THE EFFECT OF ISOELECTRIC AMINO ACIDS ON THE pH+ OF A PHOSPHATE BUFFER SOLUTION

A CONTRIBUTION IN SUPPORT OF THE "ZWITTER ION" HYPOTHESIS

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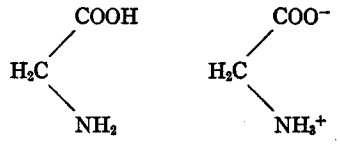
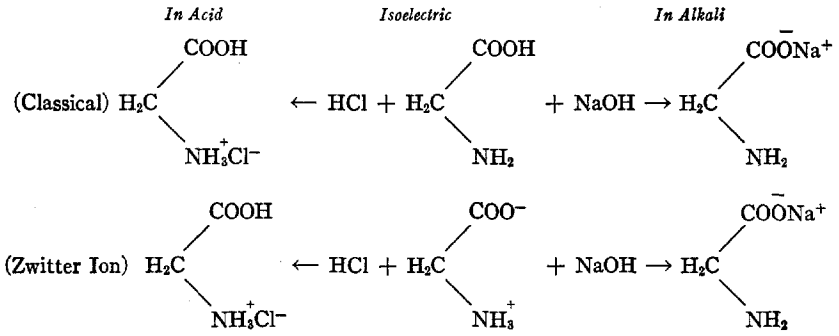
In recent years the conception in biochemistry of the ionization of amphoteric electrolytes developed by Walker (1) which is still generally accepted, has been gradually losing ground. The tendency has been to replace it with the hypothesis of Bredig (2), Adams (3) and Bjerrum (4), commonly designated as the "Zwitter Ion" hypothesis. The difference between the two hypotheses is represented by the following two formulae for isoelectric glycine.



I II

Formula I represents the current conception of the isoelectric condition of an amphoteric electrolyte. At the isoelectric point the molecule is not dissociated either as an acid or as a base (or the acid and basic dissociation are minimal and equal to each other). According to Formula II which represents the Zwitter Ion conception of isoelectric glycine, the molecule is, of course, also neutral, but this neutrality is due to the complete dissociation simultaneously of the acid and of the basic groups.

The changes which occur in acid and in alkali, according to the two conceptions, are represented by the following formulae.



As the above formulae show, there is no disagreement between the two hypotheses regarding the forms resulting from the addition of acid and of alkali. The difference is in the mechanism. According to the classical view, the effect of increasing alkalinity is to permit the ionization of the carboxyl group, which is so weak an acid that it dissociates only in alkaline reactions. Similarly the addition of acid permits ionization of the amino group, which is so weak a base that it can dissociate only in acid solutions. In alkaline solutions the buffering is due to the ionization of the weakly acid group, and in acid solutions to the ionization of the weakly basic group.

According to the Zwitter Ion hypothesis the effect of the addition of base is to depress the ionization of the amino group (both groups being completely dissociated at the isoelectric point), thus leaving the carboxyl group free to combine with the cation of the base added. Similarly in acid solution the dissociation of the carboxyl group is depressed, leaving the already ionized amino group free to form a salt with the acid. The buffering in alkaline solution is not due to the ionization of the acid group, but to the depression of the ionization of the amino group; and in acid solution to the depression of the dissociation of the acid group, rather than to the ionization of the amino group.

The essential difference between the two hypotheses consists in the strengths to be ascribed to the acid and basic groups. According to the older view the acid and basic groups are enormously weaker than acetic acid and ammonia, while according to the Zwitter Ion hypothesis they are each slightly stronger. The values are shown in Table I.

According to the Zwitter Ion hypothesis the titration constant obtained when glycine is titrated with acid is not the titration constant

(*i.e.*, the dissociation constant modified by the ionic strength of the solution) of the amino group, but the modified hydrolysis constant of the acid group. Similarly the titration constant obtained on titration with alkali is the modified hydrolysis constant of the amino group. In other words, according to Bjerrum:

$$K_{\text{acid}} = \frac{K_W}{K_b} \text{ and } K_{\text{base}} = \frac{K_W}{K_a}$$

where K_b and K_a are respectively the dissociation constants for base and acid according to the older view.

