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ISOELECTRIC FOCUSING AS A METHOD FOR THE CHARACTERIZATION OF AMPHOLYTES

III. ISOELECTRIC POINTS OF CARRIER AMPHOLYTES AND DISSOCIATION CONSTANTS OF SOME CARBOXYLIC ACIDS AND ALKYL-SUBSTITUTED AMMONIUM IONS IN SUCROSE–WATER, GLYCEROL–WATER AND ETHYLENE GLYCOL–WATER MIXTURES

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SUMMARY

Isoelectric points at 25° and 4° of some carrier ampholytes containing different protolytic groups and dissociation constants at 25° of some carboxylic acids and alkyl-substituted ammonium ions have been determined in water and in sucrose–water, glycerol–water and ethylene glycol–water mixtures.

These data were used to evaluate the errors made in the measurement of isoelectric points of ampholytes (especially proteins) by the conventional execution of density-gradient isoelectric focusing.

A correction term, which serves to obtain isoelectric points of proteins in water from density-gradient isoelectric focusing experiments, is given at 25° and 4° as a function of solvent composition and apparent isoelectric point.

The usefulness of this correction term was checked experimentally by performing density-gradient isoelectric focusing experiments on bovine serum albumin in the three solvent systems studied. Its isoelectric point in water at 25° was found to be 4.75 ± 0.02.

INTRODUCTION

In isoelectric focusing, the pH of the fraction containing the maximum amount of a focused ampholyte is generally called the isoelectric point (p*I*) of that ampholyte. This pH value is generally measured electrometrically, *i.e.*, with the aid of a glass electrode, a (saturated) calomel electrode and a pH meter which is standardized with aqueous standard buffer solutions. However, a pH value determined in that way has no physical meaning unless the sample is also an aqueous solution with approximately the same ionic strength as that of the standard buffer solutions used[1,2]. In that case, pH values can be identified very closely (within ±0.01 pH unit) with pa_H.

In density-gradient isoelectric focusing the samples are not aqueous solutions, but solutions in a mixture of water and some non-electrolyte such as sucrose. In this instance the isoelectric point, pI_{app}, obtained has no physical meaning: the measured pH cannot be interpreted either as pa_H or as pa_H^{*}. If, by some correction (see below), the measured pH value could be converted into the value of pa_H^{*}, this value, which can be equated to pI^{*}, is characteristic for the ampholyte in the solvent mixture in question. The difference between the isoelectric points of the ampholyte in water and in the solvent mixture in question, $pI - pI^{*}$, depends not only on the solvent composition, but also on the nature and the pK value of the protolytic groups that determine the isoelectric point of the ampholyte, *i.e.*, upon its isoelectric point.

This situation reduces considerably the value of isoelectric focusing as a method for the characterization of ampholytes, as we have argued before[1]. In order to determine the importance of the errors involved, we present in this paper measurements of $pI_{app} - pI$ for several ampholytes (carrier ampholytes used in isoelectric focusing) in sucrose–water, glycerol–water and ethylene glycol–water mixtures.

It was shown in a previous paper[2] that meaningful pH values, *i.e.*, pH values that can be identified closely (within ± 0.02 pH unit) with pa_H^{*}, can be obtained by subtracting from the measured pH value a quantity δ, depending only on the solvent composition. By means of this correction we were therefore able to calculate $pI^{*} - pI$ values of the ampholytes investigated from the measured $pI_{app} - pI$ values in these solvent mixtures. In order to compare the calculated $pI^{*} - pI$ values with $pK^{*} - pK$ values of acids containing the same protolytic groups as the studied ampholytes, $pK^{*} - pK$ values of some carboxylic acids and alkyl-substituted ammonium ions were also measured in these solvent mixtures.

