For the purposes of the present study, it is unnecessary to establish any general basis of comparison for P_H measurements in different solvents.

(26) Hall, *Chem. Rev.*, **8**, 191 (1931); Michaelis and Mizutani, *Z. physik. Chem.*, **116**, 135, 350 (1925); **118**, 318, 327 (1925). See also Hammett, THIS JOURNAL, **50**, 2666 (1928); Bjerrum and Larsson, *Z. physik. Chem.*, **127**, 358 (1927); and Halford, THIS JOURNAL, **53**, 2944 (1931), who points out the limitations of the principle.

The estimated values of $N_{R\pm}/N_R$ for several amino acids in 90% alcohol are listed in Table VII.   When $pK_E{}^*$ (90% alcohol) has not been measured, it has been assumed to be equal to $(pK_E)_{water} - 0.9$; a relation which fits our data for alanine and leucine, and the data of Mizutani on the primary aliphatic amines.   It was also assumed that $pK_1{}^*$ for β-alanine is the same as for β-aminobutyric acid.

TABLE VII

RELATIVE MOLE FRACTIONS AND RELATIVE ACTIVITY COEFFICIENTS OF ZWITTERIONS
AND UNCHARGED MOLECULES IN 90% ALCOHOL

Amino acid	$pK_E{}^*$	$pK_1{}^*$	log ($N_{R\pm}/N_R$)	($N_{R\pm}/N_R$)	($f_{R\pm}/f_R$)
Glycine	6.8	3.8	3.0	1000	250
α-Alanine	6.8	4.1	2.7	500	500
β-Alanine	8.2	5.1	3.1	500	250
Leucine	6.8	4.1	2.7	1250	400
ϵ-Aminocaproic acid	9.45	6.65	2.8	600	1400

The Relative Activity Coefficients of Zwitterions and Uncharged Molecules in Alcohol–Water Mixtures.—K_Z, the activity ratio of zwitterions to uncharged molecules, is constant by definition at a given temperature and pressure.   Therefore if we compare an infinitely dilute solution of the amino acid, in water, and in another medium (denoted by the subscript M)

$$K_Z = \left(\frac{N_{R\pm}}{N_R}\right)_{H_2O} = \frac{a_{R\pm}}{a_R} = \left(\frac{f_{R\pm}}{f_R}\right)_M \left(\frac{N_{R\pm}}{N_R}\right)_M \qquad (35)$$

(f = activity coefficient = activity/mole fraction, is taken as unity in an infinitely dilute aqueous solution for both molecular forms.)

The ratio $f_{R\pm}/f_R$ gives the relative distribution coefficients[27] of zwitterion and uncharged molecule, between water and the medium M.   If M is 90% alcohol, this ratio may be calculated from Table V and the fourth column of Table VII.   The results are given in the last column of Table VII, from which it appears that the affinity of a zwitterion for water as compared to 90% alcohol is from 250 to 1400 times as great as a similar affinity ratio for the corresponding uncharged molecule.

A rough estimate of the absolute magnitude of the distribution coefficients involved may be made with the aid of the solubility data in Table VI.   The distribution coefficient of the zwitterion between water and 90% alcohol is approximately given (for aliphatic amino acids) by the relative solubilities in these two solvents[28] (that is, we assume $N_W/N_A = f_{R\pm}$ as a rough approximation).

(27) Compare Bjerrum and Larsson, Z. physik. Chem., **127**, 358 (1927).

(28) This would be exactly true (1) if the measured solubility were entirely that of the zwitterion, and (2) if the activity coefficient of the amino acid were the same for a saturated solution in a given solvent as for an infinitely dilute solution in that solvent.   The first assumption appears justified, to judge from the results of the present investigation, within considerably less than one per cent. (see Table VII).   The second assumption is certainly incorrect; for glycine in water, for example, the activity of a saturated solution may be as little as 0.25 of the mole fraction.   The figures given in the following paragraph are, consequently, mere estimates of the order of magnitude of the relations involved.   The problem of evaluating true activities from solubility data will be dealt with in a forthcoming paper by Cohn, McMeekin, Edsall and Weare (see footnote, Table VI).

Thus the glycine zwitterion is about 250 times as soluble in water as in 90% alcohol (Table VI). Likewise, from Table VII, $f_{R\pm}/f_R$ is about 250 in 90% alcohol. Hence f_R (i. e., N_W/N_A for the uncharged molecule) is of the order of magnitude of unity; in other words, the solubility of the uncharged molecule of glycine would not be very different in water and in 90% alcohol. Similar calculations have been carried out on other amino acids, and the results are tabulated in the last column of Table VI. The relative solubility in 90% alcohol as compared to water increases with the length of the hydrocarbon chain. The uncharged molecules of leucine and ε-aminocaproic acid are very similar in this respect to the aminobenzoic acids, being about fifty times as soluble in 90% alcohol as in water. These values are to be taken only as rough preliminary estimates, but they illustrate clearly the radical nature of the molecular change involved in the transformation of an uncharged molecule into a zwitterion.

Summary

1. Isoelectric amino acids may exist either as "zwitterions" (^+H_3NR-COO^-) or as "uncharged molecules" ($H_2NRCOOH$). The ratio of the activities of these two forms must be constant at a given temperature. This constant (K_Z) may be evaluated, by a method due to Ebert, from the dissociation constants of the amino acids and their esters.

2. For this purpose the dissociation constants of eight amino acid esters, and of ε-aminocaproic acid, have been determined.

3. In aqueous solution the zwitterion form predominates enormously in all the substances studied. The values of K_Z range from 40,000 (for glycylglycine) to nearly 1,000,000 (for ε-aminocaproic acid).

4. Isoelectric lysine and arginine are estimated to exist chiefly as extremely polar zwitterions, carrying a positive and a negative charge at opposite ends of the long carbon chain. The dicarboxylic amino acids exist partly in this form, but chiefly as less polar zwitterions, like the simple α-amino acids.

5. K_Z diminishes rapidly with rise of temperature, approximately 11,000 calories being evolved per mole when a zwitterion is formed from an uncharged molecule.

6. In solvents of low dielectric constant, the concentration of the highly polar zwitterions is smaller, and that of the less polar uncharged molecules is greater than in water. The zwitterion form, however, still greatly predominates, even in 90% alcohol. Relative activity coefficients for both molecular species in 90% alcohol have been estimated from potentiometric titrations and discussed in relation to solubility data.