In support of the Zwitter Ion conception Bjerrum pointed out that the chemical structure of the amino acids does not warrant the very low values for the strengths of the acid and basic groups of the amino

TABLE I

The Dissociation Constants of Acetic Acid, Ammonia and Glycine

	K_a	K_b
Acetic acid	1.8×10^{-5}	—
Ammonia	—	1.9×10^{-5}
Glycine (classical values)	1.8×10^{-10}	2.6×10^{-12}
Glycine (Zwitter Ion values)	3.9×10^{-4}	5.6×10^{-5}

acids which are assigned to them on the older view. Indeed, as Lowry (5) pointed out, the effect of the amino group should be to increase the dissociability of the COOH group. From theoretical considerations it is impossible to explain an acid dissociation constant for glycine 100,000 times weaker than acetic acid, and a basic dissociation constant 10,000,000 times weaker than ammonia. On the other hand, the experimental values calculated according to the Zwitter Ion hypothesis can be derived from the constants for acetic acid and ammonia from the same theoretical considerations which permit calculation of the constants for the polybasic acids and polyacid bases.

For these reasons confidence in the older or classical view of the ionization of amphoteric electrolytes has diminished. The present feeling regarding the two theories is conveyed by the following quotation from Kirk and Schmidt (6):

"In choosing the classical system of nomenclature, we do not wish to imply that the above equations actually represent the true state of affairs in the solution. Since the proposal by Bjerrum of the 'Zwitter Ion' theory, it has become increasingly probable that this theory is a closer approach to the true status of an ampholyte in solution than is the classical theory."

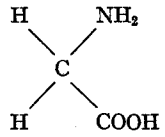
As the above quotation implies, the available evidence, favorable though it is, does not permit of a definite decision in favor of the "Zwitter Ion" theory as against the older view. All titration data can be absorbed equally well into either system. Until recently there has been no direct evidence obtained from experiments with amino acids themselves deciding in favor of either theory. It is believed that in the experiments described below this deficiency is supplied, and that the evidence which these experiments afford contributes toward a decision in favor of the "Zwitter Ion" theory.

Cohn (7) studied very thoroughly the effect of changing ionic strength upon the hydrogen ion concentration of mixtures of salts of primary and secondary phosphate solutions. His observations were that, for a given mixture of primary and secondary phosphates, the effect of increasing the ionic strength of the solution upon the activity coefficients of these two salts is to increase the hydrogen ion concentration, while decrease in the ionic strength results in a diminution of the hydrogen ion concentration. Robinson (8) observed the same effect of varying ionic strengths on the hydrogen ion concentrations of phosphate solutions when the phosphate concentration was kept constant and varying amounts of neutral salts were added.

It seems, then, that dilution of a phosphate solution whose pH was that of the isoelectric point of a given amino acid, with a solution of that amino acid at the pH of its isoelectric point might render a decision between the two theories. Since, according to the classical theory, an amino acid at its isoelectric point is dissociated neither as an acid nor as a base it may, in that condition, be compared to a non-electrolyte. Dilution of the phosphate solution with such a solution of an amino acid should change the hydrogen ion concentration of the phosphate solution in a manner similar to that effected by such non-electrolytes as urea or glucose, for example. These changes, as the experimental results below indicate, may be due, in part, to changes effected in the dielectric constant of the solutions.

On the other hand, since according to the "Zwitter Ion" hypothesis, at the isoelectric point both the acid and the basic group are completely dissociated, an amino acid molecule may, in solution in this form, be considered to resemble a strong electrolyte in the respect that it contributes to the ionic strength of the solution. However, since the ions of the isoelectric amino acid are not so free to move in solution as are the constituent ions of a strong electrolyte, it is to be expected that the effect of a given concentration of an isoelectric amino acid will be less than that of an equivalent concentration of a strong electrolyte, but much greater than that of neutral molecules such as glucose or urea. Assuming, therefore, an isoelectric amino acid, glycine, for example, to be dissociated as a monovalent electrolyte, and calculating its "ionic strength" on that basis, dilution of a phosphate solution with a solution of the amino acid of greater "ionic strength" should render the mixture more acid than the original phosphate solution, while dilution with a solution of lesser "ionic strength" should result in a less acid mixture. These changes will correspond in direction only to those obtained when the dilutions are carried out with solutions of similar ionic strengths of a strong electrolyte; but they will be distinctly greater than the effects obtained on dilution with neutral molecules.

