CXXIV. ZWITTERIONS.

I. PROOF OF THE ZWITTERION CONSTITUTION OF THE AMINO-ACID MOLECULE.

By LESLIE JULIUS HARRIS[1].

II. AMINO-ACIDS, POLYPEPTIDES, ETC., AND PROTEINS AS ZWITTERIONS, WITH INSTANCES OF NON-ZWITTERION AMPHOLYTES.

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Part I.

The suggestion that amino-acids might possess the zwitterion structure (1), $^{+}NH_3 . R . COO^{-}$ (rather than the classical structure (2), $NH_2 . R . COOH$), put forward by Adams [1916] and Bjerrum [1923], was based on a consideration of the acid and basic strengths of related compounds. Derivatives of acetic acid, etc., on the one hand, or of certain amines, etc., on the other, possessed fairly strongly acid or basic properties; and it seemed reasonable therefore to assign similar strongly acid and basic properties to glycine; which is indicated by formula (1) above (in opposition to formula (2), with its weak acid and basic properties). However, notwithstanding the force of the arguments adduced by Bjerrum, the zwitterion theory failed to receive any general acceptance, and Michaelis [1926] only reflects the almost universal view when he concludes that if any zwitterion is present it can be only "in extremely small amounts." As Michaelis cogently points out [1926], "There is no direct method up to the present time of demonstrating concretely the existence of the zwitterion...," for the methods adopted in the past for determining dissociation constants (titration curves, conductivity measurements, etc.) have been of no avail for deciding which view is correct. In the present communication[2] I am, however, able to bring forward direct evidence to show that the amino-acid molecule does possess, in preponderating proportion, the zwitterion and not the classical structure.

The implications of the new view are more far-reaching than may be apparent at first sight—the whole conception of the effects of acids and alkalis

[1] In the whole-time service of the Medical Research Council.

[2] Read before the Biochemical Society on Oct. 6th, 1928 [Harris, 1928].

on the ionisation of ampholytes has to be reversed; and new formulae must be attached to the ampholytes, and also to certain of their salts.

Effect of adding HCl and NaOH to ampholytes: implications of the zwitterion theory.

According to the classical theory addition of HCl to an amino-acid was supposed to cause ionisation of the basic NH_2-group as hydrochloride, and addition of NaOH was supposed to cause ionisation of the acid COOH-group as sodium salt. (The first of these reactions afforded a measure of k_b, the apparent basic strength of the ampholyte; and the second a measure of k_a, the apparent acid strength of the ampholyte[1].)

$$Cl^- + {}^+NH_3 . R . COOH \underset{(k_b;\ \text{low})}{\overset{+\ HCl}{\longleftarrow}} NH_2 . R . COOH \underset{(k_a;\ \text{low})}{\overset{+\ NaOH}{\longrightarrow}} NH_2 . R . COO^- + Na^+ \quad ...(1)\ \text{old view,}$$

$$Cl^- + {}^+NH_3 . R . COOH \underset{(K_A;\ \text{high})}{\longleftarrow} {}^+NH_3 . R . COO^- \underset{(K_B;\ \text{high})}{\longrightarrow} NH_2 . R . COO^- + Na^+ \quad ...(2)\ \text{new view.}$$

If, on the other hand, the *zwitterion* formula be correct additions of HCl and NaOH have just the reverse effect. For the amino-group of the free amino-acid (centre of equation (2)) is already ionised, so that addition of HCl merely serves to depress the ionisation of the carboxyl group, *i.e.* the acid carboxyl group is "replaced" by the stronger acid, HCl. And similarly with the addition of NaOH—the NaOH being a stronger base "replaces" the weaker base, *i.e.* the amino-group; and the ionisation of the carboxyl-group is left unchanged. (According to the new view, then, the addition of HCl measures the acid dissociation constant instead of the basic constant of the old view. Similarly the addition of NaOH measures the basic constant; for this addition does not affect the acid dissociation, but it depresses the basic dissociation.)

Interpretation of the HCl and NaOH titration curves of glycine according to the old and new theories respectively.

The above argument means, in brief, that the dissociation constants must be reversed if the new view is true: what was described as the basic constant according to the old view now relates to the new acid constant, and *vice versa*. The amino-acid is no longer considered as a potential weak base and a potential weak acid, but a comparatively strong acid and base simultaneously.

If we examine the corrected [Harris, 1923, 1; 1925, 1] titration curve [Henderson, 1908; Hasselbalch, 1911] of a comparatively weak acid, A_4B_4 (Fig. 1), we see that it is identical with the back titration curve of a comparatively strong base, $A_4'B_4'$. Similarly, the titration curve of a comparatively weak base $B_1'A_1'$ is indistinguishable from the back titration (replacement) curve of a comparatively strong acid B_1A_1. In general, titration curves of bases are indistinguishable from back titration curves of acids and the back

[1] For simplicity we shall use ionisation, instead of activity, notation throughout the present paper.

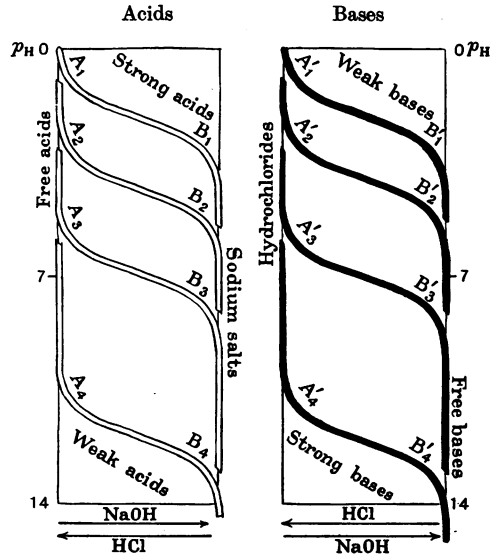


Fig. 1. Identity of titration curves of strong (weak) acids with back titration curves of weak (strong) bases, and *vice versa*. ("Corrected" titration curves.)