THEORETICAL

If certain conditions are fulfilled (see Appendix), an aqueous solution of an ampholyte has a pa_H value equal to its isoelectric point:

$$pa_H = pI \tag{1}$$

When the pH value of this solution (pH_w) is measured in the usual way, it can be interpreted as pa_H:

$$pH_w = pa_H = pI \tag{2}$$

For a solution of the same ampholyte in a partly aqueous solvent, an equation analogous to eqn. 1 holds:

$$pa_H^{*} = pI^{*} \tag{3}$$

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* An asterisk is used to denote that the quantity under consideration (here the activity of H^+ ions) is referred to the infinitely dilute solution in the same solvent. If this symbol is omitted, the quantity under consideration is referred to the infinitely dilute solution in water (or is considered in a general way).

However, when the pH of this solution (pH_s) is measured in the usual way, it cannot be interpreted readily (see Introduction):

$$pH_s \neq pa_H \neq pa_H^* \tag{4}$$

but it can be called the apparent isoelectric point, pI_{app}:

$$pH_s = pI_{app} \tag{5}$$

For the solvents used in isoelectric focusing, pH_s can be converted into pa_H^* by subtracting a quantity δ, depending on the solvent composition:

$$pH_s - \delta = pa_H^* \tag{6}$$

It follows that

$$pH_s - \delta = pI^* \tag{7}$$

Thus, measurement of the pH values of solutions of the same ampholyte in water and in a partly aqueous solvent can provide data on $pI^* - pI$, the primary medium effect upon the isoelectric point:

$$pH_s - \delta - pH_w = pI^* - pI \tag{8}$$

The pH value (pH_w) of an aqueous solution of a carboxylic acid (HA) and its conjugated base (A) can be written as

$$pH_w = pK_{HA} - \log\left[\frac{m_{HA}^0 - m_H}{m_A^0 + m_H}\right] + \log \gamma_A \tag{9}$$

For such a solution in a partly aqueous solvent, it holds that

$$pH_s - \delta = pK_{HA}^* - \log\left[\frac{m_{HA}^0 - m_H}{m_A^0 + m_H}\right] + \log \gamma_A^* \tag{10}$$

where m^0 is the molality, uncorrected for hydrolysis, m is the actual molality, γ represents the activity coefficient and

$$m_H = \text{antilog}\,(-pH_s + \delta - \log \gamma^*) \tag{11}$$

For solutions of an ammonium ion constituent (BH) and its conjugated base (B), similar equations hold:

$$pH_w = pK_{BH} - \log\left[\frac{m_{BH}^0 + \varepsilon}{m_B^0 - \varepsilon}\right] - \log \gamma_{BH} \tag{12}$$

TABLE I

VALUES OF $pI_{app} - pI$ AT 25° FOR AMPHOLINES OF VARIOUS pH RANGES AND FOR SERVALYTE OF pH RANGE 2–4

Solvent	*Ampholyte*								
	Ampholine								*Servalyte*
	pH 2.5–4, pI 2.81	*pH 3.5–5, pI 4.34*	*pH 4–6, pI 4.87*	*pH 5–7, pI 5.94*	*pH 6–8, pI 6.90*	*pH 7–9, pI 8.03*	*pH 9–11, pI 9.86*	*Interpolated* pI 3.60*	*pH 2–4, pI 3.68*
Sucrose:									
15%	−0.02	−0.02	−0.02	0	0.01	0	−0.04	–	−0.03
30%	−0.05	−0.04	−0.04	0	0.01	0	−0.09	–	−0.05
45%	−0.06	−0.04	−0.04	0 01	0.02	0.01	−0.15	–	−0.06
60%	−0.03	−0.01	0	0.07	0.08	0.07	−0.19	–	−0.03
Glycerol:									
20%	0.04	0.04	0.03	0.04	0.03	0.02	−0.04	0.07	0.03
40%	0.10	0.11	0.08	0.06	0.07	0.06	−0.07	0.14	0.07
60%	0.20	0.22	0.16	0.10	0.11	0.09	−0.09	0.26	0.16
80%	0.36	0.39	0.29	0.14	0.14	0.12	−0.10	0.46	0.28
Ethylene glycol:									
20%	0.09	0.09	0.05	0.01	0.03	0.01	−0.06	0.11	0.06
40%	0.22	0.22	0.15	0.05	0.06	0.04	−0.10	0.25	0.15
60%	0.40	0.40	0.29	0.10	0.10	0.07	−0.12	0.45	0.29
80%	0.65	0.66	0.50	0.18	0.12	0.09	−0.15	0.74	0.48

