THE AMPHOTERIC PROPERTIES OF SOME AMINO-ACIDS AND PEPTIDES.

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INTRODUCTION.

The change in the hydrogen ion concentration of a solution upon the addition of acid or alkali has been used to follow the changes characteristic of certain dissolved substances. Thus, the progressive neutralization of polybasic acids and of polyacid bases has been studied in this way.[1] Amphoteric electrolytes lend themselves readily to this method of investigation. Recently, the results obtained with a number of proteins have been presented in the form of titration curves in which amounts of standard acid and alkali were plotted along one axis and the resulting pH values of the protein solution along the other axis.[2] Such titration curves may be expected to be more or less characteristic for the acid- and base-combining properties or groups of such substances. In view of the importance and value of such studies in protein chemistry, the titration curves of some of the simple amino-acids and dipeptides are presented here as a necessary preliminary to a more satisfactory understanding of the more complex bodies.

Experimental Methods.

The following substances were studied; glycine, alanine, α-amino-butyric acid, leucine, glycyl-glycine, alanyl-glycine, and alanyl-alanine. Data were obtained also for solutions of sodium chloride, acetone,

[1] Hildebrand, J. H., *J. Am. Chem. Soc.*, 1913, xxxv, 847.

[2] *Cf.* Cohn, E. J., Gross, J., and Johnson, O. C., *J. Gen. Physiol.*, 1919–20, ii, 145. Cohn, E. J., Wolbach, S. B., Henderson, L. J., and Cathcart, P. H., *J. Gen. Physiol.*, 1918–19, i, 221. Sörensen, S. P. L., *Compt. rend. lab. Carlsberg*, 1915–17, xii. Clark, W. M., The determination of hydrogen ions, Baltimore, 1920, especially pp. 34 and 211, for general discussion and references.

acetamide, urea, acetic acid, and aceturic acid. The substances were prepared by the usual methods and showed satisfactory states of purity except for the alanyl-alanine, for which the nitrogen content was found to be 1 per cent less than the calculated value. Attempts at purification did not remedy this, so that evidently an impurity, possibly alanine, was present.

The solutions studied were prepared so that 1 liter of the final mixture contained in every case 0.05 gram molecule of the amino-acid or peptide or other substance, and 0.05 gram molecule of sodium chloride, while the amounts of hydrochloric acid or sodium hydroxide (0.1 N) added were varied. The water used was distilled first from sulfuric acid, then from alkaline permanganate, and condensed in a block tin condenser.

The concentration of the ampholyte or other substance and of the sodium chloride in the final volume (50 cc. as made up and used) was the same in all cases. The only variable was the amount of acid or alkali which was added, or the ratio between this and the concentration of solute.

The hydrogen ion concentrations of the solutions were determined by electromotive force measurements using calomel electrodes with 0.1 M potassium chloride solution, saturated potassium chloride bridge, unknown solution with hydrogen electrode (platinum or gold coated with palladium black), and a potentiometer of Leeds and Northrup type "K" in conjunction with a type "H" galvanometer with a loading coil to decrease its period. Cells of the rocking type described by Clark[3] were used with the modification of having an adapter of about 25 cc. capacity fused on the inlet tube.[4] Hydrogen was obtained compressed in cylinders and was purified by passage through (electrically) heated copper gauze and then bubbled through water.

The customary precautions in purifying the materials, and standardizing the various parts of the apparatus and the solutions were taken. The hydrogen ion concentrations in terms of pH were calculated from the experimental measurements by the formula[5]

[3] Clark, W. M., *J. Biol. Chem.*, 1915, xxiii, 475.

[4] The writers desire to thank Professor F. S. Lee of the Department of Physiology of Columbia University for presenting to them the excellent rocking apparatus used in this work.

[5] Sörensen, S. P. L., *Compt. rend. lab. Carlsberg*, 1909, viii, 22, 29.

$$\text{pH} = \frac{\pi - 0.3377}{0.0577 + ((t - 18)\,0.0002)}$$

in which π = reading in volts; 0.3377 = potential of calomel electrode; and $0.0577 + ((t - 18)0.0002)$ = correction factor for temperature $t°$, as a thermostat was not used.

In a number of cases the hydrogen ion concentrations were also determined colorimetrically. Where this was done, the indicators and standard solutions described by Clark and Lubs were used.[6]

The isoelectric points of the various ampholytes were also determined by the method described by Michaelis[7] and developed by Sörensen.[8] This consists of adding the pure ampholyte solution to a series of solutions of progressively changing hydrogen ion concentration containing the indicator and noting where no change in color occurred on mixing the unknown ampholyte solution and standard solution.

Experimental Results.

The results obtained for the pH values for the solutions of the various substances in the presence of acid and alkali are given in Table I. Although not stated in the table, it may be recalled that all the solutions contained sodium chloride of the concentration 0.05 M. Column 1 headed "Water" contained no added solute but the sodium chloride and acid or alkali. The headings of the remaining columns indicate the solute present, 0.05 M in every case.