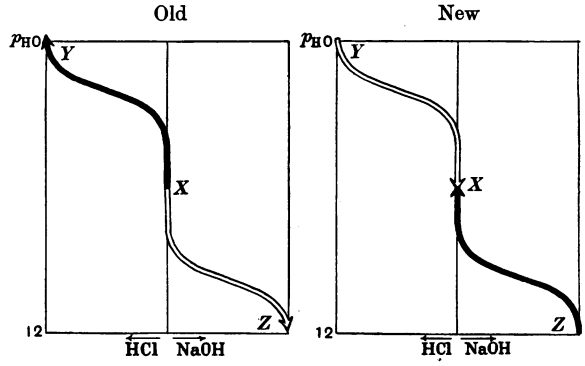


Fig. 2. Alternative interpretations of the experimental titration curve of glycine. (Position of arrow on curve shows end-point.)
═, acid group ▬, basic group.

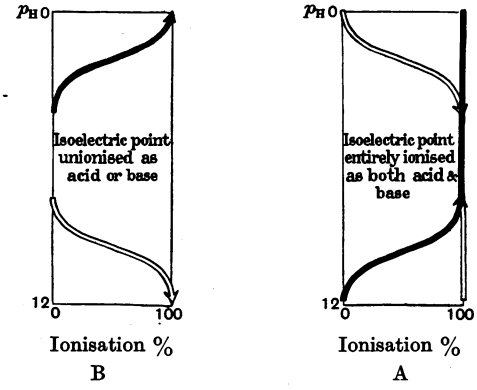


Fig. 3. Ionisation curves of glycine according to (A) new, and (B) old, views.

titration curves of bases from the titration curve of acids; in fact they coincide when the p_k (= p_H mid-point) is the same.

In the case of glycine, the experimental titration curve [Harris, 1923, 1] is shown in Fig. 2. This may be interpreted in two different ways, and we are unable to say by inspection of the curve which is correct. Either (1) XY is the titration curve of the basic amino-group and XZ is the titration curve of the acid carboxyl group, or (2) XY is the replacement curve of the acid carboxyl group and XZ is the replacement curve of the basic group. The first alternative implies that the amino-acid possesses the classical structure, and the second the zwitterion structure. If the first alternative is true, ionisation is at a maximum at highly acid and alkaline p_H values, and at a minimum at the isoelectric point. If the second alternative is true, on the contrary, ionisation is at a maximum at the isoelectric point (simultaneously as acid and base) (Fig. 3).

In earlier papers these two alternatives were recognised, but it was pointed out that it was immaterial from a practical point of view, in carrying out titrations for estimating acid and basic groups, which view was correct. With no effect on the practical accuracy, one could define the HCl curve (XY), either by the symbol k_b for an apparent basic group, or by the symbol [Bjerrum, 1923] K_A for the back titration of an acid group; and similarly the NaOH curve XZ, might be defined by an apparent acid constant, k_a, or by a zwitterion basic constant K_B [Harris, 1923, 1, p. 441; 1925, 2, p. 381].

If one could discover whether XY is a basic titration curve or an acid back-titration curve (or alternatively, whether XZ is an acid titration curve, or a basic back-titration curve) one would be in a position to decide which of the two theories is correct, classical or zwitterion.

Practical.

According to the new view, the amino-acid solution may be compared with a solution of ammonium acetate (*i.e.* moderately strong base plus moderately strong acid). According to the old view it may be compared with a solution of very weak base and very weak acid (*e.g.* aniline plus boric acid). That is, in the first instance the HCl and NaOH curves are back titration curves, of acid and basic groups respectively (*i.e.* acetate replaced by HCl and ammonium by NaOH); in the second they are direct titration curves of basic and acid groups respectively (*i.e.* aniline converted to hydrochloride, and boric acid to sodium salt)[1].

$$\mathrm{NH_4^+ + CH_3COOH \overset{+\,HCl}{\underset{K_a}{\longleftarrow}} NH_4^+ + CH_3COO^- \overset{+\,NaOH}{\underset{K_b}{\longrightarrow}} NH_3 + CH_3COO^-}$$

$$\mathrm{C_6H_5NH_3^+ + H_3BO_3 \overset{}{\underset{K_b}{\longleftarrow}} C_6H_5NH_2 + H_3BO_3 \overset{}{\underset{K_a}{\longrightarrow}} C_6H_5NH_2 + H_2BO_3^-.}$$

The technique of the present study involves the determination of acid-base combination curves (corrected titration curves) in the presence of form-

[1] In the case of the amino-acid, the two charges are of course carried by the same molecule; in the analogues cited by separate ions.

aldehyde, and a comparison of the results so obtained with those in water. Experimental details of the method have already been described at length [Harris, 1929].

Results.

When various carboxylic acids are titrated in dilute formaldehyde it is found that there is, in general, no significant shift in the position of the titration curve compared with that in water. On the other hand, when nitrogenous bases such as ammonia and amines are so titrated there is, in general, a very considerable shift of the titration curve in the direction of diminished basicity, owing to the formation of more weakly basic compounds of the type of methylene derivatives in equilibrium with the original base. The extent of the shift depends on the relative excess of formaldehyde present. We therefore find a very wide generalisation that a curve which shows a marked shift to diminished basicity, in presence of formaldehyde, relates to a basic group, and that acid groups are not so shifted.

Experimental results with ammonium acetate (0·1 N sol.) are shown in Fig. 4. Here the NaOH curve XZ is already known to relate to the basic

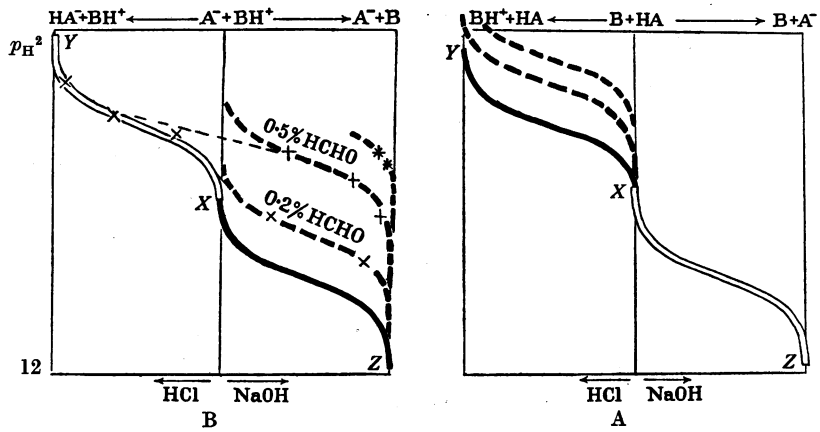


Fig. 4. Effect of small additions of formaldehyde on titration curves of (A) aniline and boric acid, (B) ammonium acetate.

