T H E S I S

on

The Effect of Salts on the Apparent Isoelectric Point of Amphoteric Electrolytes

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The problem of the effect of salts on the apparent isoelectric point of amphoteric substances was brought to our attention by some very interesting work of Baly (1) on sewage colloids. He found, by means of measurements on the optimum flocculation of the colloids with negatively charged Bentonite suspensions, that the addition of sodium chloride up to 3% caused successive shifts of the point of minimum stability (optimum precipitation) amounting to several pH units toward the alkaline side. His work was substantiated by Ghosh (2) who studied the phenomenon as related to gelatin and haemoglobin.

As a consequence of these observations the present work was undertaken to determine, if possible, the underlying cause of such a shift, by observing the behavior of other amphoteric materials, both colloidal and non-colloidal in nature, and by correlating these results with those obtained by other investigators.

Kondo and Hayashi (3) investigated the effect of salts on the apparent isoelectric point of rice glutelin, finding, in opposition to general results obtained by Baly and Ghosh, that the isoelectric point was shifted to the acid side, the effect of the individual ions agreeing with the Hofmeister series. They reported similar effects on casein and globulin. They attributed their results to the power of the different ions to keep the protein in the ionic form even when the hydrogen ion activity of the so-

lution was identical with the theoretical isoelectric point of the protein. They further concluded that this ionizing power would cause the point of minimum ionization or maximum flocculation of the protein to differ from the theoretically calculated isoelectric point, in the presence of salts, and that the shift in the point of optimum precipitation of a difficultly soluble ampholyte is only an apparent shift in the isoelectric point.

During the progress of the present investigation articles have appeared by H. Andrzejewski (4) bearing on the effect of salts on the isoelectric point of gelatin; by Przylecki (5), and by Siedroyé (6) concerning similar effects on caseinogen and serum albumin. All of these investigators determined the minimum amount of alcohol required to precipitate the protein at definite pH values. By keeping the pH constant at given values and adding salt, they found that at the isoelectric point and below it, added salts increased the minimum amount of alcohol required, to an extent diminishing as the pH value of the solutions was decreased. Above the isoelectric point the amount of alcohol increased as the pH was raised. This may be interpreted as involving a shift of the apparent isoelectric point to the acid side (in this case, the point at which a minimum of alcohol was required to precipitate the protein), but the authors, not being interested in the aspects of the problem presented in this thesis, drew no

such conclusions.

THEORY

Michaelis (7) defines the isoelectric point of an amphoteric electrolyte as the hydrogen ion concentration at which the sum of the cations and anions of an ampholyte is at a minimum, and at which the concentration of the cations is equal to that of the anions. As a result of this minimum of ionization, other properties, such as conductivity, solubility, viscosity, osmotic pressure, swelling of gels, etc., will also exhibit minimum or maximum values at the isoelectric point. Michaelis defines a value which he calls the dissociation residue, ρ, as the ratio of the concentration of the undissociated portion, X, to the total concentration, A, of the ampholyte, and it naturally follows that at the point of minimum dissociation this value will be at a maximum. Thus, the isoelectric point of an ampholyte is the hydrogen ion concentration at which the dissociation residue is a maximum.

If X^+ represents the concentration of cations, X^- the concentration of anions, K_a the acid dissociation constant and K_b the basic dissociation constant, an expression may be derived which represents the hydrogen ion concentration at the isoelectric point. The dissociation of an amphoteric electrolyte in solution is expressed, according to classical views, by the equations:

$$X \rightleftharpoons X^- + H^+ \qquad K_a = \frac{X^- \cdot [H^+]}{X}$$

$$X + HOH \rightleftharpoons X^+ + OH^- \qquad K_b = \frac{X^+ \cdot [OH^-]}{X}$$

The dissociation may be also expressed in the following manner: $A - X^+ - X^- = X$

Substituting values obtained for X^+ and X^-

$$X = A - K_b \frac{X}{[OH^-]} - K_a \frac{X}{[H^+]} = \frac{A}{1 + \frac{K_a}{[H^+]} + \frac{K_b}{[OH^-]}}$$

By definition

$$\rho = \frac{X}{A} = \frac{1}{1 + \frac{K_a}{[H^+]} + \frac{K_b}{[OH^-]}}$$

Substituting for $[OH^-]$ its value, $\frac{K_w}{[H^+]}$

$$\rho = \frac{1}{1 + \frac{K_a}{[H^+]} + \frac{K_b}{K_w} \cdot [H^+]}$$

Since the dissociation residue is a maximum at the isoelectric point, its reciprocal is a minimum at the same point, and has the value

$$\frac{1}{\rho} = 1 + \frac{K_a}{[H^+]} + \frac{K_b}{K_w} \cdot [H^+]$$

Differentiating with respect to $[H^+]$, yields

$$\frac{d(\frac{1}{\rho})}{d[H^+]} = -\frac{K_a}{[H^+]^2} + \frac{K_b}{K_w}$$

Equating the expression to zero to obtain the minimum value of $\frac{1}{\rho}$, and solving,

$$[H^+] = \sqrt{\frac{K_a}{K_b} \cdot K_w}$$

Thus, at the isoelectric point, (the point of maximum dissociation residue), the hydrogen ion concentration is expressed in terms of the acid and basic dissociation constants and the ion product for water. The above expression holds for a solution of an ampholyte, either colloidal or non-colloidal in nature, in the absence of true salts of the ampholyte, since it represents the hydrogen ion concentration at the point where there is a maximum of undissociated molecules.