The titration curves plotted from these results are given (Figs. 1, 2, and 3). The pH values are given as abscissæ, and as ordinates are shown the number of cc. of 0.1 N hydrochloric acid or sodium hydroxide added to 25 cc. of 0.1 M solution of the indicated solute (also 0.1 M with respect to sodium chloride), and the whole diluted to 50 cc. The numbering of the curves corresponds to the numbering of the columns in Table I. Since the results of the amino-acids are so nearly alike, only one curve (Curve 6, Fig. 2) is given. Similarly for the dipeptides, only one curve (Curve 10, Fig. 3) is presented.

[6] Clark, W. M., and Lubs, H. A., *J. Bact.*, 1917, ii, 1, 109, 191.

[7] Michaelis, L., *Biochem. Z.*, 1912, xlvii, 251. Hasselbalch, K. A., *Biochem. Z.*, 1916, lxxviii, 129.

[8] Sörensen,[2] p. 150.

TABLE I.

Milliequivalents of HCl or NaOH per liter of solution containing 50 milliequivalents of solute.	(1) Water.	(2) Urea.	(3) Acetamide.	(4) Acetone.	(5) Acetic acid.	(6) Glycine.	(7) Alanine.	(8) α-Aminobutyric acid.	(9) Leucine.	(10) Glycylglycine.	(11) Alanylglycine.	(12) Alanylalanine.	(13) Aceturic acid.
						HCl							
50	1.32	1.29	1.29	1.29	1.27	1.94	1.84	1.75	1.85	2.26	2.21	2.18	1.23
44						2.02	1.95	1.85	1.97	2.48			
40	1.45	1.39	1.39	1.39		2.11	2.05	1.97	2.05	2.65	2.74	2.55	
34						2.26	2.18	2.08	2.21	2.89	2.83		
30	1.55	1.50	1.50	1.50	1.49	2.32	2.32	2.25	2.30	3.00	2.97	2.75	1.46
24						2.51	2.49	2.42	2.44	3.29	3.15	3.09	
20	1.68	1.68	1.69	1.67		2.62	2.61	2.54	2.55	3.34	3.28	3.21	
18						2.70	2.68	2.62	2.63	3.42	3.39		
14	1.85					2.85	2.82	2.76	2.77	3.58	3.53	3.59	
10	2.07	1.97	2.00	1.97	1.95	3.02	3.01	2.92	2.94	3.75	3.71	3.83	1.88
8						3.13	3.11	3.04	3.07	3.88	3.84	3.90	
6	2.23				2.09	3.28	3.25	3.16	3.21	4.02	3.99	4.03	2.03
4						3.46	3.43	3.35	3.42	4.21	4.19	4.35	
2	2.71				2.52	3.75	3.72	3.63	3.71	4.65	4.42	4.58	2.24
1	2.96					4.06	4.04	3.92	4.05	4.82	4.74	5.16	

NaOH													
0	4.31	4.82	5.00	4.31	2.88	5.39	5.34	4.75	6.12	5.64	5.21	5.59	2.36
1	10.31			10.63		8.04	8.02		7.89	6.58	6.43	6.48	2.39
2	10.98			11.05	3.25	8.34	8.35	(7.14)	8.19	6.93	6.76	6.84	2.49
4						8.70	8.71	8.48	8.53	7.24	7.10	7.17	
6	11.64			11.70	3.67	8.83	8.85	8.73	8.71	7.45	7.32	7.33	2.69
8						9.07	9.00	8.94	8.86	7.61	7.53	7.50	
10	11.90	11.92	11.88	11.93	3.93	9.16	9.13	9.10	9.01	7.71	7.59	7.61	2.90
14	12.04					9.34	9.31	9.32	9.19	7.88	7.83	7.80	
18						9.50	9.47	9.51	9.35	8.03	7.93	7.93	
20	12.20	12.23	12.21	12.27	4.34	9.55	9.54	9.57	9.43	8.11	8.01	8.01	3.28
24						9.71	9.78	9.73	9.56	8.26	8.23	8.16	
30	12.38	12.40	12.34	12.43	4.69	9.89	9.98	9.92	9.77	8.43	8.39	8.36	3.64
34						10.02	10.12	10.08	9.89	8.61	8.55	8.53	
40	12.50	12.51	12.45	12.59	5.10	10.28	10.37	10.27	10.12	8.85	8.75	8.77	4.12
44						10.49	10.58	10.46	10.35	9.08	9.05	9.08	4.50
50	12.59	12.54	12.65	12.64	6.51	10.86	10.93	10.88	10.81	9.78	9.81	10.23	(10.05)