═, acid curve. ▬▬, basic curve. ▬ ▬, basic curve in HCHO.

ion (p_K for ammonia being 9·3) and HCl curve XY to the acid ion (p_K for acetic acid being 4·7). As anticipated, the HCl curve is not appreciably shifted (since it relates to an acid group); and the NaOH curve is very considerably shifted (since it relates to a basic group). In the case of a weak acid-weak base mixture (analogue of classical ampholyte formula) the reverse is the case. The HCl curve, as expected, is shifted since it relates to the basic group, and the NaOH curve, which related to the acid group, is comparatively unaffected[1].

[1] With certain weak acids (SH, and boric acid) it is possible to detect the existence of a reaction with formaldehyde and some resulting shift in p_k value. In the case of boric acid, the shift is still relatively slight even in high concentrations of formaldehyde, and so remains in contrast with the large shift shown by bases in quite low concentrations of formaldehyde. With SH the shift is in the opposite direction to that of a base (see Part II, Fig. 18).

The result with glycine is shown in Fig. 5. The full experimental values for this and five other amino-acids have already been given elsewhere, where the conclusions here adduced were briefly summarised, and the analytical applications discussed in detail [Harris, 1929]. As was earlier predicted would be the case [Harris, 1923, 2], there is a considerable increase in k_a (= decrease in K_B), the constant for the apparent acid group, titratable by NaOH [Harris, 1925, 2]. The HCl curve, on the other hand, is not appreciably changed.

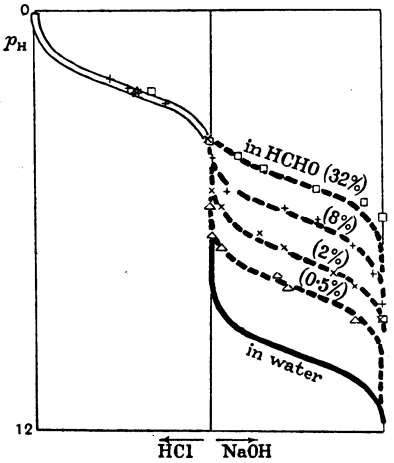


Fig. 5. Titration curves of glycine in presence of increasing concentrations of HCHO.

□, 32 % HCHO. +, 8 % HCHO.
×, 2 % HCHO. △, 0·5 % HCHO.

The fact that the NaOH curve is shifted towards diminished basicity, in the manner characteristic of basic groups, and that the HCl curve remains virtually unaffected, as is customary with acid groups, forces one to the conclusion that the former is the basic (back titration) curve and the latter the acid (back titration) curve, *i.e.* that the amino-acid has the zwitterion and not the classical constitution. Any other conclusion would involve ridiculous assumptions. The alternative interpretation, that there is an immense increase in the acid strength and virtually no measurable change in the basic strength, would obviously be meaningless. The formaldehyde is, in fact, added with the express purpose of combining with the NH_2-group and weakening its basicity, a property which is made use of in elementary analytical operations. And it would be equally ludicrous to imagine that so great an increase (*e.g.* ten-thousand fold) in the real strength (as opposed to k_a) of the carboxyl group could result from a slight modification in, maybe, a distant amino-group[1]. The entire parallel between the amino-acid and the zwitterion-analogue (ammonium acetate) and the unmistakable contrast between the amino-acid and the classical analogue (weak acid plus weak base) in their reactions to titration in formaldehyde, form sufficiently striking evidence in themselves.

Additional confirmation will be apparent (1) if it is shown for a compound containing x amino-groups and y carboxyl groups, that x of its curves are shifted by formaldehyde, and y remain virtually stationary; and also (2) if examples of certain ampholytes predicted to be of the classical and not of the zwitterion type are examined, and it is shown that here, in contrast with glycine, there is a shift not of the NaOH but of the HCl curve. Such confirmation is given in the next section.

[1] *E.g.* in the case of polypeptides (see Part II) where the distance of the amino- from the carboxyl group precludes the possibility of such an influence.

PART II.

Technical details. The technique was the same as that described earlier except that instead of making a separate colorimetric determination for each fresh point we used the hydrogen-electrode (in conjunction with a calomel-half-cell) for determining continuous p_H curves. With the exception of arginine and histidine which were titrated at 23°, and *p*-aminophenol at 25°, all titrations were carried out at room temperature, 14–19°. E.M.F. was measured by a Cambridge-Paul portable potentiometer. Our absolute p_H standard was 0·1 *N* HCl ($p_H = 1·07$) and the calomel cell and potentiometer were standardised against it at frequent intervals. In practically all cases titrations were carried out at a concentration of 0·1 *M*, either 2 or 5 cc. of the solution being used. In special cases, namely tyrosine, *p*-aminobenzoic acid and glycyltryptophan, concentrations as dilute as 0·01 *M* had to be used owing to the insolubility of the ampholyte and its methylene derivative at the isoelectric point. Glycyltryptophan behaved peculiarly: its methylene derivative although soluble on the alkaline side of the isoelectric point appeared to be practically insoluble on the acid side even in high concentrations of HCl.

In order to avoid any appreciable change in volume during titration *N* HCl and NaOH were used, being added from a micro-burette graduated in 0·01 cc. To save the necessity of performing two separate titrations, the requisite number of equivalents of acid or alkali were added to the free amino-acid so as to bring the p_H to the extreme end of either the acid or the alkali curve, and a single back titration was then carried out with either alkali or acid. Small concentrations of formaldehyde, 0·25–2 %, were found to be sufficient for obtaining an appreciable shift of the basic curve. The presence of formaldehyde in such concentration did not appear to affect the working of the hydrogen-electrode to any extent—since hardly any change in E.M.F. (1 m.v. or less) was observed when a small volume of water which had been brought to a given p_H was mixed with large excess of 5 % formaldehyde at the same p_H. Approximate blank corrections were made by determining the amount of acid or alkali required to bring the same volume of aqueous or dilute formaldehyde solvent to the same p_H in absence of the solute. The blank correction on the acid side is practically the same for dilute formaldehyde as for water. On the alkaline side, however, the blank for formaldehyde becomes considerable above p_H 10·5. Strictly speaking, the blanks obtained in this way are slightly too large, for the amino-acid uses up an appreciable proportion of the formaldehyde to form the methylene derivative.