Professor Pauling has pointed out to one of us that isoelectric glycine, even considered from the point of view of the classical conception, possesses a considerable electrical moment; and that more appropriate controls than glucose or urea would be compounds with electrical moments corresponding to that calculated for the molecule



From the values assigned to the NH_2 and COOH groups by Debye (9) the maximum electrical moment of isoelectric glycine, if it exists in the above form, is less than 1.9×10^{-18}. The additional controls chosen accordingly were ethyl alcohol, acetone, acetonitrile and phenol, with dipole moments of 1.7×10^{-18}, 2.7×10^{-18}, 3.6×10^{-18} and 1.7×10^{-18}, respectively. Phenol, whose acid dissociation constant is of the order of magnitude of 10^{-10}, at the hydrogen ion concentrations obtaining here, 10^{-6}, may be considered as a neutral molecule.

Two amino acids were employed, glycine and alanine. They were purified by recrystallization at their isoelectric points; and their purity was established by determinations of the relative and absolute values of total and free amino nitrogen. Solutions of these amino acids were made with ionic strengths, calculated on the basis of the "Zwitter Ion" hypothesis, equal to, greater than, and less than that of a phosphate solution. Dilution with amino acid solutions of the same ionic strength changed the hydrogen ion concentration very little. When the phosphate solution was diluted with an amino acid solution of greater ionic strength, the pH of the solution decreased; when the dilution was made with an amino acid solution of lesser ionic strength, the pH, of course, became greater. Similar results were obtained when the phosphate solution was diluted with solutions of potassium sulphate and of potassium chloride. On the other hand, when a phosphate solution was diluted with water or with solutions of glucose, ethyl alcohol, acetone, acetonitrile or urea, the pH of the resulting mixture was higher than that of the original phosphate solution. Phenol, on the other hand, induces an increase in acidity nearly as great as that due to glycine. It seems improbable that the similar effects of the amino acids and of phenol on the hydrogen ion concentration of a phosphate solution are due to the same cause, in the light of the divergent effects of ethyl alcohol, acetone, acetonitrile and urea, and also on account of the differences in structure and physical properties. Among the possible explanations which suggest themselves is that phenol in aqueous solution possesses a high degree of hydration.

The experimental results, with the exception of those with phenol, are in accordance with the predictions from the "Zwitter Ion" hypothesis, and are correspondingly difficult to interpret from the point of view of the classical theory.

The Zwitter Ion hypothesis receives very strong support from the observations made in the last few years on the dielectric constants of aqueous solutions of the amino acids. Hedestrand (10) found that the dielectric constants of solutions of glycine and of alanine increase with increasing concentrations of the amino acids, and that this increase is a linear function of the concentration of the amino acid. The dielectric constants of solutions of glycine and of alanine were found

also to be the same. Similar observations have been made by Walden and Werner (11) with alanine and betaine, by Fürth (12) with glycine, and by Blüh (13) with leucine. Aqueous solutions of alcohol, acetone, acetonitrile and glucose have markedly lower, and of urea slightly higher dielectric constants than that of water (14, 15).

Blüh, Walden and Werner, and Hedestrand consider the increased dielectric constants of aqueous solutions of the amino acids to be evidence in favor of their Zwitter Ionic constitution. In view of the present uncertainty in this field of the physical chemistry of solutions it is premature to consider this evidence alone as decisive. This increase in dielectric constant may be due simply to the increased concentration of molecules with a much higher dipole moment than that of water; or to the decomposition by the charges of the ionized poles of the amino acid of aggregates of water molecules whose dipole moment is low, into single molecules with higher electrical moment; or to the formation of large polarized complexes about each of the ionized poles of the amino acid, in the manner postulated for ions. On the other hand, the burden of explanation is definitely upon the classical theory as the observed effect of amino acids on the dielectric constants of aqueous solutions is in the expected direction only if they are considered as existing in solution as Zwitter Ions.

Recently methods have been devised for measuring the dielectric constants of relatively strong solutions of strong electrolytes (16). It has been found that at concentrations above 0.01 normal the values of the dielectric constants increase very quickly and at concentrations as low as 0.02 normal they considerably exceed that of water. In this respect also, therefore, the behavior of isoelectric amino acids is similar to that of strong electrolytes.

These observations on the effect of strong electrolytes strengthen the criticism of the theoretical foundations of the Debye-Hückel (17) activity theory, that the actual macroscopic dielectric constant of the solution should be employed in the equation for the activity of an ion instead of that of the pure solvent. The introduction later of the b term, and of the "salting out" term, both derived from the experimental data and both varying with each system, indicates this fundamental theoretical weakness in the theory, especially as even with the employment of these two "constants" the agreement between observed

and calculated values for activity coefficients over any considerable range of moderately high concentrations is only approximate.