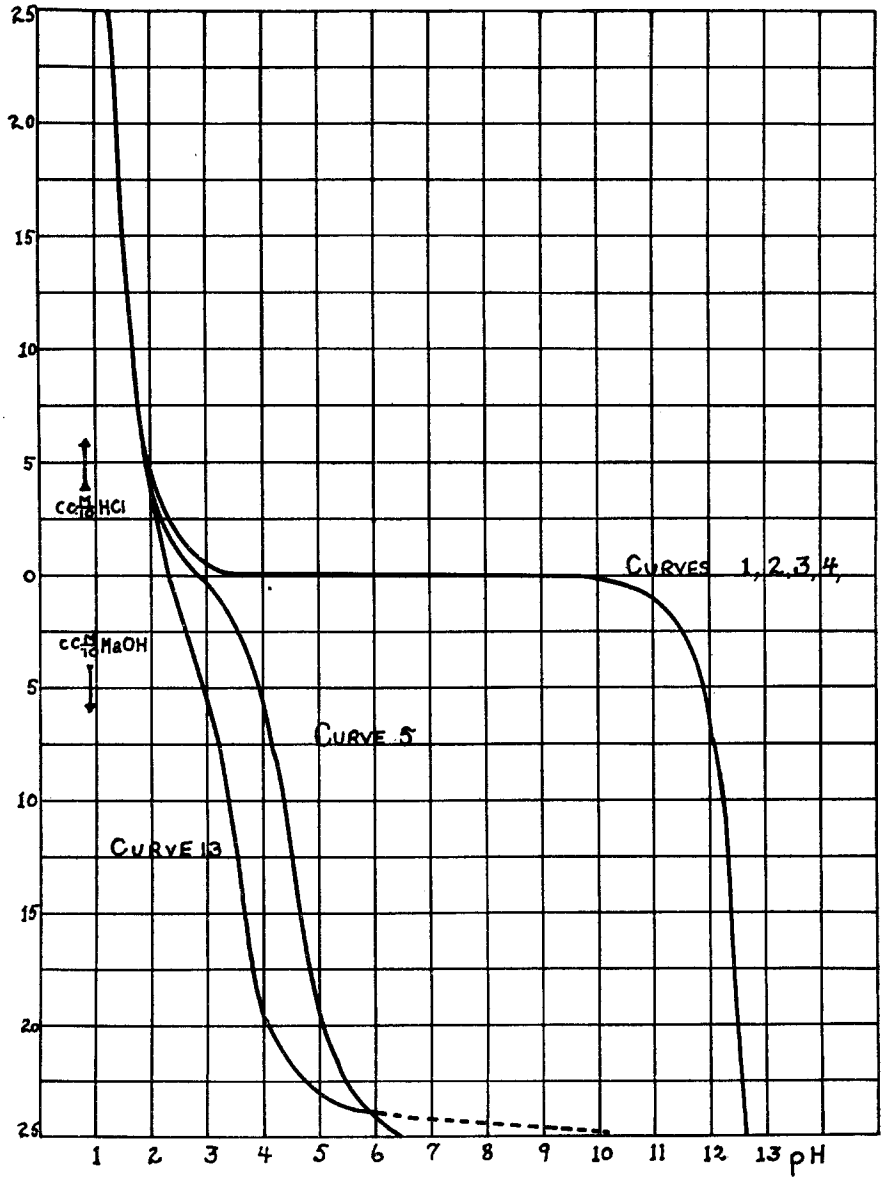

FIG. 1. Titration curves of water (1), urea (2), acetamide (3), acetone (4), acetic acid (5), and aceturic acid (13).