In the case of *p*-aminobenzoic acid, the HCl curve has its p_k at a highly acid reaction (p_H 2) and the extreme insolubility of the methylene derivative necessitated the use of a large volume of solvent, so that there was a very large blank correction, and it became difficult to measure accurately the extent of the shift in formaldehyde. To overcome this difficulty, a 0·1 *M* solution of the ampholyte was made up with sufficient HCl to bring it to p_H 1·75, 18 cc. of a 5 % solution of formaldehyde were brought to the same p_H, and then 2 cc. of the ampholyte solution were added. The p_H dropped to 1·42. The amount of NaOH required to bring the p_H back to 1·75 was then determined (see Fig. 16). Controls were done by adding 2 cc. of ampholyte to 18 cc. of H_2O at p_H 1·75, and by diluting 18 cc. of 5 % formaldehyde with 2 cc. of H_2O at the same p_H. No appreciable shift in p_H was observed in either case. Similar points were determined on the NaOH curve. The same method was used with *p*-aminophenol.

The gelatin curve was obtained as follows. 5 cc. of a 1 % solution of gelatin were taken for each point and varying amounts of acid or alkali were added to each sample. In the presence of formaldehyde two of the p_H values (7·4 and 7·65) were determined colorimetrically because the formation of a gel interfered with the ready use of the hydrogen-electrode. The first NaOH curves for tyrosine and aspartic acid in presence of 16 % formaldehyde were also done colorimetrically as previously described [Harris, 1929].

1. DIBASE-MONOACIDS (HISTIDINE, LYSINE, ARGININE).

Figs. 6 and 7 show the results with histidine and lysine. It will be seen that two curves are considerably shifted and one remains virtually stationary, in the anticipated manner. The fact that the NaOH curve is shifted implies

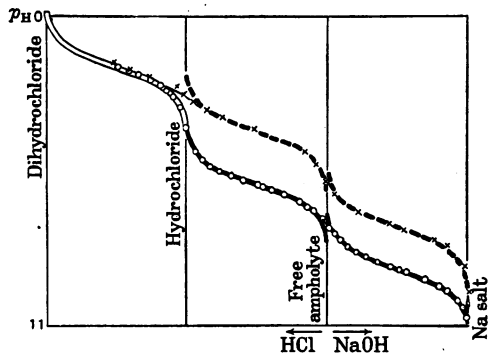


Fig. 6. Titration curves of histidine, in water and in HCHO.
O, in water. ×, in HCHO (1 %).

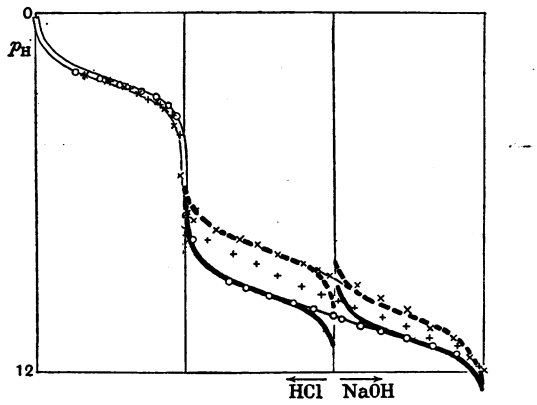


Fig. 7. Titration curves of lysine, in water and HCHO respectively.
O, in water. +, in HCHO (1 %). ×, in HCHO (2 %).

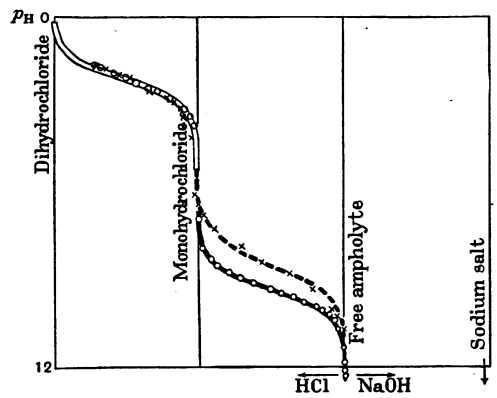
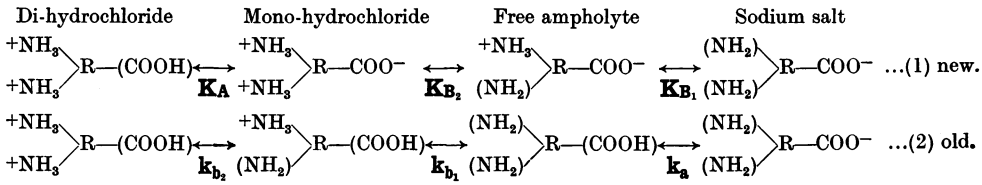


Fig. 8. Titration of arginine in water and HCHO.
O, in water. ×, in HCHO (0·25 %).

that histidine and lysine possess the zwitterion structure. Therefore the addition of NaOH does not represent neutralisation of the carboxyl-group as formerly supposed, but back titration of the stronger basic group. The first addition of HCl causes the ionisation as hydrochloride not of the stronger, but of the weaker of the two basic groups; while addition of the second equivalent of HCl contrary to former belief, represents not neutralisation of the weaker basic group but back titration of the fairly strong acid (COOH) group. Similar results are obtained with arginine except that the guanidine group is so strongly basic that its titration curve occurs in an inaccessibly alkaline zone and that the determination of a shift is rendered difficult by the very large blank correction.

Equation (1) shows the new view of the compounds formed when HCl and NaOH are added to these ampholytes; equation (2) representing the old view. (Addition of HCl, right to left; addition of NaOH, left to right.)