* See footnote on p. 167.

$$pH_s - \delta = pK^*_{BH} - \log\left[\frac{m^0_{BH} + \varepsilon}{m^0_B - \varepsilon}\right] - \log \gamma^*_{BH} \quad (13)$$

with

$$\varepsilon = \text{antilog}\,(pH_s - \delta - pK^*_s - \log \gamma^*) \quad (14)$$

where K^*_s is the autoprotolysis constant of the solvent.

Thus, measurement of the pH values of these solutions in water and in a partly aqueous solvent provides, by means of eqns. 9–14, data on $pK^* - pK$, the primary medium effect upon the dissociation constant.

EXPERIMENTAL

Measurements of isoelectric points of carrier ampholytes

Solutions (2%, w/v) of carrier ampholytes in water and in partly aqueous solvents were prepared by weighing, using commercially available stock solutions of Ampholines (LKB, Stockholm, Sweden) for various narrow pH ranges and of Servalytes (Serva, Heidelberg, G.F.R.) for the pH range 2–4.

pH values were determined at 25° and 4° with a combined glass/silver–silver chloride/3 *M* potassium chloride electrode (Ingold Type 401), using a Radiometer Type 4 pH meter, which was calibrated with aqueous NBS standard solutions.

The chemicals used were sucrose (Baker, Phillipsburgh, N.J., U.S.A.; analysed grade), glycerol (Merck, Darmstadt, G.F.R.; p.a. grade) and ethylene glycol (Merck; p.a. grade).

pI and pI^* values were calculated by means of eqns. 2 and 7, δ values being taken from a previous paper[2].

Measurements of dissociation constants of acids

About equimolal solutions of carboxylic acids and their conjugated bases were prepared by dissolving weighed amounts of the acids in appropriate weighed amounts of a 0.005 molal sodium hydroxide solution in the solvents in question. The acids used were formic acid (Fluka, Buchs, Switzerland; p.a. grade), acetic acid (Merck; p.a. grade) and propionic acid (Fluka; p.a. grade).

About equimolal solutions of amines and their conjugated acids were prepared by dissolving weighed amounts of the corresponding ammonium chlorides in appropriate weighed amounts of a 0.005 molal sodium hydroxide solution in the solvents in question. The chlorides used were methylammonium chloride (Merck; p.a. grade), ethylammonium chloride (Fluka; puriss. grade) and propylammonium chloride (Fluka; puriss. grade).

pH values at 25° were determined as described above. pK and pK^* values were calculated by means of eqns. 9–14, $\log \gamma$ and $\log \gamma^*$ being calculated as indicated in a previous paper[2] and K^*_s values being taken from the literature[3,4].

Determination of the isoelectric point of bovine serum albumin

Aliquots of 10 mg of bovine serum albumin, Cohn fraction V (Sigma, St. Louis, Mo., U.S.A.), were focused in an electrofocusing column (LKB 8100-1) at a temperature of the cooling water of 4°. The concentration of Ampholines (pH range