The application of the Debye theory to solutions of weak electrolyte presents an even more complicated problem. With changing dielectric property of the solution, as occurs when the concentration of electrolyte changes, not only are variations induced in the activities of the ions, but also in the equilibrium between the ionized and unionized moieties, *i.e.*, in the equilibrium constant. Michaelis and Mizutani observed, for example, with increasing concentrations of alcohol, *i.e.*, with diminishing dielectric constant, that the pK′ (*i.e.*, the pH of a 50 per cent neutralized solution) of a weak acid progressively increases (20). If one attempts to calculate the pH in this case with such an equation as that of Cohn for aqueous phosphate solutions (7), taking into account only the effect of the changed dielectric constant on the activities of the ions, the calculation yields the reverse result, *i.e.*, the pH progressively diminishes with diminishing dielectric constant. The opposite result actually observed by Michaelis and Mizutani is due to the greater effect of the changing dielectric property of the solution on the dissociation equilibrium, than on the opposing effect on the activities of the ions.

In the case of the addition of an isoelectric amino acid to a phosphate buffer solution, it is not possible at present to determine how much of the observed decrease in pH is due to the resultant, of the effect of the changed dielectric property of the solution, of increasing the dissociation constant of the second hydrogen of phosphoric acid, on the one hand, and of its opposing effect of increasing the activities of the ions on the other; and how much to the increased ionic strength resulting from the addition of the amino acid. If, for whatever reason, the dissociation constant remains unchanged, then the whole responsibility for the decrease in pH of the phosphate amino acid mixture must be carried by the increase in ionic strength. Regardless of the extent to which these three factors contribute to the observed decrease in pH, the explanation can, it seems, be derived only from the conception of isoelectric amino acids as Zwitter Ions.

The effect postulated above of the changing dielectric constant of the solvent on the dissociation constants of weak acids, weak bases, and ampholytes, provides a qualitative explanation for some of the anom-

alous effects (*i.e.*, from the point of view of the Debye theory) of neutral salts on the ionization of weak electrolytes observed by Simms (24). Simms states that "the direction of all the deviations observed in this paper (with the exception of the effect of the SO_4 ions) is to render the solutions more acid than expected." Michaelis and Mizutani observed, as mentioned above, that in solutions of diminishing dielectric constant the dissociation of weak acids diminishes, whereas the dissociation of ammonia increases. The slope of the curves representing the acid constants is quite steep, while that of basic constants is nearly horizontal. Since, in the range of concentrations employed, increasing salt concentration increases the dielectric constant the predictable effect of the addition of strong electrolytes, extrapolating the curves into the zone of increasing dielectric constants, is to render a solution of a weak acid more acid, and a solution of a weak base slightly less basic, *i.e.*, more acid. In the case of an amphoteric electrolyte, such as glycine, the effect of addition of salt is to increase the acid dissociation constant considerably, and to depress slightly the basic dissociation constant. Both effects tend to move the isoelectric point of the amino acid toward the acid side. This prediction is confirmed by the observation of Simms that "isoelectric points drop with increase in ionic strength." A similar explanation may account for the gradually increasing values with increasing concentration, of the dissociation constants of such moderately strong acids as the first hydrogen of phosphoric acid and of hydrosulfate, observed by Sherrill and Noyes (25).

It is important that the similarity in behavior of isoelectric amino acids to strong electrolytes, which is predictable from the Zwitter Ion and not from the classical conception, is independent of theoretical considerations of electrolyte solutions. While the Debye theory accounts qualitatively for the experimental facts, the explanation of the quantitative differences between amino acids and strong electrolytes, and between different non-electrolytes (Table IX) requires a development of the theory which has not yet been attained.

Professor Pauling has pointed out that whatever be the electrical moments of the amino acids, the moment of a protein molecule is probably low, on account of the probable even distribution of free carboxyl and amino groups about the periphery of the large molecule. This prediction is supported by the findings that the dielectric con-

stants of aqueous solutions of albumin and of gelatine are distinctly lower than that of water (17). These determinations of the dielectric constants of the amino acids and proteins cannot be accepted without reservation, because, apparently, no precaution was taken to ensure that the substances employed were in their isoelectric condition and that the solutions were salt-free. It is probable that they were all more or less in the salt form. However, in the case of the amino acids, the increase in dielectric constant is so large, and in the case of the proteins the decrease is so great, that it is unlikely, if the determinations were made with these ampholytes in isoelectric, salt-free solutions, that the direction, or even the magnitude of the values would be significantly different. The magnitude of the dissociation constants of the free carboxyl and free amino groups on the periphery of the protein molecule would, of course, still be of the order of magnitude of acetic acid and ammonia.