Michaelis (8) further utilizes this conception of maximum dissociation residue in an attempt to explain the behavior of ampholytes in the process of true salt formation. It is evident that if ρ is plotted as the ordinate against pH as the abscissa there will be a maximum for ρ at the pH of the isoelectric point. This curve is called the ρ curve for the ampholyte, and in its construction it is assumed that the pH changes are brought about by buffers which do not form true salts with the ampholyte. If the addition of a salt causes the formation of two salts of the ampholyte which may not be completely ionized, one with the anion and one with the cation, having dissociation constants differing from those of the pure ampholyte and from each other, both the ordinate and the abscissa of the ρ curve will be shifted. The ordinate values will be lowered at all points probably because of the fact that the salts formed will have higher degrees of dissociation at all pH

values than the pure ampholyte. The abscissa values will be shifted to the right or left because of the difference between the dissociation constants of the salts formed. If superficial consideration only were given to this effect it would appear that there had been an actual shift of the isoelectric point by the addition of the salt. However, by defining the isoelectric point once more, in terms of degree of charge, or the difference between the concentration of the cation and the anion, it is found that the isoelectric point is the hydrogen ion concentration at which the degree of charge is zero. By calculating from expressions involving ρ, the degree of charge, the dissociation constants of the ampholyte and the dissociation constants of the true salts of the ampholyte, it is found that the original expression for the hydrogen ion concentration at the isoelectric point may be derived regardless of the presence of true salts of the ampholyte. Consequently, it is concluded that true salt formation does not deflect the actual isoelectric point, but since the point of maximum ρ is deflected, the pH values at maximum ρ and at the isoelectric point are not identical in the presence of true salts of the ampholytes. This might be, then, a logical explanation for the apparent shift of the isoelectric point on the addition of salt to an ampholyte, and is used by Kondo and Hayashi in a manner essentially the same but differing in mode of explanation ; (see page 2).

The present investigation was not planned to settle the extremely difficult question as to whether the addition of salts actually causes a shift in the true isoelectric point, but was planned to be devoted to the study of the effect of salts in shifting the pH concurrent with a maximum value of the dissociation residue, and to attempt a further explanation of the observed and correlated facts.

Examination of the literature shows one or two calculations such as that of Michaelis (see above) which might possibly explain a shift in the point of minimum stability (apparent isoelectric point) upon addition of neutral salts. In addition, some of the more recent developments in the theory of solutions, due to Brønsted, Bjerrum, and Debye and Hückel may be applied to this particular problem in such a manner as to offer hope of a general solution. This treatment follows:

According to the classical theory of the ionization of ampholytes, the ampholyte in solution is regarded as existing in three forms, the undissociated molecule, the cation, and the anion. For the electrolytic dissociation of such a substance in aqueous solution, the following equations are postulated:

$$NH_2 \cdot R \cdot COOH \rightleftharpoons NH_2 \cdot R \cdot COO^- + H^+$$

$$NH_2 \cdot R \cdot COOH + HOH \rightleftharpoons NH_3^+ \cdot R \cdot COOH + OH^-$$

If the same designations be used as in the proof of the iso-

electric point formula, the same relations will exist:

$$X \rightleftharpoons X^- + H^+ \qquad K_a = \frac{X^- \cdot [H^+]}{X} \qquad 1$$

$$X + HOH \rightleftharpoons X^+ + OH^- \qquad K_b = \frac{X^+ \cdot [OH^-]}{X} \qquad 2$$

A more modern manner of representing the dissociation of amino acids has been given by Bjerrum (9). He shows evidence that the undissociated electrolyte exists almost entirely in the form of the "Zwitter" ion, or amphoteric ion which is neutral but carries both a positive and a negative charge. The Zwitter ion is postulated as having the following formula: $NH_3^+ \cdot R \cdot COO^-$

The formulation of K_a and K_b is essentially the same as in the classical theory.

The extended theory of acids and bases developed by Brønsted, (10), which has proved to be of outstanding value in the interpretation of the chemistry of solutions, defines an acid as any substance capable of giving up a proton, and a base as any substance which is capable of accepting a proton. It leads to a somewhat modified expression for the dissociation constant of a base as is shown in what follows. Walker (11) has offered an explanation of the dissociation of amino acids which, when modified to be in accord with the inter-ionic attraction theory, Brønsted's extended theory, and Bjerrum's conception of the Zwitter ion, leads to the following expression:

$$NH_3^+ \cdot R \cdot COO^- + H^+ \rightleftharpoons NH_3^+ \cdot R \cdot COOH$$
$$\text{Base} + H^+ \rightleftharpoons \text{Acid}$$
$$NH_3^+ \cdot R \cdot COO^- \rightleftharpoons NH_2 \cdot R \cdot COO^- + H^+$$
$$\text{Acid} \rightleftharpoons \text{Base} + H^+$$