2. Monobase-diacids (aspartic acid, glutamic acid, tyrosine, cysteine, Figs. 9, 10 and 11).

In the case of aspartic and glutamic acids (Fig. 9), we have one curve showing the typical shift and two virtually stationary, corresponding with one NH_2- and two COOH-groups. (Tyrosine and cysteine (Figs. 10 and 11) with their very weakly acid OH- and SH-groups are similar, apart from the distinctive behaviour of the SH-group towards formaldehyde[1].) Addition of HCl represents not titration of NH_2 as in the old view, but back titration of a fairly strongly acid COOH-group, viz. the stronger of the two COOH-groups in glutamic and aspartic acid. Addition of the first equivalent of NaOH represents, in the cases of glutamic and aspartic acids, titration of the weaker of the two COOH-groups and not the stronger, as in the old view; in the cases of tyrosine and cysteine, it represents back titration of the NH_2-group, instead of titration of the stronger COOH as in the old view. Addition of the second equivalent of NaOH represents, in the cases of glutamic and aspartic acids, back titration of the fairly strong NH_2-group, in place of titration of the very weak second COOH-group on the old view; in the cases of tyrosine and cysteine it no doubt represents titration of the very weak OH- and SH-groups respectively[1].

Cannan and Knight [1927], it will be recalled, were unable to decide which

[1] It will be seen (Fig. 11) that there is evidence of a reaction occurring between the SH-group of cysteine and HCHO which results in a perceptible weakening of its acidity and a measurable shift of the p_k to increased basicity [cf. Pirie and Pinhey, 1929] very distinct from the typical shift to diminished basicity characteristic of a basic group.

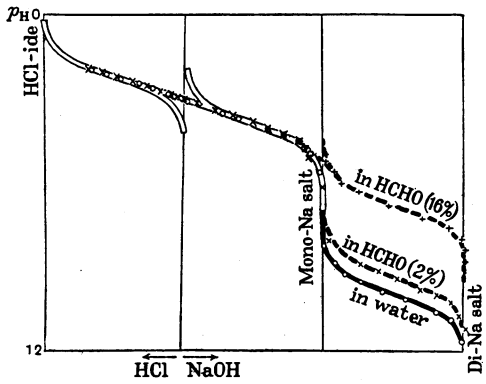


Fig. 9. Titration of aspartic acid in water and HCHO.
○, in water. ×, in HCHO (2 %). +, in HCHO (16 %).

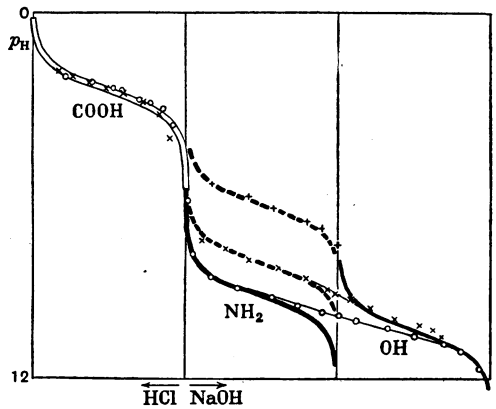


Fig. 10. Titration of tyrosine in water and HCHO.
○, in water. ×, in HCHO (2 %). +, in HCHO (16 %).

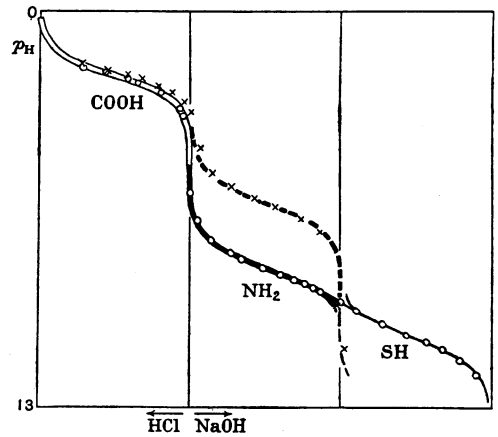
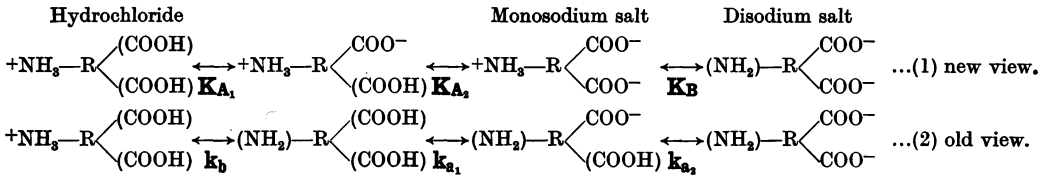


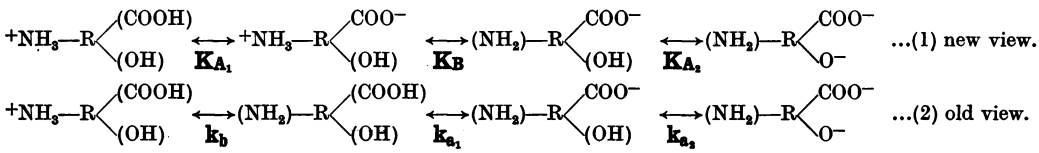
Fig. 11. Titration of cysteine in water and HCHO. ○, in water. ×, in HCHO (1·5 %).

arrangement was probable for cysteine, since, with the evidence then available "plausible argument could be made for either allocation."

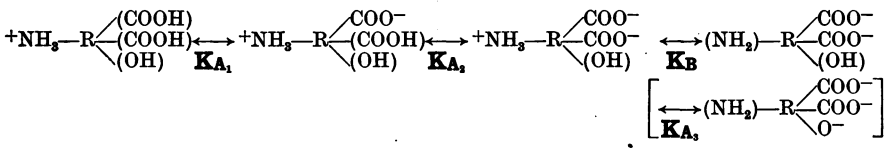
For glutamic and aspartic acids.



For tyrosine (and cysteine)[1].