On account of these variations in the dielectric constants of aqueous solutions of electrolytes, amino acids and proteins, such quantities as dissociation constants, and activity coefficients, determined in simple aqueous solutions, may, it seems, be employed without modification in considerations of physiological conditions, only very tentatively, until more data are available.

The work of Pauli and his co-workers (18) on the behavior of electrolyte-free proteins supports the Zwitter Ion hypothesis and suggests that it may be significant in the explanation of protein behavior *in vivo*. Pauli and his collaborators found that albumin may behave as a cation in hydrogen ion concentrations considerably removed from the isoelectric point. This behavior, and certain observations on the conductivity of serum albumin solutions saturated with CO_2 were best accounted for by assuming the existence of Zwitter Ionic protein. By a similar explanation Pauli and his collaborators accounted for the behavior of electrolyte-free protein toward neutral salts and strong acid.

The values of the dissociation constants of the substitution compounds of acetic acid are also in better accord with the Zwitter Ion hypothesis.

Lowry, quoting the observations of Vorländer on the strength of the carboxyl groups in the substituted anilino-acetic acids, points out

that the amino group is an acylous substituent, *i.e.*, that its effect is to increase the strength of an adjacent acid group. Table XI shows that this effect of the amino group on the COOH group in glycine is demonstrable only when its value is calculated from the point of view of the Zwitter Ion hypothesis. The classical theory gives an anomalous, very low value for the COOH group of glycine and forces upon the amino group the property of markedly weakening the acid group. Similar evidence is contained in the values for the acid and basic constants of the substituted amino acids. Whereas the negative logarithm of the acid dissociation constant of glycine according to the classical theory is 9.75, this value for acetylaminoacetic acid is 3.64, and for anilino-acetic acid 4.4. The classical value for the negative logarithm of the basic dissociation constant of glycine is 11.59, for amino-acetic acid ethyl ester it is 7.01 (19).

Michaelis and Mizutani (20) found that the dissociable groups of glycine titrated in acid solution varied, with increasing alcohol content, in the same manner as COOH groups, while the constant titrated in alkaline solution behaved like ammonia. Michaelis and Mizutani wrote "Dieser Befund steht in schöner Übereinstimmung mit der neueren Auffassung von der Dissoziation des Glykokoll."

Experimental Procedure and Results

The glycine used was recrystallized twice from an aqueous solution at pH 6.0. After drying over sulfuric acid to constant weight the hydrogen ion concentration of a 3 per cent solution in CO_2-free water was at pH 6.27. The isoelectric zone of glycine extends from approximately pH 4.5 to pH 7.5, and its isoelectric point is at pH 6.08 (6). The close proximity of the hydrogen ion concentration of the solution of glycine in water to that of its isoelectric point was taken to indicate that no significant amount of the amino acid was present in the form of a salt. The free amino nitrogen corresponded to 18.96 per cent of the weight of the glycine. The theoretical per cent of total nitrogen and free amino nitrogen in glycine is 18.65 per cent.

The alanine used was twice recrystallized. The pH of a 3.56 per cent solution in CO_2-free water was 6.09. The isoelectric point of alanine is at pH 6.04; the isoelectric zone extends, as in the case of glycine, from pH 4.5 to pH 7.5. The free amino nitrogen corresponded to 15.61 per cent of the weight of the dissolved alanine. The theoretical per cent of total and free amino nitrogen is 15.70.

The phosphate solutions were prepared from KH_2PO_4 and K_2HPO_4 according to the data of Cohn. It was not deemed necessary to obtain the phosphate salts in the same degree of purity as the amino acids as the results to be obtained were

to consist of differences in hydrogen ion concentration before and after dilution. It is probable that they were not perfectly dry and that the K_2HPO_4 salt contained a small amount of impurities. The hydrogen ion concentrations of the phosphate mixture do not coincide absolutely with, but are close to, the values obtained by Cohn. 0.5 molar solutions of the two salts were prepared. These were diluted as required. In a later series of experiments mixtures of KH_2PO_4 and Na_2HPO_4 were employed. Solutions with a molecular ratio of 4 of the acid to the basic salts were used. The hydrogen ion concentrations which were obtained were very close to the theoretical isoelectric point of glycine.