If K_A represents the acid dissociation constant, K_B the basic dissociation constant, $X^{\pm}$ the concentration of Zwitter ions and X^+ and X^- the concentration of the cation and anion respectively,

$$K_A = \frac{X^- \cdot [H^+]}{X^{\pm}} \qquad 3$$

$$K_B = \frac{X^+}{X^{\pm} \cdot [H^+]} \qquad 4$$

If the ampholyte does not exist in the form of a Zwitter ion, which is probably the case with the amino benzoic acids, a similar expression may be formulated on the basis of Brønsted's work, but omitting the use of the Zwitter ion.

$$NH_2 \cdot C_6H_4 \cdot COOH + H^+ \rightleftharpoons NH_3^+ \cdot C_6H_4 \cdot COOH \qquad 6$$
$$\text{Base} + H^+ \rightleftharpoons \text{Acid}$$
$$NH_2 \cdot C_6H_4 \cdot COOH \rightleftharpoons NH_2 \cdot C_6H_4 \cdot COO^- + H^+ \qquad 5$$
$$\text{Acid} \rightleftharpoons \text{Base} + H^+$$

Using the same terminology, but indicating the concentration of undissociated molecules by X:

$$K_B = \frac{X^+}{X \cdot [H^+]} \qquad 6$$

$$K_A = \frac{X^- \cdot [H^+]}{X} \qquad 5$$

Since it is immaterial, for the purpose of calculating the dissociation constants, whether the undissociated portion is represented by Zwitter ions or by undissociated mole-

cules, 5 and 6 are identical with 3 and 4. The undissociated portion will hereafter be designated by X. If 6 is multiplied by K_W,

$$K_B \cdot K_W = \frac{X^+ \cdot [H^+] \cdot [OH^-]}{X \cdot [H^+]} = \frac{X^+ \cdot [OH^-]}{X} = K_b$$

So expression 6 and 2 are related. This is a general relationship holding in all cases between K_B as defined by Brønsted and K_b, the classical dissociation constant. The dissociation constants derived above are not true constants but are subject to variations with changes in salt concentration.

However, if the thermodynamic expressions for the dissociation constants be used, the constants so derived are true constants under all conditions. If K_A' and K_B' represent the thermodynamic dissociation constants of acid and base respectively, f represents the activity coefficient of the substance, and a represents the activity, the following expressions may be derived.

$$K_A' = \frac{a_{X^-} \cdot a_{H^+}}{a_X} = \frac{X^- \cdot [H^+]}{X} \cdot \frac{f_{X^-} \cdot f_{H^+}}{f_X} = K_A \cdot \frac{f_{X^-} \cdot f_{H^+}}{f_X} \qquad 7$$

$$K_B' = \frac{a_{X^+}}{a_X \cdot a_{H^+}} = \frac{X^+}{X \cdot [H^+]} \cdot \frac{f_{X^+}}{f_X \cdot f_{H^+}} = K_B \cdot \frac{f_{X^+}}{f_X \cdot f_{H^+}} \qquad 8$$

The variation of the activity coefficient with salts is given, at least qualitatively, by the limiting law of the Debye-Huckel inter-ionic attraction theory (12),

$$-\log f = 0.5\, z_1 z_2 \sqrt{\mu} = 0.5\, z^2 \sqrt{\mu}$$

where μ is the ionic strength of the solution, and z_1 and z_2 are the valences of the ions affected by the salt addition.

In the case of the ampholytes used in this paper, z_1 and z_2 can be assumed equal and are probably unity in value. Consider again the general case from which equations 7 and 8 were derived (10):

$$Acid \rightleftharpoons Base + H^+ \qquad 9$$

Let C_A, C_B and C_{H^+} represent the concentration of acid, base and hydrogen ion respectively, let f_A, f_B, and f_{H^+} represent the corresponding activity coefficients, and let K_A be used as previously. Computing for the individual activity coefficients,

$$\log f_B = -0.5\, z_B^2 \sqrt{\mu}$$
$$\log f_{H^+} = -0.5\, z_{H^+}^2 \sqrt{\mu}$$
$$\log f_A = -0.5\, z_A^2 \sqrt{\mu}$$

Using 7

$$\log \frac{f_B \cdot f_{H^+}}{f_A} = \log f_B + \log f_{H^+} + \log f_A = -0.5\sqrt{\mu}\left(z_B^2 + z_{H^+}^2 - z_A^2\right)$$

Valence of H^+ is one, so

$$-\log \frac{f_B \cdot f_{H^+}}{f_A} = \left(z_B^2 - z_A^2 + 1\right)$$

From stoichiometric equation

$$z_A = z_B + 1$$

By algebraic substitution this reduces to

$$\log \frac{f_B \cdot f_{H^+}}{f_A} = z_B \sqrt{\mu}$$

Consequently

$$\frac{f_B \cdot f_{H^+}}{f_A} = 10^{z_B \sqrt{\mu}}$$

$$K_A = \frac{c_B \cdot c_{H^+}}{c_A} = K_A' \cdot 10^{-z_B \sqrt{\mu}}$$

This expression may be used to predict the effect of salt addition as applied to this problem. When reaction 5 is considered in connection with 7 and 9, it is apparent that the base has a negative valence and that an increase in salt concentration would increase the value of K_A. If reaction 6 and equation 8 are similarly analyzed the base is seen to have zero valence, and K_B will consequently remain constant with addition of salt, at fairly low concentration. Therefore, according to the formula