TABLE II

VALUES OF $pI_{app} - pI$ AT 4° FOR AMPHOLINES OF VARIOUS pH RANGES AND FOR SERVALYTE OF pH RANGE 2-4

Solvent	*Ampholyte*								
	Ampholine								*Servalyte*
	pH 2.5–4, pI 2.85	*pH 3.5–5, pI 4.43*	*pH 4–6, pI 5.01*	*pH 5–7, pI 6.22*	*pH 6–8, pI 7.28*	*pH 7–9, pI 8.46*	*pH 9–11, pI 10.41*	*Interpolated* pI 3.60*	*pH 2–4, pI 3.77*
Sucrose:									
15%	−0.02	−0.02	−0.02	0.01	0.03	0.01	−0.04	–	−0.03
30%	−0.05	−0.04	−0.03	0.02	0.04	0.02	−0.10	–	−0.05
45%	−0.05	−0.04	−0.02	0.05	0.06	0.04	−0.16	–	−0.04
60%	−0.02	0.02	0.03	0.14	0.14	0.08	−0.19	–	0.02
Glycerol:									
20%	0.04	0.04	0.03	0.04	0.04	0.04	−0.04	0.05	0.02
40%	0.10	0.11	0.11	0.11	0.10	0.11	−0.05	0.12	0.07
60%	0.21	0.23	0.19	0.16	0.16	0.13	−0.06	0.25	0.17
80%	0.39	0.42	0.34	0.21	0.21	0.17	−0.08	0.47	0.33
Ethylene glycol:									
20%	0.08	0.10	0.06	0.05	0.05	0.03	−0.04	0.11	0.04
40%	0.20	0.23	0.14	0.09	0.11	0.09	−0.04	0.25	0.14
60%	0.40	0.43	0.30	0.15	0.16	0.15	−0.05	0.47	0.30
80%	0.68	0.70	0.52	0.22	0.22	0.19	−0.06	0.78	0.54

* See footnote on p. 167.

3.5–10) was 2% (w/v). Density gradients, produced with a gradient mixer (LKB 8121), were carefully layered on the anolyte [0.25 *M* orthophosphoric acid in 60% (w/w) sucrose]. The catholyte was an aqueous 0.25 *M* sodium hydroxide solution. After focusing at constant power (5 W) for 48 h (LKB 2103 power supply) the contents of the column were collected in 1-ml fractions.

The extinction at 280 nm of these fractions was measured using a Uvichem Type H1620 spectrophotometer, the pH value at 25° of the fractions with maximum UV extinction was measured as described above and the solvent composition was determined by measurement of the refractive index (n), taking into account a correction for the contribution by the mean concentration of Ampholines [$\Delta n = 0.0041$ at 25° for a 2% (w/v) solution].

RESULTS

Isoelectric points of carrier ampholytes

The results are given in Tables I and II. As an example, Fig. 1 gives a plot* of $pI_{app} - pI$ *versus* pI_{app} in ethylene glycol–water mixtures at 25°.

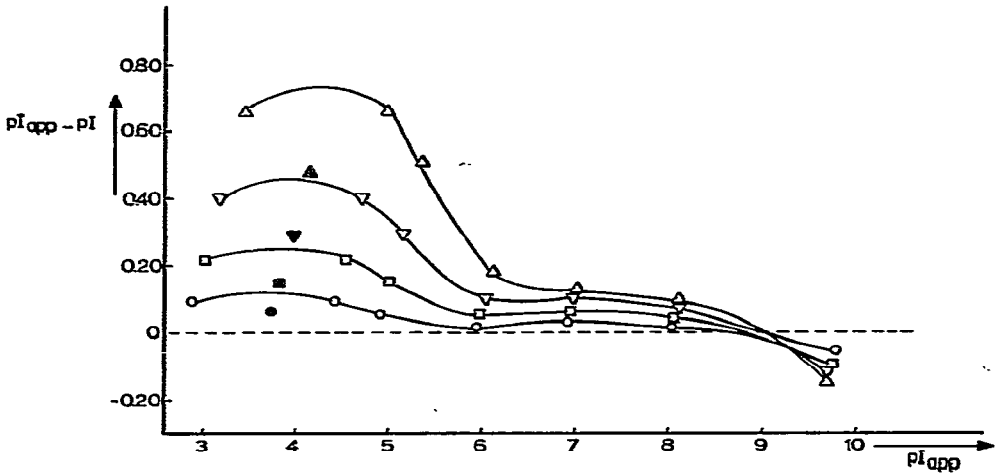

Fig. 1. Plot of $pI_{app} - pI$ *versus* pI_{app} for Ampholines (open symbols) and Servalyte (closed symbols) at 25° in ethylene glycol–water mixtures containing 20 (○), 40 (□), 60 (▽) and 80% (w/w) (△) of ethylene glycol.

Dissociation constants of acids

In Table III, the pK and pK* values are given, together with literature[6,7] values for pK.