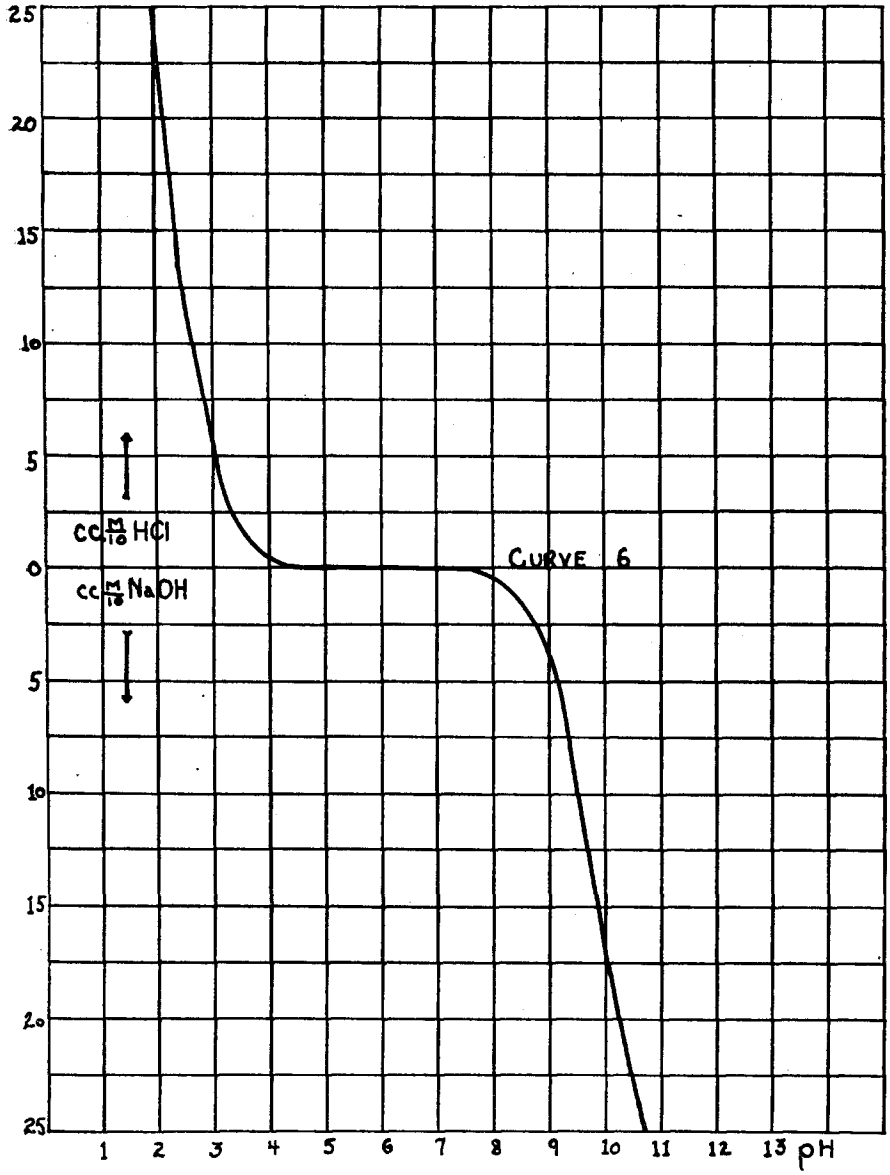

FIG. 2. Titration curve of glycine. Practically identical with titration curves of alanine, α-amino-butyric acid, and leucine.

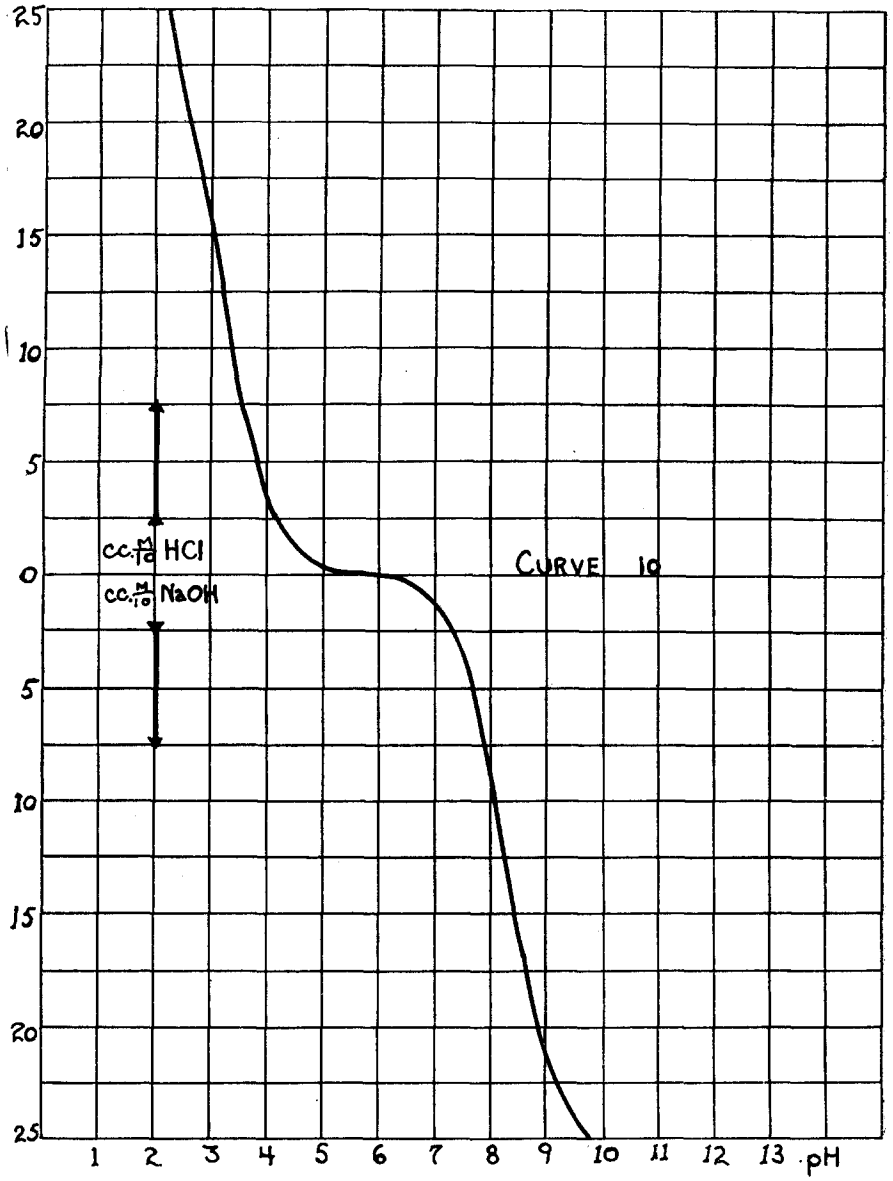

FIG. 3. Titration curve of glycyl-glycine. Practically identical with titration curves of alanyl-glycine and alanyl-alanine.