$$[H^+] \text{ at I.E.P.} = \sqrt{\frac{K_a}{K_b} \cdot K_w}$$

which, since $K_a = K_A$, and $K_b = K_B \cdot K_w$, becomes

$$[H^+] \text{ at I.E.P} = \sqrt{\frac{K_A}{K_B}}$$

the addition of salt should increase the value of K_A while K_B automatically maintains itself constant, and the hydrogen ion concentration should be increased. Thus, if the assumptions made above are essentially correct, the addition of neutral salts to an ampholyte should cause a shift in the apparent isoelectric point to the acid side. This is contrary to the observations of Baly, but agrees in every case with the observations of other investigators as well as with those recorded in the experimental part of this paper. A critical test of this theory should involve the use of both colloidal and non-colloidal ampholytes, with various buffers, and in particular, an ampholyte having an isoelectric point on the alkaline side of neutrality. The excessive cost of these latter materials has

prevented tests of this part of the theory, and all the tests made have offered considerable experimental difficulty.

EXPERIMENTAL

The method which appears to be most applicable for the purpose of determining the isoelectric point of colloidal ampholytes in the presence of salts is that suggested by an experiment in Holmes, (13), in which alcohol is used to coagulate albumin sols whose pH is maintained at varying values by means of acetic acid-acetate buffers. The pH of the solution in which maximum flocculation occurs is taken as the isoelectric point.

Two other methods based on minimum solubility may be used for difficultly soluble ampholytes. The first consists of determining the solubility of the ampholyte in a series of buffered solutions of varying pH. The pH of the solution showing minimum solubility of the ampholyte should be concurrent with the isoelectric point. Another method of this type has been investigated by Michaelis , (14). If, to samples of an alkaline solution of the ampholyte in different test tubes, gradually increasing amounts of acid are added, the maximum precipitation will occur in the tube where the pH resulting from the reaction of the acid and the base most nearly approaches that of the isoelectric point of the ampholyte. Michaelis has successfully applied this method to para and meta-amino benzoic

acid and to aspartic acid.

Another method of determining the isoelectric point, based on a different principle, applies equally well to colloidal and non-colloidal ampholytes and has been investigated by Michaelis (15). This method is based on the fact that if a base is added to a dilute buffer of definite pH, the pH rises, and vice versa. An ampholyte in solution will behave as a base as long as the pH of the solution is below that corresponding to its isoelectric point, and as an acid when the opposite is true. Thus if an ampholyte is added to the dilute buffer solution in sufficient concentration, it will increase the pH when the buffer is below the isoelectric point of the ampholyte, and decrease it if the pH is above. At the isoelectric point, there will be no deflection of the pH of the buffer on the addition of the ampholyte. Michaelis used this method successfully upon phenylalanine and with a fair degree of success upon glycine. The method fails to be sensitive, however, when the ionization constants of the ampholyte are too small.

Kondo and Hayashi (3) have quoted Sørenson as stating that there should be no effect of salts on the apparent isoelectric point of albumin. Sørenson's original paper, (16) however, merely states that he found no effect on the isoelectric point of egg albumin caused by ammonium sulfate when the isoelectric point was determined by the method of

Michaelis (15). Preliminary tests indicated that, contrary to the statement of Kondo and Hayashi, added salts had a pronounced effect on the apparent isoelectric point of egg albumin, so it was decided to make a thorough investigation of this question with highly purified material as a starting point.

Jirgenson, (17), in some work on sensitivity of albumin sols to organic substances, used Merck's purified egg albumin, by dissolving in water, dialyzing, and using only freshly made sols. This method of preparing the sols was tried, and it was found that regardless of the time and conditions of the dialysis, the resulting sol was decidedly cloudy and would continually flocculate immediately after the cessation of dialysis. Consequently it was thought best to prepare the albumin from egg whites by the method of La Rosa (18), a modification of Sorenson's original method (16). The egg whites were mixed with saturated ammonium sulfate solution to dissolve the albumin, from which the globulin was centrifuged. The albumin was precipitated with ammonium sulfate. The final water solution of albumin was electrodialyzed until its conductivity was equal to that of the distilled water used in the process. About one liter of 1.9% albumin sol was obtained as a final product from thirty-six eggs. The dialysis was carried out in an electric refrigerator at 5°C., using toluene as a preservative, and the product was kept in the

refrigerator throughout the time of use. The flocculation of this sol was extremely slight during this period.

The method of precipitating with alcohol or acetone was chosen as the method best suited to the determination of the isoelectric point of this protein, because a close control could be kept on the salt concentration, and because it obviated the necessity of using such a dilute buffer that pH measurements would be inaccurate. The procedure followed in making the runs is as follows: 0.5 ml. of the sol was added to each of a series of test tubes, followed by 2 ml. of McIlvane's phosphate citric acid buffer mixtures of the desired pH. The desired amount of salt solution was then added, and the solutions were diluted to 8.5 or 9.0 ml., depending upon the amount of alcohol to be used. These solutions were divided in two equal portions. To one portion was added enough alcohol to make the volume 5 ml., and to the other was added an equal volume of water. This quantity of alcohol varied from 0.5 to 0.75 ml. over the pH range used, but was constant for any one run over a small range. The tube in which maximum precipitation occurred was observed and the pH of the corresponding solution containing water instead of alcohol was measured by means of the quinhydrone electrode. The pH of the solution could not be determined in the tube containing alcohol because of an effect of organic substances of that nature on the quinhydrone electrode, an effect for

which no corrections are available. Correction of the pH for the salt error and protein error inherent in the quinhydrone electrode was not necessary since neither was present in sufficient quantity to cause an appreciable error.