* Preliminary experiments were performed with Ampholine solutions of narrower pH ranges. These solutions had been prepared by fractionating the commercially available stock solutions on a flat bed[5] of Sephadex G-75 beads swollen in water. Solutions in partly aqueous solvents were subsequently obtained by adding aliquots of the organic component, thereby diluting the Ampholines. In view of the dilution effect, dealt with in the Appendix, these data are not tabulated in Tables I and II, but they have been used to estimate the curvature of graphs of $pI_{app} - pI$ *versus* pI_{app} (such as those in Fig. 1) for $3 < pI_{app} < 5$. In order to enable the reader, who might wish to use the data for correction purposes (see under Discussion), in constructing such graphs, interpolated data at $pI = 3.60$ have been added in an extra column of Tables I and II.

TABLE III

pK AND pK* VALUES AT 25° FOR SOME CARBOXYLIC ACIDS AND SUBSTITUTED AMMONIUM IONS IN SUCROSE–WATER, GLYCEROL–WATER AND ETHYLENE GLYCOL–WATER MIXTURES

Acid or ion	*pK*		*pK**											
	This work	*Lit.*[6,7]	*Sucrose (%, w/w)*				*Glycerol (%, w/w)*				*Ethylene glycol (%, w/w)*			
			15	*30*	*45*	*60*	*20*	*40*	*60*	*80*	*20*	*40*	*60*	*80*
Formic acid	3.74	3.75	3.83	3.93	4.05	4.25	3.89	4.08	4.35	4.81	3.93	4.13	4.43	5.03
Acetic acid	4.75	4.76	4.83	4.93	5.05	5.28	4.93	5.10	5.41	5.92	4.96	5.22	5.55	6.20
Propionic acid	4.87	4.87	4.95	5.06	5.19	5.44	5.06	5.27	5.60	6.13	5.10	5.40	5.77	6.42
Methylammonium	10.61	10.63	10.64	10.75	—	—	10.64	10.79	10.96	11.31	10.60	10.62	10.58	10.87
Ethylammonium	10.61	10.63	10.67	10.77	—	—	10.66	10.81	10.96	11.31	10.60	10.61	10.61	10.94
Propylammonium	10.52	10.53	10.58	10.66	—	—	10.55	10.70	10.86	11.17	10.49	10.54	10.48	10.91

TABLE IV

VALUES OF pI_{app} AT 25° OF BOVINE SERUM ALBUMIN, DETERMINED IN THREE DIFFERENT DENSITY GRADIENTS

Solvent system	*Concentration of organic component (%, w/w)*	pI_{app}	$pI_{app} - pI$	pI
Sucrose–water	36.4	4.67	−0.05	4.72
Glycerol–water	59.1	4.93	0.17	4.76
Ethylene glycol–water	62.5	5.12	0.34	4.78

Isoelectric point of bovine serum albumin

The experimental data are given in the first three columns of Table IV.

DISCUSSION

As can be seen from the results for Ampholines in Tables I and II, the difference between the apparent isoelectric point, pI_{app}, and the pI value in water depends on the nature and concentration of the organic component in the solvent mixture and on the pI value of the Ampholine. These differences range from zero in water to −0.2 pH unit for basic Ampholines in 60% sucrose and +0.7 pH unit for acidic Ampholines in 80% ethylene glycol. They are rather small in sucrose–water mixtures over a wide range of sucrose concentrations: for Ampholines with $3 < pI < 9$ the values of $pI_{app} - pI$ are within ±0.1 pH unit for sucrose concentrations ranging from 0 to 60%.

As proteins contain essentially the same protolytic groups as Ampholines, it is reasonable to suppose that the above-mentioned statements also hold for $pI_{app} - pI$ values of proteins in these solvent systems. Hence, for proteins with $3 < pI < 9$, the $pI_{app} - pI$ values in sucrose–water mixtures are probably also within ±0.1 pH unit. This may be the reason why the unsuitability of pI_{app} as a characteristic quantity has never been detected experimentally, as sucrose is mostly used in density-gradient isoelectric focusing.