The values for the isoelectric points of some of the substances are given in Table II. The significance of these points will be discussed in detail later in this paper. The values calculated by means of the expression

$$I = \sqrt{k_w \frac{k_a}{k_b}}$$

in which k_a and k_b represent the ionization constants of the ampholyte substance acting as an acid and as a base, and k_w the ionization constant of water (very nearly 10^{-14} at the temperatures used) are given and also the values determined experimentally by means of the potentiometer (values from Table I), and by the indicator methods.

TABLE II.

Isoelectric Points of Some Amino-Acids and Peptides.

	k_a*	k_b*	Isoelectric points.		
			Calculated.	Potentiometer.	Indicator.
			pH	*pH*	*pH*
Glycine	1.8×10^{-10}	2.7×10^{-12}	6.1	5.4	6.0
Alanine	1.9×10^{-10}	5.1×10^{-12}	6.2	5.3	6.15
Leucine	1.8×10^{-10}	2.5×10^{-12}	6.1	6.1	7.0
Glycyl-glycine	1.8×10^{-8}	2.0×10^{-11}	5.5	5.6	5.5
Alanyl-glycine	1.8×10^{-8}	2.0×10^{-11}	5.5	5.2	5.15
Alanyl-alanine				5.6	6.15

* Winkelblech, K., *Z. physik. Chem.*, 1901, xxxvi, 546. Euler, H., *Z. physiol. Chem.*, 1907, li, 219.

Before discussing these results, a number of points connected with the experimental measurements will be taken up briefly; first for the results given in Table I, then for those in Table II.

Recently it was stated[9] that "There is hardly any use attempting the measurement of unbuffered solutions, if indeed there would be any significance to the measurement were it accurate." This statement apparently necessitates further explanation, possibly basing the definition of the term buffer in some way upon the slope of the titration curve. Otherwise, solutions of hydrochloric acid, etc., would be

[9] Clark,[2] p. 184. *Cf.* also a similar statement on p. 34.

included among those for which accurate determinations are not possible.

This raises the question of possible inaccuracies in the determinations. Aside from such questions as the presence of small amounts of impurities at or near the isoelectric points of ampholyte solutions where the buffer action is found to be small,[10] certain points connected with the relative accuracies of the chemical and electrical measurements are involved. Comparing, for example, the determination of the hydrogen ion concentration of 0.1 N hydrochloric acid (pH approximately 1.0) and of 0.1 N sodium hydroxide (pH approximately 13.0), the electrical measurement is much more sensitive for the latter. The component determined in both cases is the hydrogen ion. In the preparation of the solutions, the alkaline solution is more readily influenced or changed (perhaps due to the accidental nature of the surroundings) as regards its hydrogen ion concentration. Alkaline solutions may therefore require the presence of buffer mixtures in order to obtain constant and reproducible results under conditions with which analogous acid solutions apparently give accurate values with no buffer present.

The presence of sodium chloride of the given concentration had no appreciable influence on the determinations as shown by the work of Harned[11] and of Fales and Nelson[12] on mixtures of acid and neutral salts, of Sörensen[13] who compared ampholyte solutions with and without salt, and of Tague[14] who measured amino-acid solutions without salt.

Some determinations by others may be compared with the results given here, although in most cases the results are not comparable for a whole series. The solutions with no added solute besides the acid and alkali agree satisfactorily with such results[15] except that Tague appeared to be more successful in excluding carbon dioxide. The results for glycine agreed well with those of Sörensen[13] and of Tague[14] where comparison is possible.

[10] *Cf.* Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914, for a careful and systematic discussion of these relations.

[11] Harned, H. S., *J. Am. Chem. Soc.*, 1915, xxxvii, 2460.

[12] Fales, H. A., and Nelson, J. M., *J. Am. Chem. Soc.*, 1915, xxxvii, 2773.

[13] Sörensen, S. P. L., *Compt. rend. lab. Carlsberg*, 1909, viii.

[14] Tague, E. L., *J. Am. Chem. Soc.*, 1920, xlii, 173.

[15] *Cf.* Frary, F. C., and Nietz, A. H., *J. Am. Chem. Soc.*, 1915, xxxvii, 2264.