The isoelectric point of the albumin in the absence of salts other than those present in the buffer was found to be at a pH of 4.33, a somewhat lower value than that obtained by Sørenson (16), possibly due to the presence of the buffer salts. Extrapolation of the curves in Fig. 3 gives a value of 4.6 at zero salt concentration, a figure more nearly approaching that found by Sørenson, 4.79-4.82.

Experiments were carried out using varying concentrations of sodium chloride, potassium chloride, potassium iodide, barium chloride, and sodium sulfate, with the results shown in Table 1, and Figures 2 and 3.

Table 1

Salt M.	Na_2HPO_4 M.	Total C M.	μ Total	$\sqrt{\mu}$	pH at I.E.P.
		NaCl			
0.00	0.0160	0.0160	0.0160	0.125	4.33
0.05	0.0148	0.0648	0.0648	0.255	3.97
0.10	0.0132	0.1132	0.1132	0.335	3.65
0.15	0.0106	0.1606	0.1606	0.400	3.31
0.20	0.0074	0.2074	0.2074	0.455	2.95
0.30	0.0016	0.3016	0.3016	0.550	2.45
		KCl			
0.00	0.0160	0.0160	0.0160	0.125	4.33
0.05	0.0128	0.0628	0.0628	0.250	3.76
0.10	0.0098	0.1098	0.1098	0.330	3.33
0.15	0.0058	0.1558	0.1558	0.395	2.98
0.20	0.0008	0.2008	0.2008	0.450	2.61

Salt M.	Na_2HPO_4 M.	Total C M.	μ Total	$\sqrt{\mu}$	pH at I.E.P.
		KI			
0.00	0.0160	0.0160	0.0160	0.125	4.33
0.02	0.0148	0.0348	0.0348	0.186	4.06
0.05	0.0122	0.0622	0.0622	0.250	3.53
0.07	0.0082	0.0782	0.0782	0.280	3.21
0.10	0.0008	0.1008	0.1008	0.315	2.78
		Na_2SO_4			
0.00	0.0160	0.0160	0.0160	0.125	4.33
0.02	0.0136	0.0336	0.0736	0.270	3.86
0.03	0.0114	0.0416	0.1014	0.320	3.59
0.04	0.0074	0.0474	0.1274	0.360	3.23
0.05	0.0016	0.0516	0.1516	0.390	2.92
		$BaCl_2$			
0.00	0.0160	0.0160	0.0160	0.125	4.33
0.02	0.0160	0.0360	0.0760	0.275	3.90
0.04	0.0142	0.0542	0.1342	0.365	3.56
0.06	0.0128	0.0728	0.1928	0.440	3.20
0.10	0.0082	0.1082	0.3082	0.550	2.67

A very pronounced shift in the point of minimum solubility was caused by the addition of all salts investigated. While Kondo and Hayashi (3) found, with rice glutelin, a shift to the acid side on the addition of salts, the shift was not so large as observed in this work on albumin. Furthermore, while they found evidence that the salt effect was related to the Hofmeister series, no clear deductions of a similar nature can be made for albumin. Since the method of treatment adopted in this thesis was based on ideas of ion activities and their change with ionic strength, it has seemed more logical to study this salt effect from the standpoint of ionic strength. When the pH of the apparent isoelectric point is plotted against the square root of μ including (a point which has apparently

been overlooked by previous investigators) the concentration of the buffer salt as well as that of the added salt, certain interesting regularities appear which do not appear when salt concentration only is taken into account. These results are shown graphically in Figures 2 and 3. In the curves for pH against the square root of μ it will be noted that in solutions of low ionic strength the effect of all the salts approaches a common tangent which, when extrapolated to zero ionic strength, closely approximates the true isoelectric point of the albumin. At low concentrations, therefore, it would appear so far as these results can be trusted, that the deciding factor is the ionic strength. However, as the solution becomes more concentrated individual ion effects naturally become more noticeable. The potassium salts are found to show a slightly different approach to the common tangent from the other salts, and the sulfate and iodide ions show strong individual effects. The results of Kondo and Hayashi, shown in Fig. 1, while not so consistent, show the sodium chloride and barium chloride close together and similar in their effect, and the sulfate and iodide ions apart from the others just as in the curves for albumin.

From the standpoint of a broad test of the theory it was desired to investigate not only the colloidal ampholytes, but the non-colloidal ones as well, and since it was also desirable to work with one whose basic dissocia-

tion constant is greater than its acid dissociation constant, the possibilities were examined and caffein was chosen as the best available example of such a substance, since the cost of amino acids having these characteristics was prohibitive. K_a for caffein is ca. 10^{-14}, and K_b is ca. 10^{-10}. The isoelectric point, therefore, should be in the alkaline range at a pH value of about 9.0.