For hydroxyglutamic acid (not yet examined), the reactions may be predicted as follows, with a vanishingly small K_{A_3}:



It will be seen that new formulae are given for the monohydrochloride and the free amino-acid, in the case of the monoacid-dibasic ampholytes; the dihydrochloride and sodium salt retaining the old constitution. With glutamic and aspartic acids, the monosodium salt and free ampholyte are given new constitutions, the other salts remaining unchanged; but with tyrosine and cysteine, only the free amino-acid is changed and both the sodium salts are unaltered.

3. Polypeptides as zwitterions.

In Figs. 12 and 13 are shown typical results with a di- and a tri-peptide, glycyltryptophan, and glycylglycylglycine. It will be observed that these compounds behave similarly to the simple amino-acids with the same number of basic and acid groups from which they are derived. The constants of peptides show [Harris, 1923, 1] a slight decrease in value of K_A and K_B compared with the corresponding amino-acid as, of course, is to be anticipated, from the removal of the influence of the neighbouring ionised group. The constants of glycyltryptophan not hitherto known are reported elsewhere [Birch, 1930].

[1] For cysteine put SH for OH in equations following.

4. Taurine.

The constants of taurine in themselves afford very suggestive evidence, by analogy, for the zwitterion theory. Taurine contains one amino-group,

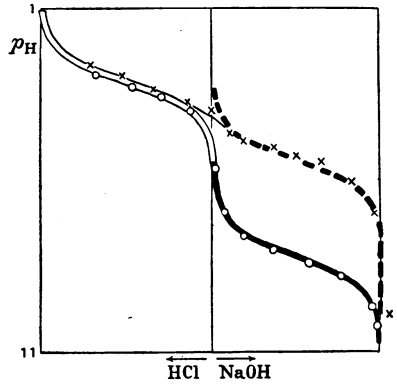


Fig. 12.

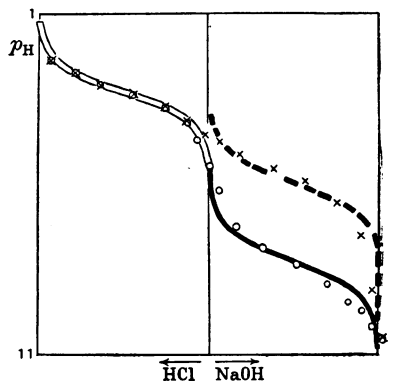


Fig. 13.

Fig. 12. Titration curve of a dipeptide, glycyltryptophan, in water and HCHO.
○, in water. ×, in HCHO (1 %).

Fig. 13. Titration curve of a tripeptide, glycylglycylglycine, in water and HCHO.
○, in water. ×, in HCHO (1 %).

therein resembling an ordinary α-monoaminomonocarboxylic acid; but the usual carboxyl group is replaced by sulphonic acid. According to the classical view the acid dissociation constant of taurine is quite small, corresponding in fact with that of other amino-acids; but the basic dissociation constant is vanishingly small. This view is from the first difficult to accept, because one would expect a very high acid constant as in other sulphonic acids, and a basic constant similar to that of an ordinary α-amino-acid or peptide.

The constants fall into line, however, if the new view is accepted, K_A being very high, as in other sulphonic acids; and K_B being of the same order as K_B for analogous compounds (*i.e.* fairly high). The NaOH curve, that is, is presumed to be the displacement curve of the NH_2-group and not the titration curve of the sulphonic acid group.

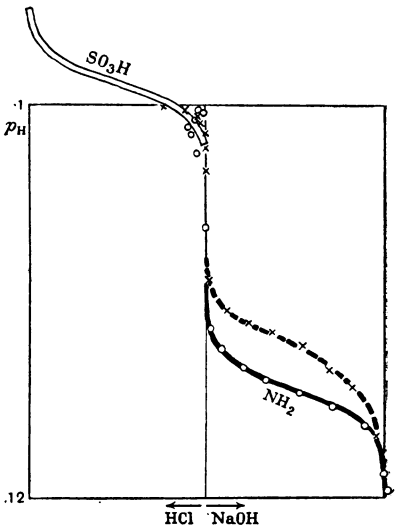


Fig. 14. Titration curves of taurine as *experimentum crucis*.
○, in water. ×, in HCHO (2 %).

Our formaldehyde titration curve of taurine (Fig. 14) confirms very decisively the zwitterion hypothesis. The NaOH curve is markedly shifted, in the manner

characteristic of a base or the basic groups of the zwitterion amino-acids. It would be impossible to picture formaldehyde causing a very marked increase in the acid constant of the sulphonic acid group, which would be the alternative interpretation.

5. Demonstration of non-zwitterion constitution of certain ampholytes.

Interpreting the glycine titration curve in the zwitterion fashion, as we are now entitled to do, the acid and basic curves are seen to fall in the same relative positions as in the combined curves of acetic acid plus ammonia (*i.e.* the HCl curve refers to the acid group, and the NaOH curve to the basic group)[1] (see Part I, Figs. 2 and 4). When, in contrast, we consider the combined curves of a weak base such as aniline and a weak acid such as phenol we see that they possess, to an extreme degree, the formal appearance of the predicted curves of the non-zwitterion type of electrolyte (that is, the HCl is the basic curve and the NaOH the acid curve). Hence we are led to suppose that the ampholyte derived from these two substances, that is aminophenol, will have the non-zwitterion form (Fig. 15). To test the accuracy of our supposition, we have determined the titration curves of *p*-aminophenol in water and in formaldehyde. The result confirms our prediction and definitely establishes the non-zwitterion nature of this ampholyte. For, in direct contrast with glycine, the HCl curve shows the characteristic displacement to a lowered p_H, and the NaOH curve is virtually unchanged. Hence, the HCl curve

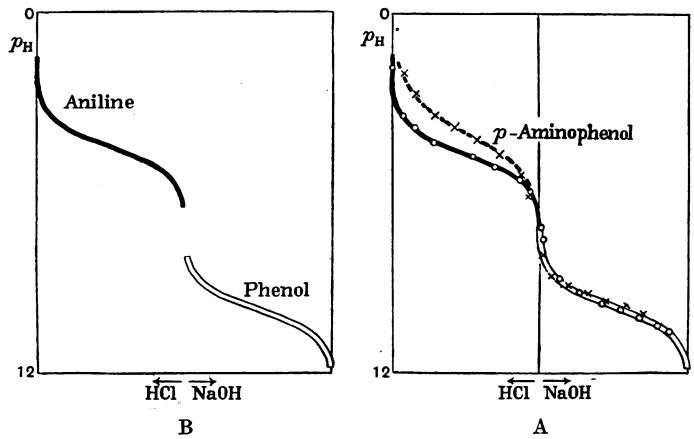


Fig. 15. Titration curves (A) of *p*-aminophenol and (B) of parent substances, aniline and phenol.
○, in water. ×, in HCHO (1·5 %).