The results have been presented without introducing the correction used by Tague in his work in which the amount of acid or alkali required to bring the solution without solute to a definite hydrogen ion concentration was subtracted from the amount required with the solute present. This corrected value would give a more nearly accurate measure of the reaction between amino-acid, etc., and acid or alkali. For the results given here, this correction is negligible except for the largest additions of acid or alkali. If desired, the data given in Column 1 of Table I will permit the introduction of these corrections.

With regard to the isoelectric point results given in Table II, the determinations by the potentiometer method, that is the determination of the hydrogen ion concentrations of the solutions with neither acid nor alkali added, cannot lay claim to any great degree of accuracy for the amino-acids since the buffer action is so slight and since a broad zone exists on the curve where traces of acid or alkali may change the results appreciably. With the dipeptides this zone is narrower and the results should be correspondingly more accurate. The indicator method appears to give somewhat more accurate results as fewer manipulations are required. However, too much stress must not be placed upon their exactness.

Discussion of Results.

The data given refer to one concentration (0.05 M) for each substance. This naturally limits for the present the general applicability of the conclusions.

Michaelis[10] and others[16] have developed the theoretical interpretation of such titration curves and applied their conclusions to the results of a number of substances. Among other applications, they have considered the relations to the acid and basic dissociation constants of the ampholyte, to conditions for the smallest and greatest buffer actions, etc. In this paper, the discussion will be limited to questions which have been discussed briefly or not at all by others.

[16] *Cf.* Clark[2] for the general treatment and for complete references.

The following conclusions may be drawn from the results given in Table I and shown in the curves.

(*a*) The results for water, urea, acetamide, and acetone are practically identical. There is therefore at the dilutions used no combination involving the amino groups of urea or acetamide and hydrochloric acid, or enolization of urea, acetamide, or acetone accompanied by reaction with alkali which would be manifested by a change in hydrogen ion concentration.

(*b*) The results for the four amino-acids are nearly the same, showing only minor differences. Thus, on the acid side, alanine and leucine are identical, glycine and α-amino butyric acid differing slightly and in opposite directions; on the alkaline side glycine and alanine are the same differing slightly from the other two. There is a broad zone near the point at which no acid or alkali was added where practically no buffer action is shown.

(*c*) The results for the dipeptides, except for a few apparently minor differences, are strikingly similar throughout.

(*d*) As compared with the amino-acids, there was a very much smaller range where little or no buffer action was shown by the dipeptides. Aside from this, the curves for the two series were practically parallel. The vertical differences between the two sets of curves show the differences in the amounts of acid and of alkali required to bring the substances to the same pH values. Much more acid or alkali was required for the dipeptides than for the amino-acids, the difference being due evidently to the $-CO-NH-$ group of the dipeptides. The differences increase with increasing quantities of acid or alkali added, reach a maximum, and then decrease again. The chemical nature of the $-CO-NH-$ group readily accounts for these properties, acid combining with the $-CO-NH-$ group, alkali bringing about enol-lactim rearrangement and accompanying reaction.

(*e*) Aceturic acid on the acid addition side showed the same results as acetic acid and water. The influence of the highly ionized hydrochloric acid predominated over any effects of the weaker acids. On the alkaline side, the pH values found for aceturic acid were smaller than those for acetic acid with the same amount of alkali. This difference is evidently connected with the presence of the $-CO-NH-$ group and analogous to the difference between dipeptides and amino-

acids. The vertical differences between the two curves also show the extent of base-combining property or enol-lactim tautomerism.

The method of plotting pH values against the amounts of acid and alkali added is also open to question, but it appears to be the best method which is available. Placing the emphasis on the hydrogen ion concentration gives a one-sided chemical perspective, especially for the alkaline solutions. The suggestion of Wherry[17] to use pH 7.0 as the zero point and to calculate values from this as a logarithmic function is not practicable as indicated by Clark.[18] The suggestion may be made to determine a zero point for each substance, this point to be identical with the isoelectric point, and to measure acidity and alkalinity from this point logarithmically in terms similar to the pH scale. This would fix a characteristic property for each substance as the starting point. The practical difficulty lies in the determination of the isoelectric point, as its experimental measurement is probably the least accurate point of most titration curves.

The general chemical nature of amphoteric electrolytes will be taken up briefly here. These substances, depending upon conditions, ionize or react as salts in which the complex ampholyte component acts as the positive constituent or as the negative constituent. Loeb[19] has recently pointed out clearly and convincingly, and has presented considerable experimental evidence to show, that the positive ampholyte ion (of gelatin, for example) is fundamentally different from the same ampholyte as negative ion. The relative acidity and alkalinity of the solution govern these changes, the ionization taking the different courses in more acid or more alkaline solutions (the transition point will be taken up presently). In order to account for the different processes of ionization, it appears to be necessary to assume that the molecule (unionized) is different in the two cases. In order to ionize so that the complex is part of the cation, the molecule must possess a structure different from that of the molecule which ionizes with the complex as part of the anion. This difference in structure may be due to an isomeric rearrangement, possibly tautomeric, to a difference in the action of the solvent involving hydration, or to some

[17] Wherry, E. T., *J. Washington Acad. Sc.*, 1919, ix, 305.