The solubility of caffein is approximately 1.5 g. per 100 g. of water at room temperature, so the choice of applicable methods for the determination of the isoelectric point is more or less limited to solubility methods and Michaelis' method of the pH shift of buffers (15). The solubility method was tried with Merck's C.P. Caffein Alkaloid, using Sørenson's Borate-HCl-NaOH mixtures for the buffer. Samples of caffein were rotated with buffer mixtures of varying pH in a constant temperature both for thirty minutes. Samples were drawn, the caffein was extracted with chloroform, fractions of the chloroform solution were evaporated and the residue weighed. However, no point of minimum solubility was found between pH 8, and pH 11.5, but the solubility decreased steadily with increasing pH, indicating that the acidic properties were too weak to show any effect in dilute buffers.

The method of Michaelis was then tried. The buffer solutions used in an attempt to find the optimum concentration were 0.00125M., 0.0025 M. and 0.005 M. with respect

to borate and contained varying small quantities of HCl or NaOH according to the desired pH. The pH of the buffer was measured with the hydrogen electrode in an electrode vessel so constructed as to keep the solution and space above it saturated with hydrogen and free from carbon dioxide, a necessary precaution with such dilute buffers. A portion of the same buffer was prepared and diluted with caffein solution instead of water so that the buffer concentration was identical with that of the first portion, but the solution was 0.05 M. with respect to caffein. The data obtained by this method were not reproducible probably because of the limited solubility of the caffein and the necessarily low buffer concentration.

By measuring the pH of the buffer solution, adding solid caffein to saturate the solution and remeasuring the pH, one set of data was obtained from which it was hoped to show a salt effect. Table 2 shows that the caffein apparently behaves as a base on the acid side of the isoelectric point but fails to have any effect on the basic side. These results were not closely reproducible, however, and the addition of sodium chloride up to 0.4 M. failed to give results which were sufficiently reproducible to allow any prediction of salt effect.

Table 2

Ph before addition of caffein	Ph after addition of caffein	Ph difference
7.56	7.64	+0.08
8.23	8.28	+0.05
8.54	8.57	+0.03
8.99	8.99	0.00
9.37	9.37	0.00
10.48	10.48	0.00
10.79	10.79	0.00
11.99	11.99	0.00

The borate used for these results was 0.00125 M.

It was concluded that it was impossible to find the isoelectric point of caffein by these methods or to observe any salt effect. The explanation of the difficulty encountered may lie in the weakness of caffein as an acid, in the possibility that it is not soluble enough to affect even the weakest buffer used, or in the fact that caffein is supposedly only a pseudo acid and intra-molecular shifts are necessary in order for it to act as an acid. Michaelis found that the related compound, theobromine, failed to give good results with any method which he employed, so further tests on caffein were discontinued.

Very little appears to be known regarding the isoelectric point and the effect of high salt concentrations in the preparation of cystine by the so-called isoelectric precipitation method. It was decided to work on this important amino acid in hopes that some light might be shed on its isoelectric behavior in the presence and in the absence of salts. Since the solubility of cystine is but

0.01 g. per 100 g. of water the two minimum solubility methods for determining the isoelectric point were considered and used. By rotating in a constant temperature both with phosfate-citric acid buffers ranging in pH from 3.4 to 5.8, drawing samples and determining the cystine nitrogen by the micro-Kjeldahl method described by Williams (19), the results shown in table 3 were obtained. The cystine used was carefully purified material having a specific rotation of -194 degrees.

Table 3

pH	mg. Nitrogen in sample
3.4	0.50
4.0	0.35
4.6	0.35
5.2	0.28
5.8	0.25

This range includes the accepted value for the isoelectric point, 4.3, and the calculated value, 5.06. It is evident that since these runs, although not exactly reproducible, show the same trend as in other runs not tabulated, the isoelectric point cannot be found in this manner, the solubility differences being too slight and showing no sign of a minimum value.

The method used by Michaelis for difficultly soluble ampholytes should also be applicable to cystine. The following procedure was carried out employing this method. A two percent solution of cystine in 0.5 N. HCl was made up. To equal parts of this solution in a series of test

tubes, di-sodium phosphate was added as a buffer and varying quantities of 1 N. NaOH were added, covering a large pH range. The cystine was soluble below a pH of 1.80 and above 6.60, and showed maximum precipitation at pH 6.60. More dilute solutions yielded the same results, and more concentrated solutions would contain such a high concentration of salt as to be of no value in finding the isoelectric point if a salt effect exists. The addition of sodium sulfate up to 0.4 molar had no effect on this point of maximum precipitation. These methods of treatment apparently fail to give conclusive information regarding the isoelectric behavior of cystine or any salt effect on this ampholyte.

It was next decided to use a non-colloidal ampholyte whose isoelectric point is well known and easily obtainable. O-aminobenzoic acid (anthranilic acid) fits this description and since its solubility is but 0.35 g. per 100 g. of water the isoelectric point may be determined by the method of Michaelis used above. A 6% solution of anthranilic acid in 0.5 N. NaOH was made for the solution from which to precipitate the ampholyte. The quantity of this solution was maintained constant throughout the experiments and the variation in pH was effected by the addition of 3.75 N. acetic acid. Appropriate quantities of water were added to the various runs, so that the maximum concentration of sodium acetate was 0.15 M. for any run.