[1] For a quantitative prediction of the precise p_k values it would be necessary to pay regard to the electrostatic effect of the simultaneously ionised neighbouring groups; and the ampholyte is more accurately to be compared not with the parent substances themselves, acetic acid and ammonia, but with their substituted derivatives.

relates to the basic, and the NaOH curve to the acid group. The action of HCl and NaOH is to be represented

$$^{+}NH_3 . R . (OH) \xleftarrow{HCl} (NH_2) . R . (OH) \xrightarrow{NaOH} (NH_2) . R . O^{-}.$$

p-*Aminobenzoic acid.* In the case of *p*-aminobenzoic acid the comparative weakness of the parent substances (aniline and benzoic acid) again renders the non-zwitterion structure more likely. In this instance, however, the prediction might at first sight seem somewhat less secure, because benzoic acid is by no means so weak an acid as is phenol in the case just cited. The known constants for *p*-aminobenzoic acid are 2·0 and 4·8. If 2·0 is the basic and 4·8 the acid constant, *p*-aminobenzoic acid will be a non-zwitterion. If, conversely, 2·0 is the acid and 4·8 the basic constant, *p*-aminobenzoic acid will be a zwitterion. The constants of the parent substances benzoic acid and aniline are

$$\text{benzoic acid, } p_k \quad \ldots \quad = 4{\cdot}2,$$
$$\text{aniline, } p_k \quad \ldots \quad \ldots \quad = 4{\cdot}6;$$

a combination of which values would represent a bare border-line case of zwitterion. However, it is more accurate to take as types not the parent substances, benzoic acid and aniline, but comparable *para*-substituted derivatives. We are indebted to Mr J. B. S. Haldane for the suggestion that *p*-aminobenzoic acid may be predicted to have an acid constant near to that of *p*-methyl (or hydroxy)-benzoic acid and a basic constant near to that of *p*-nitroaniline. The values in question are

$$p\text{-nitroaniline, } p_k \quad \ldots \quad \ldots \quad \ldots \quad = 2{\cdot}0,$$
$$p\text{-methyl (or hydroxy)-benzoic acid, } p_k = 4{\cdot}4\text{–}4{\cdot}5.$$

That is, the predicted values of *p*-aminobenzoic acid are very close to the known values, provided the latter be taken in the non-zwitterion order. Our experimental results appear to indicate that *p*-aminobenzoic acid does indeed possess the non-zwitterion form, to a preponderating extent. For we find that the HCl curve is shifted by HCHO in the manner typical of a basic group and the NaOH curve is not significantly changed (Fig. 16).

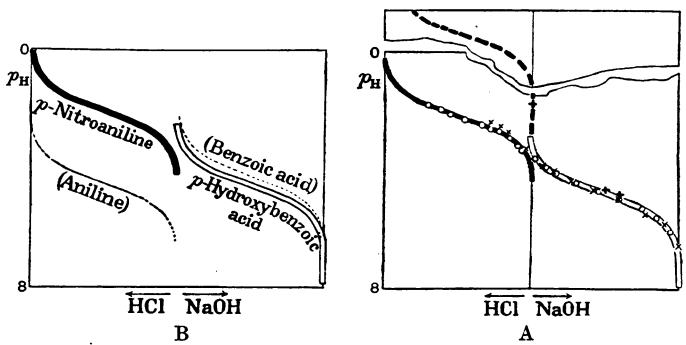
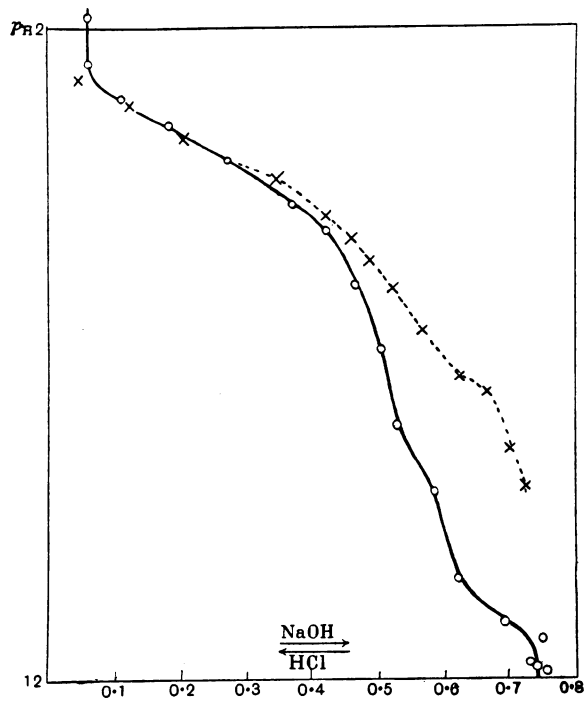


Fig. 16. Titration curves of (A) *p*-aminobenzoic acid in water and in HCHO and (B) combined titration curves of parent substances and related derivatives.
○, in water. ×, in HCHO (0·5 %). +, in HCHO (5·0 %).