Two preparations of glucose were employed, one a recrystallized specimen, the other Pfanstiehl's C.P. anhydrous d-glucose, with an ash content of 0.05 per cent. The aqueous solutions of both samples were neutral. The phenol was a Merck "blue label" preparation, and the urea Baker's analyzed; the ethyl alcohol and acetone had been redistilled, the acetonitrile was a specimen obtained from Professor Lucas. The strong electrolytes employed were a pure sample of potassium sulfate, and Merck's "blue label" potassium chloride. The determinations of the hydrogen ion concentration were carried out electrometrically with a hydrogen electrode and a saturated calomel half cell. A Leeds and Northrup hydrogen ion potentiometer was employed and the error for any one observation was not more than 0.0002 volts. Observations were repeated on each solution with change of gas for each reading until three consecutive pH readings were obtained whose difference was not greater than 0.0002. The type of hydrogen electrode employed was that devised by Moloney (21). With this apparatus determinations made on the same solution at different times coincided within the limits of ± 0.01 pH.

No attempt was made in the earlier experiments to carry out the observations at constant temperature. Observations of the temperature of the calomel half cell and of the solution were made for each reading. All the readings were then standardized by deducting the E.M.F. of the saturated calomel half cell from the E.M.F. observed. The resulting value, which was the E.M.F. of the solution, was corrected for temperature by calculating the corresponding E.M.F. at 25°C. To this value was added the E.M.F. of the saturated calomel electrode at 25°C. The pH was then calculated from this value. The values for the calomel cell are those obtained by Michaelis, quoted by Clark (22).

The experiments in which the effects were compared, of compounds with known electrical moments with those due to isoelectric glycine, were performed later with different preparations of the phosphate salts, and recrystallized glycine. The measurements of the hydrogen ion concentration were made with a Leeds and Northrup Type K potentiometer, in an air bath maintained at 25°C. The duplicate readings here agreed to within 0.0001 volts; and each of the values given represents concordant duplicate observations with each of two electrodes.

If isoelectric glycine in water is in the form of a Zwitter Ion, dilution of the phosphate solution with a glycine solution of greater ionic

TABLE II

The Effect of Dilution of a Phosphate Solution with Isoelectric Glycine, Potassium Chloride, and Water

Final ionic strength of phosphate solution	pH^+ in various solvents		
	Water	0.25 molar isoelectric glycine	0.25 molar KCl
0.087	6.25	6.08	6.01
0.022	6.38	6.20	6.04
0.0055	6.49	6.26	6.05

TABLE III

The Effect of Dilution of a Phosphate Solution with Solutions of Isoelectric Glycine of Greater "Ionic Strengths"

Phosphate solution		Glycine solution		E.M.F.	Temperature		pH
Volume	Ionic strength	Volume	"Ionic strength"		Of calomel half cell	Of solution	
cc.		*cc.*		*volts*	°C.	°C.	
30	0.1	—	—	0.6489	23	23	6.85
10	0.1	20	0.4	0.6451	25.6	25.8	6.74
10	0.1	20	0.3	0.6482	25.6	24.5	6.83
10	0.1	20	0.2	0.6507	25.6	25.5	6.84
10	0.1	20	0.1	0.6512	25.6	25.5	6.84
10	0.1	20	water	0.6560	25.6	24.5	6.96

TABLE IV

The Effect of Dilution of a Phosphate Solution with Solutions of Isoelectric Glycine of Lesser "Ionic Strengths"

Phosphate solution		Glycine solution		E.M.F.	Temperature		pH
Volume	Ionic strength	Volume	"Ionic strength"		Of calomel half cell	Of solution	
cc.		*cc.*		*volts*	°C.	°C.	
30	0.4	—	—	0.6391	25	24.5	6.67
10	0.4	20	0.4	0.6379	25	23.5	6.66
10	0.4	20	0.3	0.6413	25	24.5	6.70
10	0.4	20	0.2	0.6433	25	24.8	6.73
10	0.4	20	0.1	0.6451	25	24.5	6.77
10	0.4	20	water	0.6473	25	25.0	6.79

strength may be expected to cause a decrease in the pH of the mixture, while dilution with a solution of lower ionic strength may be expected to increase the pH. The results of such an experiment are shown in Tables II, III and IV.