[18] Clark,[2] p. 28.

[19] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 39, 237, 363, 483, 559; 1919–20, ii, 87.

other cause. An equilibrium relation between the two (or more) unionized forms would exist as a rule. This conception complicates somewhat the theoretical treatment of amphoteric electrolytes presented by Bredig,[20] Walker,[21] and others by the addition of an equation representing such an equilibrium and by introducing the concentration of the suitable molecular species in the equations representing the ionization constants. In strongly acid or alkaline solution, however, practically only one form would be present.

The difference in the ionization of the ampholyte is not always brought out clearly. For example, with a protein, the two kinds of ions are sometimes indicated as Na^+ (Protein$^-$) and (Protein$^+$) Cl^-. As a matter of fact, rarely, if ever, do the ions (Protein$^-$) and (Protein$^+$) have even the same chemical composition. This may be indicated by taking the simple case of glycine. Here, the ions are $Cl^-(NH_3CH_2CO_2H)^+$ or $Cl^-(C_2H_6O_2N)^+$, and $(NH_2CH_2CO_2)^-Na^+$ or $(C_2H_4O_2N)^-Na^+$, or $(HONH_3CH_2CO_2)^-Na^+$ or $(C_2H_6O_3N)^-Na^+$, omitting possible hydration of the ions.

The consideration of the transition point spoken of in connection with the different kinds of ionization is a more difficult one. It can best be treated from the side of the isoelectric points of the amphoteric substances.

The definitions of isoelectric point given by various workers have been contradictory at times. The most satisfactory definition appears to be that hydrogen ion concentration at which the properties such as electrical conductivity, viscosity, solubility, etc., when studied over extended ranges of acidity in moderately dilute solutions, show a point of inflexion.[19] Chemically this would be interpreted as stating that in more acid solutions the substance ionizes with the complex as part of the cation, in more alkaline solutions, as part of the anion. At the isoelectric point combination with added acid or base and accompanying ionization is a minimum, or the substance is in a maximum uncombined state. The method of cataphoresis in which the hydrogen ion concentration is determined at which the ampholyte does not migrate in solution or migrates in both directions under the influence

[20] Bredig, G., *Z. Elektrochem.*, 1899–1900, vi, 33.

[21] Walker, J., *Proc. Roy. Soc. London, Series B*, 1904, lxxiii, 155; 1905, lxxiv, 271; *Z. physik. Chem.*, 1904, xlix, 82; 1905, li, 706.

of an electric current has been used to determine the isoelectric points of a number of proteins and other complex bodies.[10, 22]

The equation for calculating the isoelectric point of an amphoteric electrolyte

$$I = \sqrt{\frac{k_a}{k_b} k_w}$$

was deduced by Michaelis and Mostynski.[23] Their deduction does not appear to be altogether clear in so far as the assumptions involved are concerned. The following deduction brings out perhaps more satisfactorily these assumptions. It includes the view that the concentration of the unionized molecule in each case is given by the total number of molecules, not separate molecular species as indicated above.

$$k_a\,(\mathrm{HAOH}) = (\mathrm{H^+})\,(\mathrm{AOH^-})$$

$$k_b\,(\mathrm{HAOH}) = (\mathrm{OH^-})\,(\mathrm{HA^+})$$

$$\frac{k_a}{k_b} = \frac{(\mathrm{H^+})(\mathrm{AOH^-})}{(\mathrm{OH^-})(\mathrm{HA^+})}$$

$$= \frac{(\mathrm{H^+})^2}{k_w} \cdot \frac{(\mathrm{AOH^-})}{(\mathrm{HA^+})}$$

If

$$(\mathrm{AOH^-}) = (\mathrm{HA^+})$$

$$(\mathrm{H^+}) = \sqrt{\frac{k_a}{k_b} k_w} \qquad (1)$$

This derivation assumes the equalities of (HAOH) in the two ionization equations and of (AOH^-) and (HA^+). In the Michaelis and Mostynski original deduction, the assumptions were stated to be the equality of the complex anion and cation concentrations and a minimum total ionization of the ampholyte.

The following deduction involves the view of different molecular species ionizing as acid and as basic salts.

[22] Hardy, W. B., *J. Physiol.*, 1898, xxiv, 288. Cohn, Gross, and Johnson,[2] p. 149.