6. Proteins as zwitterions.

Having shown that amino-acids possess the zwitterion structure and then extended our observation to embrace di- and tri-peptides, it became almost a foregone conclusion that proteins also would be found, when we came to apply the same technique, to belong to this category. In the case of one protein, gelatin, we have already pointed out [Harris, 1925, 2] that it was impossible to accept the previously universal view that the isoelectric molecule possessed no ionic charges, because the p_H difference between the acid end-point at p_H 2·4 and the isoelectric point at p_H 4·7 is less than the minimum of 4 p_H units, which is necessary to cover the conversion (98 %) of even one charged group into its non-charged state. Therefore, since isoelectric gelatin possesses no net charge, positive or negative, it was necessary to assume that there was an equal number of negative charges to balance the positive, unless, of course, the accepted values for the acid end-point and isoelectric point could be shown to be in error. But the foregoing argument, while demonstrating that isoelectric gelatin is no longer to be regarded as non-ionised, does not go so far as to indicate that the titration of the principal acid groups occurs in the acid p_H range and that of the principal basic groups in the alkaline range, which



Cc. of $N/1$ acid or alkali per 0·5 g. crude protein

Fig. 17. Titration curves of gelatin in water and in HCHO.
○, in water. ×, in HCHO (1 %).

is the full implication of the zwitterion results in the case of amino-acids and peptides. This we now show to apply to gelatin by our formaldehyde titrations which are summarised in Fig. 17. The ionisation of gelatin is therefore to be represented mainly as

$$(^{+}NH_3)_x \,.\, \mathrm{Pr} \,.\, (COOH)_y \xleftarrow{+\,HCl} (^{+}NH_3)_x \,.\, \mathrm{Pr} \,.\, (COO^{-})_y \xrightarrow{+\,NaOH} (NH_2)_x \,.\, \mathrm{Pr} \,.\, (COO^{-})_y.$$

7. Controls with various acids and bases.

The general behaviour of single bases and acids with HCHO is summarised in Fig. 18. Acetic and other carboxylic acids are not significantly affected. Ammonia, various amines, etc., show considerable shifts with very small additions of formaldehyde. With boric acid a shift can be detected but is comparatively small even with large amounts of formaldehyde.

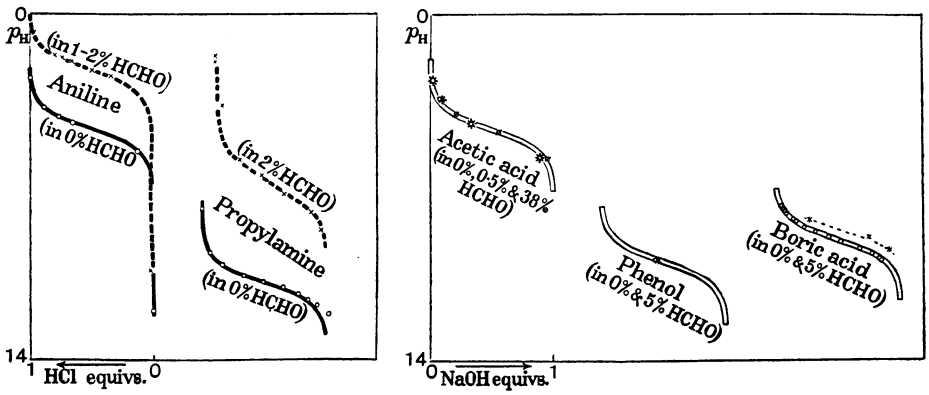


Fig. 18. Effect of formaldehyde on titration curves of various bases and acids.
○, in water. ✱, in 38 % HCHO, colorimetric. ∗, in 5 % HCHO.
×, in 1–2 % HCHO. /, in 0·5 % HCHO.

8. Addendum on the temperature coefficient method.

Elsewhere [Birch and Harris, 1930] we have referred to a further method which enables us to distinguish between a carboxyl and an amino-group in the titration curve of a given amino-acid, and hence to obtain evidence for or against the zwitterion structure. Briefly it depends on the observation that the p_k of a carboxyl group shows little change with temperature when calculated as an acid constant on the p_H scale, while the p_k of an aliphatic amino-group shows little change with temperature when calculated as a basic constant on the p_{OH} scale. The relative allocation of curves to carboxyl and amino-groups as deduced from this method agrees with the conclusions to be drawn from the formaldehyde titration method; and we have described the application of the method to the identification of the three constitutions of the titration curve of histidine and of arginine.

Summary.

Part I.

The HCl and NaOH titration curves of glycine (measured by p_H indicator virages) have been determined in the presence of increasing concentrations of formaldehyde. The weakening of the basicity of the NH_2-group caused by the formation of a methylene derivative is found to result in a displacement not of the HCl curve (*i.e.* the apparent basic curve), but of the NaOH curve (*i.e.* the apparent acid curve). It follows that the HCl and NaOH titration curves of the amino-acid represent, not neutralisation of NH_2 and COOH respectively, as in the classical view, but "replacement" (back titration) of COOH and of NH_2 by a stronger acid and a stronger base, HCl and NaOH respectively.

Direct evidence is therefore available of the accuracy of the zwitterion (ionised internal salt) theory.

That the shift with HCHO to lowered basicity is characteristic of a basic curve is shown by similar investigations with free bases and ammonium salts, etc., and with other amino-acids and ampholytes.

Part II.

With ampholytes containing more than one NH_2- or COOH-group the number of curves characteristically shifted is shown to be equal to the number of basic groups, and those virtually unaffected to the COOH-groups.

The method has been used for determining whether a given ampholyte possesses the zwitterion or non-zwitterion constitution. By its aid it is also possible to determine which are the basic and which the acid components of the titration curves.

New formulae must be ascribed to the monohydrochlorides and the free amino-acids in the case of diaminomonocarboxylic acids, and to the monosodium salts and the free amino-acids in the case of dicarboxymonoamino-acids. The stages in the ionisation of cysteine, tyrosine, and other amino-acids and their salts are similarly set out.

The apparent acid constant of taurine is demonstrated to relate to the basic group.

p-Aminophenol and *p*-aminobenzoic acid are demonstrated to exist as non-zwitterions, HCHO giving a significant shift of the HCl and not the NaOH curve.

Polypeptides are shown to be zwitterions. Similarly with a protein (gelatin) the COOH-groups titrate in the acid and the NH_2-groups in the alkaline p_H range.

COOH- and NH_2-groups in titration curves may be identified also (and hence the zwitterion theory tested) by a new "temperature coefficient" method which depends on the fact that the dissociation constants of NH_2-groups are found to show only small temperature changes when expressed on

the p_{OH} scale and large changes on the p_H scale, and *vice versa* for COOH-groups.

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