TABLE V

The Effect of Dilution of a Phosphate Solution with Solutions of Isoelectric Alanine of Greater and Lesser "Ionic Strengths"

Phosphate solution		Alanine solution		E.M.F.	Temperature		pH
Volume	Ionic strength	Volume	Ionic strength		Of calomel half cell	Of solution	
cc.		*cc.*		*volts*	*°C.*	*°C.*	
10	0.4	20	0.4	0.6362	19.0	19.0	6.68
10	0.4	20	0.2	0.6407	20.25	21.5	6.71
10	0.4	20	0.08	0.6418	20.5	21.5	6.73
10	0.4	20	water	0.6398	18.75	18.6	6.75
5	0.4	25	water	0.6421	18.5	19.25	6.77
15	0.16	35	0.36	0.6361	20.25	21.25	6.63
15	0.16	35	0.26	0.6389	20.5	21.25	6.69
15	0.16	35	0.09	0.6412	20.5	21.0	6.73
15	0.16	35	water	0.6460	20.5	20.25	6.83

TABLE VI

The Effect of Dilution of a Phosphate Solution with Solutions of Potassium Sulfate of Various Ionic Strengths

Phosphate solution = 0.4	Potassium sulphate solution		E.M.F. observed	Temperature		pH
	Volume	Ionic strength		Of calomel half cell	Of solution	
cc.	*cc.*		*volts*	*°C.*	*°C.*	
30	—	—	0.6402	23.5	23.9	6.66
10	20	0.4	0.6380	23.0	23.8	6.65
10	20	0.3	0.6391	22.25	23.35	6.66
10	20	0.2	0.6399	22.5	22.5	6.70
10	20	0.1	0.6436	22.5	23.2	6.75
10	20	water	0.6462	22.0	22.7	6.80

Tables II and III show that dilution of a phosphate solution with a glycine solution of greater ionic strength causes an increase in the hydrogen ion concentration. Dilution with a glycine solution of

lower ionic strength, Table IV, brings about a decrease in the hydrogen ion concentration.

Similar results were obtained when a phosphate solution was diluted with an isoelectric solution of alanine. These results are given in Table V.

Dilution of the phosphate solution with solutions of potassium sulfate similar to those of the amino acid solutions used, gave similar changes in hydrogen ion concentration. This is shown in Tables VI and VII.

TABLE VII

The Effects of Dilution of a Phosphate Solution with Potassium Chloride Solutions of the Same, Lesser, and Greater Ionic Strengths

Phosphate solution		Potassium chloride solution		E.M.F.	Temperature		pH
Volume	Ionic strength	Volume	Ionic strength		Of calomel half cell	Of solution	
cc.		*cc.*		*volts*	*°C.*	*°C.*	
30	0.4	—	—	0.6391	25	24.5	6.67
5	0.4	25	0.4	0.6368	26.5	25.5	6.62
10	0.4	20	0.4	0.6382	26.4	25.0	6.65
15	0.4	15	0.4	0.6395	26.0	25.5	6.66
20	0.4	10	0.4	0.6400	26.6	26.0	6.66
10	0.4	20	0.1	0.6459	25.7	26.2	6.75
10	0.4	20	0.2	0.6426	26.0	26.0	6.70
10	0.4	20	0.3	0.6402	26.2	25.8	6.67
10	0.4	20	0.4	0.6382	26.4	25.0	6.65
10	0.4	20	0.6	0.6355	27.2	26.5	6.58
10	0.4	20	0.8	0.6330	26.7	26.0	6.54

Table VIII shows the effect of dilution of a solution of phosphate with a glucose solution whose "ionic strength" was calculated on the same basis as that of the amino acid solutions. The magnitude of the change, however, is less than that obtained with water, and depends to some extent upon the concentration of glucose. Randall and Failey (23) found that the activity coefficients of non-electrolytes are affected by the presence of electrolytes. As the results in Tables VIII and IX show, the activity of electrolytes is affected by the presence of non-electrolytes. This phenomenon, nevertheless, is not