The maximum precipitation in these solutions was found to be at pH 4.05, a value differing from the calculated value of 3.55. The addition of sodium chloride affected the point of maximum precipitation very little as is shown in table 4. This effect, however, was at least as great as observed by Kondo and Hayashi for rice glutelin. The addition of sodium sulfate affected the point of maximum crystallization to a greater extent.

Table 4

Salt M.	pH at maximum crystallization	Shift	μ	$\sqrt{\mu}$
		NaCl		
0.0	4.05		0.15	0.39
0.2	4.02	-0.03	0.35	0.59
0.4	3.97	-0.05	0.55	0.74
		Na_2SO_4		
0.0	4.05		0.15	0.39
0.2	3.92	-0.13	0.75	0.86
0.4	3.75	-0.17	1.35	1.16

These results, when graphically represented in the same manner as with the albumin, give curves of much the same type, but insufficient data were obtained to justify quantitative treatment. The above tabulated results were fairly closely reproducible.

CONCLUSION

The results of the work described in this thesis indicate in general, that with the ampholytes for which a sharply defined isoelectric point has been found in the above experiments, there is a definite although varying shift of the apparent isoelectric point to the acid side,

caused by the addition of neutral salts. This has been definitely shown with egg albumin and with anthranilic acid. For both of these substances the acid dissociation constant is greater than the basic dissociation constant.

Although from theoretical considerations the shift should be independent of the relative size of K_a and K_b, and dependent only on the added salt and the nature of the charge on the substance which is considered by Brønsted's definition to be a base, it still remains to be proved experimentally that the shift of the apparent isoelectric point with salt addition will be in the same direction with ampholytes where K_a is less than K_b in order to settle the question in a more general manner.

The results obtained with egg albumin and those obtained with anthranilic acid do not agree with Baly's work on sewage colloids or with the work of Ghosh on gelatin, since these investigators claim a shift of the pH to the alkaline side, but they are in agreement with the experiments of Andrzejewski, Przylecki, and Siedroyé on gelatin, caseinogen, and serum albumin, and with those of Kondo and Hayashi on glutelin. It is possible that the reason for the discrepancy lies in the method of determining the apparent isoelectric point or in the effect of salts on the Bentonite used by Baly and Ghosh.

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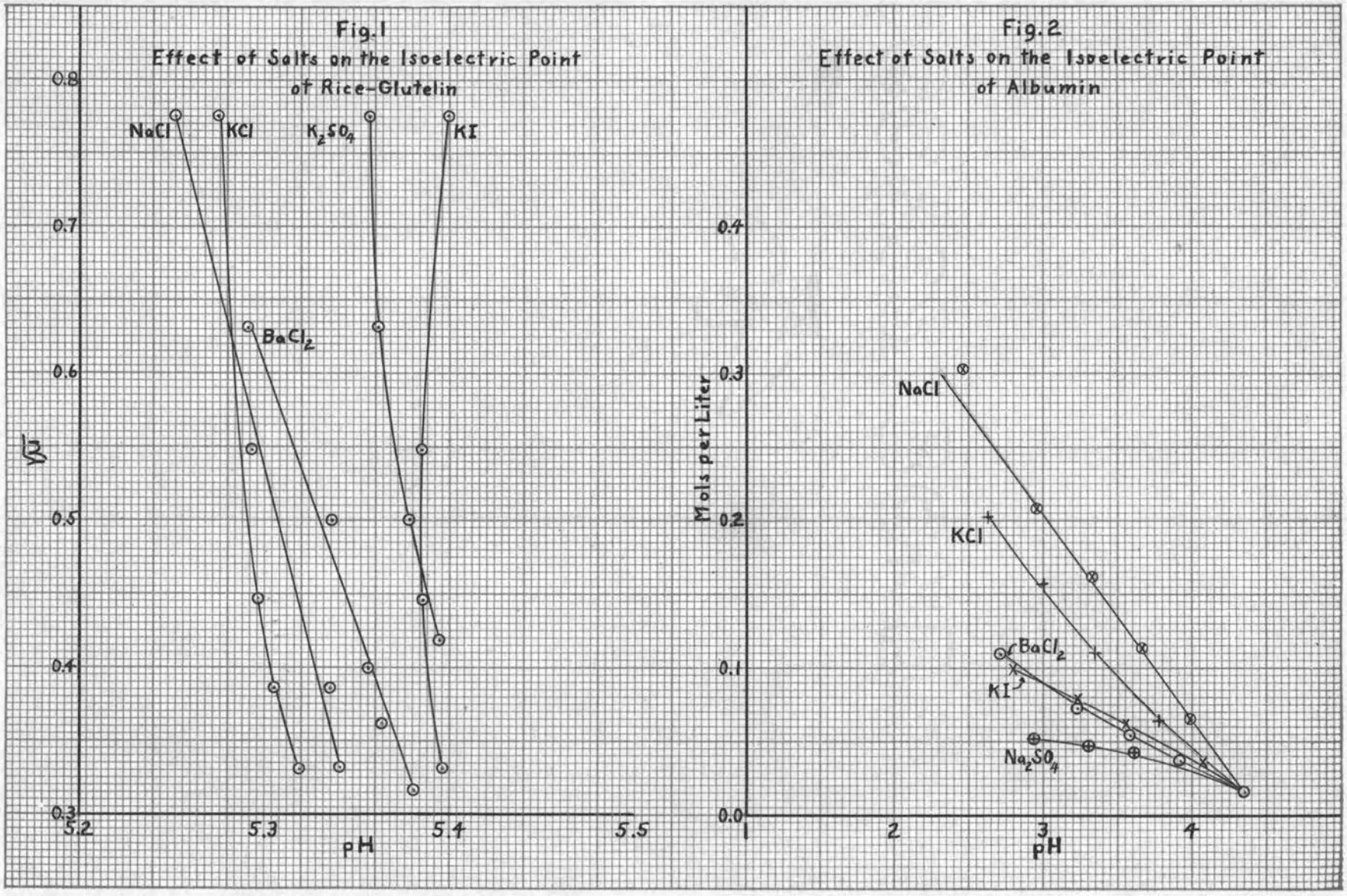
Fig. 1
Effect of Salts on the Isoelectric Point of Rice-Glutelin
NaCl
KCl
K_2SO_4
KI
$BaCl_2$
0.8
0.7
0.6
0.5
0.4
0.3
5.2
5.3
5.4
5.5
pH
Fig. 2
Effect of Salts on the Isoelectric Point of Albumin
NaCl
KCl
$BaCl_2$
KI
Na_2SO_4
Mols per Liter
0.4
0.3
0.2
0.1
0.0
1
2
3
4
pH