[23] Michaelis, L., and Mostynski, B., *Biochem. Z.*, 1910, xxiv, 79.

$$k_a\,(\mathrm{HAOH}) = (\mathrm{H^+})\,(\mathrm{AOH^-})$$

$$k_b\,(\mathrm{HA_1OH}) = (\mathrm{OH^-})\,(\mathrm{HA_1^+})$$

$$\frac{k_a}{k_b} = \frac{(\mathrm{H^+})\,(\mathrm{AOH^-})\,(\mathrm{HA_1OH})}{(\mathrm{OH})\,(\mathrm{HA_1^+})\,(\mathrm{HAOH})}$$

$$= \frac{(\mathrm{H^+})^2\,(\mathrm{AOH^-})\,(\mathrm{HA_1OH})}{k_w\,(\mathrm{HA_1^+})\,(\mathrm{HAOH})}$$

$$(\mathrm{H^+}) = \sqrt{\underset{(a)}{\frac{k_a}{k_b}\,k_w} \cdot \underset{(b)}{\frac{(\mathrm{HA_1^+})}{(\mathrm{HA_1OH})}} \cdot \underset{(c)}{\frac{(\mathrm{HAOH})}{(\mathrm{AOH^-})}}} \qquad (2)$$

Equation (2) differs from equation (1) in containing the additional terms (*b*) and (*c*). For the two equations to give the same isoelectric point in any given case either of the following relations must hold (these are fundamentally the same with the terms arranged differently).

$$\frac{(\mathrm{HA^+})}{(\mathrm{AOH^-})} = \frac{(\mathrm{HA_1OH})}{(\mathrm{HAOH})} \qquad (3)$$

$$\frac{(\mathrm{HA_1^+})}{(\mathrm{HA_1OH}) + (\mathrm{HA_1^+})} = \frac{(\mathrm{AOH^-})}{(\mathrm{HAOH}) + (\mathrm{AOH^-})} \qquad (4)$$

From equation (4) it is evident that the degrees of ionization of the two molecular species are the same. This states nothing in regard to the concentrations of the ions or of the molecular species from which they are derived. Also, from equation (3), the ratio of the ampholyte ion concentrations is equal to the ratio of the corresponding unionized molecular species concentrations. That is to say, a greater ionic concentration of the ampholyte as acid species is accompanied by a greater relative concentration of the corresponding unionized molecular species. Obviously, also, equations (1) and (2) are identical if $(\mathrm{HA_1^+}) = (\mathrm{AOH^-})$ and $(\mathrm{HA_1OH}) = (\mathrm{HAOH})$, the assumptions under which equation (1) was deduced.

Equations (1) and (2) do not give the same isoelectric point, if the reciprocal of (*c*) is larger than (*b*), or the ionization as acid is greater than as base. The product of (*b*) and (*c*) will then be less than unity and the value obtained by means of equation (2) will be less than that given by equation (1). The reverse relation holds similarly.

Because of insufficient data, the application of these relations is possible only in isolated cases. For simple substances, including amino-acids, etc., the isoelectric point as defined is probably identical with the hydrogen ion concentration of the pure substance dissolved in water. For more complex substances such as proteins, with a number of different acid- and base-combining groups, there will probably ordinarily be an overlapping of actions. The isoelectric point will then be the hydrogen ion concentration which involves a minimum combination with added acid or alkali, where the protein exists most nearly uncombined. The isoelectric point of a substance obviously shows the relative strengths of the substance acting as an acid and as a base.

Equation (1) requires that the isoelectric point of an ampholyte does not change with change in concentration.[24] Some results with glycine and asparagine show definite if small changes in the hydrogen ion concentrations of solutions of these substances on dilution.[25] The use of equation (2) may help to explain these variations.

The agreement between the isoelectric points calculated by means of equation (1) and those found experimentally is surprisingly close in many cases. For substances such as glycine, etc., where the values of the acid and basic dissociation constants are not far removed from each other and the isoelectric points in the neighborhood of the hydrogen ion concentration of the solvent, this is not unexpected. For a substance like aspartic acid, for which $k_a^{26} = 1.5 \times 10^{-4}$ and $k_b^{26} = 1.2 \times 10^{-12}$, the calculated isoelectric point according to equation (1) is very nearly $(H^+) = 10^{-3}$N. The value found by the indicator method was $(H^+) = 10^{-2.9}$N. This can only mean that there is some sort of compensation with terms (*b*) and (*c*) of equation (2) resulting in the calculated values differing to only minor extents from those given by equation (1). It must be remembered, however, that this compensation is not a necessary conclusion in every case as far as known at present, but that differences may be shown by the two equations.

[24] *Cf.* Tizard, H. T., *J. Chem. Soc.*, 1910, xcvii, 2490.

[25] Quoted by Clark,[2] p. 30, from results of S. P. L. Sörensen.

[26] Winkelblech, K., *Z. physik. Chem.*, 1901, xxxvi, 546. Lundén, H., *Z. physik. Chem.*, 1906. liv, 532; *J. Biol. Chem.*, 1908, iv, 287.

SUMMARY.

The titration curves of solutions of glycine, alanine, α-amino-butyric acid, leucine, glycyl-glycine, alanyl-glycine, alanyl-alanine, acetone, acetamide, urea, acetic acid, and aceturic acid were determined and some of the relations as dependent upon the chemical structures discussed.

The isoelectric points of some of the amphoteric electrolytes were found experimentally. The definition of isoelectric point, its theoretical significance, and method of calculation were considered in some detail.