TABLE VIII

The Effect of Dilution of a Phosphate Solution with a Glucose Solution

Phosphate solution		Glucose solution		E.M.F.	Temperature		pH
Volume	Ionic strength	Volume	Mols. per litre		Of calomel half cell	Of solution	
cc.		*cc.*		*volts*	°C.	°C.	
30	0.4	—	—	0.6391	25	24.5	6.67
25	0.4	5	0.4	0.6372	21.8	21.2	6.68
20	0.4	10	0.4	0.6371	21.8	20.0	6.70
15	0.4	15	0.4	0.6398	22	21.2	6.72
10	0.4	20	0.4	0.6405	22	20.5	6.75
5	0.4	25	0.4	0.6413	21.7	20.6	6.76
15	0.4	15	water	0.6421	22.5	21.8	6.75
5	0.4	25	water	0.6465	22.0	21.5	6.83
30	0.1	—	—	0.6489	23.0	23	6.85
15	0.1	15	0.4	0.6493	22.5	22.5	6.86
10	0.1	20	0.4	0.6503	22.3	22.0	6.88
5	0.1	25	0.4	0.6525	22.5	22.0	6.92

TABLE IX

The Effects of Glycine, Ethyl Alcohol, Acetone, Acetonitrile, Urea and Potassium Chloride on the C_{H+} of a Phosphate Solution

Substance dissolved in phosphate solution	Electrical moment of substance dissolved $\times 10^{18}$	Dielectric constant of the aqueous solution of substance dissolved	pH
0.0165 Molar Phosphate alone	—	—	6.35
Ethyl alcohol 1 Molar	1.63–1.74	50 (ca.)	6.43
Acetone 1 Molar	2.63–2.97	45 (ca.)	6.51
Acetonitrile 1 Molar	3.4–3.94	100 per cent Acetonitrile 36 (19)	6.84
Urea 1 Molar	—	82 (ca.)	6.37
Isoelectric glycine 1 Molar	Calculated for classical structure 1.85	104	6.05
Potassium chloride 1 Molar	—	—	5.84

significant here. The results obtained on dilution with glucose are slight compared to those obtained with glycine and alanine.

In Table IX are recorded the hydrogen ion concentrations of 0.0165 molar phosphate (μ = 0.022) solutions containing in molar concentra-

TABLE X

The Effects of Potassium Chloride, Isoelectric Glycine, and Phenol on the C_{H+} of a Phosphate Solution

Substance dissolved in 0.0165 molar phosphate μ = .022	Molar concentration of substance dissolved	pH
0.0165 molar phosphate alone	—	6.35
KCl	0.2	6.09
	0.5	5.96
	1.0	5.84
Isoelectric glycine	0.2	6.26
	0.4	6.17
	0.6	6.14
	0.8	6.09
	1.0	6.05
Phenol	0.2	6.24
	0.4	6.27
	1.0	6.11

TABLE XI

Dissociation Constants of Acetic Acid and of Some of Its Substitution Compounds

Substance	pK_a
$H\text{-}CH_2COOH$	4.74
$COOCH_3\text{-}CH_2\text{-}COOH$	4.34
$O{=}CH\text{-}COOH$	3.74
$NO_2\text{-}CH_2\text{-}COOH$	3.34
$NH_2\text{-}CH_2\text{-}COOH$	9.75 Classical value
	3.41 Zwitter Ion value

tion, isoelectric glycine, ethyl alcohol, acetone, acetonitrile and urea. As we were concerned here with directional changes only no corrections are introduced for the vapor tensions of the ethyl alcohol, ace-

tone or acetonitrile. In every case the direction of the change indicated electrometrically was confirmed by a colorimetric determination.

These results in Table IX show the different effect of glycine on the hydrogen ion concentration of a phosphate solution from that of substances with electrical moment corresponding to that calculated for the classical form of isoelectric glycine.

Tables II and X show the comparative effects on the hydrogen ion concentration of a phosphate solution of potassium chloride, isoelectric glycine, and phenol. The behavior of phenol as mentioned above may be due to its high degree of hydration; but in the absence of data it constitutes a definite flaw in the evidence.

SUMMARY

The relative merits of the classical conception and of the Zwitter Ion conception of the dissociation of amphoteric electrolytes are discussed, and the following data are presented which confirm the Zwitter Ion hypothesis of Bjerrum, and which are not in accord with the classical view.

1. Amino acids in the isoelectric form resemble strong electrolytes in that they contribute to the ionic strength of the solution.

2. The dielectric constants of aqueous solutions of amino acids, like those of solutions of strong electrolytes greater than 0.02 normal, are considerably greater than that of pure water.

3. The magnitude of the dissociation constants of substituted acetic acids and of glycine, are more easily accounted for with the Zwitter Ion than with the classical conception.

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