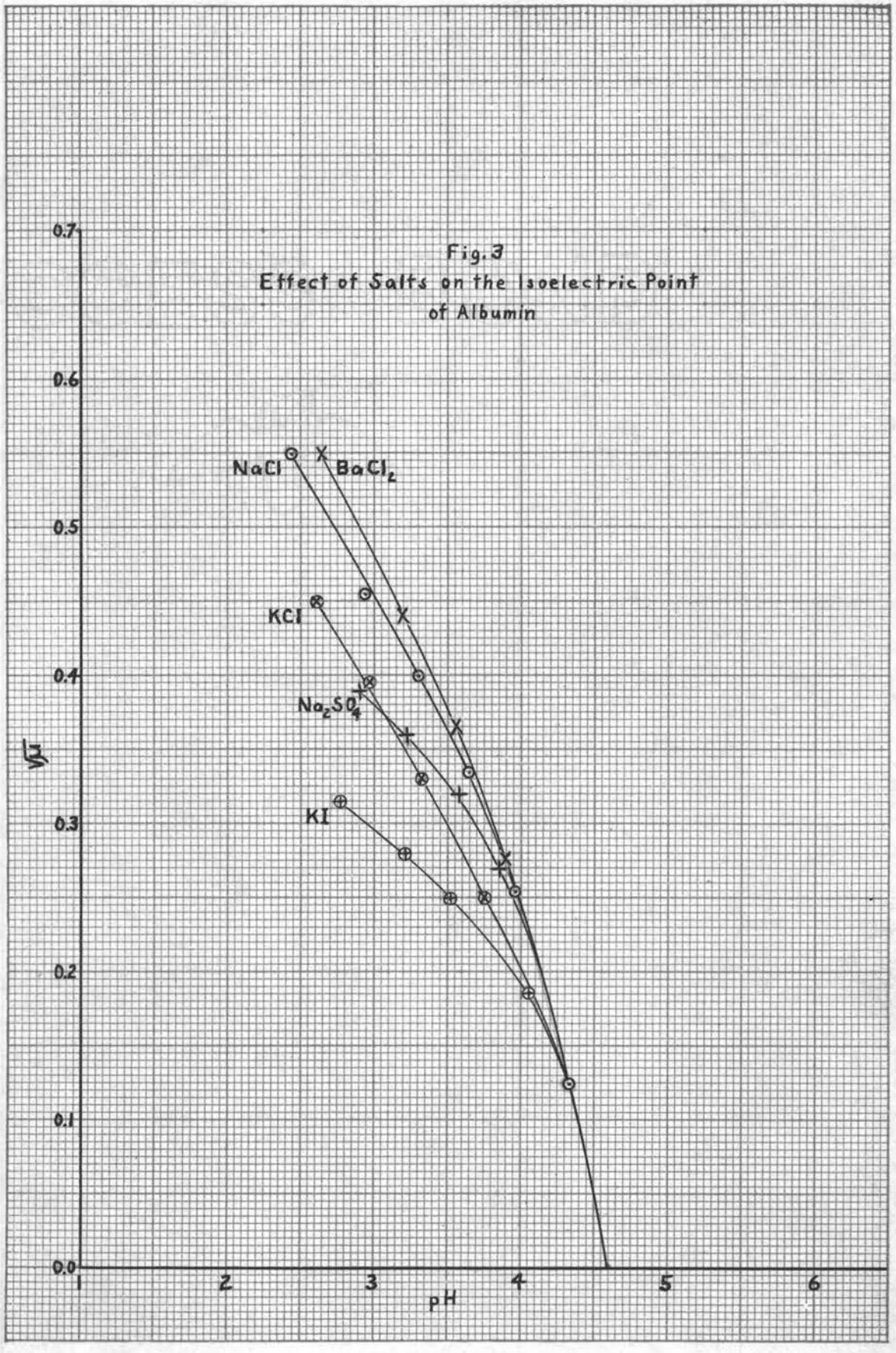

Fig. 3
Effect of Salts on the Isoelectric Point of Albumin