A comparison of the data in Tables I and II for Ampholines and Servalyte (also illustrated in Fig. 1) reveals the influence of the nature of the protolytic groups in the ampholyte upon its $pI_{app} - pI$ value: Servalytes differ from Ampholines in that part of the carboxyl groups is replaced by phosphonic and sulphonic acid groups[8]. Thus, whereas $pI_{app} - pI$ values for Ampholines probably can be used for correction of pI_{app} values of proteins, they are not valuable for other classes of ampholytes.

From the data for pI (in water) at 25° (Table I) and those at 4° (Table II), values of the temperature coefficient, $(\Delta pI/\Delta T)_{pI}$, holding for a mean temperature (about 15°) can be calculated. They are plotted in Fig. 2 against pI, together with the 95% interval of the distribution of $(dpK_a/dT)_{pK_a}$ values for carboxyl, amine and pyridine groups, given in a previous paper[1]. The present data appear to be situated in the centre of this distribution, which substantiates our earlier discussion[1].

As has been pointed out under Theoretical, $pI_{app} - pI$ values can be converted into $pI^* - pI$ values by combining the former with values of δ, previously determined[2]

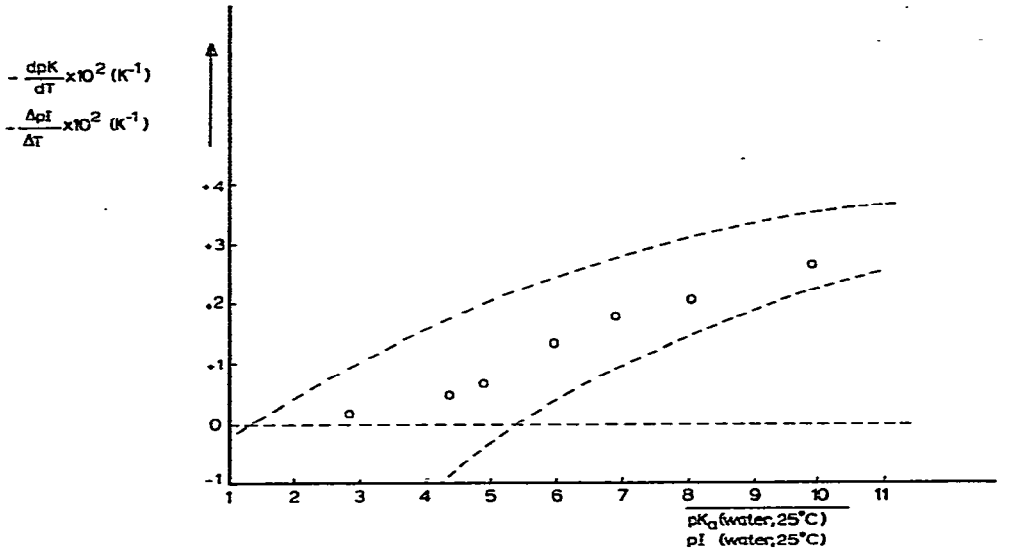

Fig. 2. Values of $\Delta pI/\Delta T$ of Ampholines (○) for the temperature range 4–25° and the 95% interval (broken lines) of the distribution of dpK/dT values at 25° for carboxyl, amine and pyridine protolytic groups.

for the solvent mixtures used. The resulting values of $pI^* - pI$ are depicted in Fig. 3 for acidic and basic Ampholines, together with the δ values.

In fact, one would expect values of $pI^* - pI$ for Ampholines to range between the $pK^* - pK$ values of carboxylic acids and of alkyl-substituted ammonium ions. Therefore, the $pK^* - pK$ values of propionic acid and propylammonium ion are included in Fig. 3. It can be seen that the above expectation is realized to a good approximation.

A comparison of Fig. 3 with the data in Table I clearly demonstrates that the characterization errors, $pI_{app} - pI$, encountered in isoelectric focusing, are the resultant of two opposing effects, *viz.*, the error $pI^* - pI$ (the primary medium effect

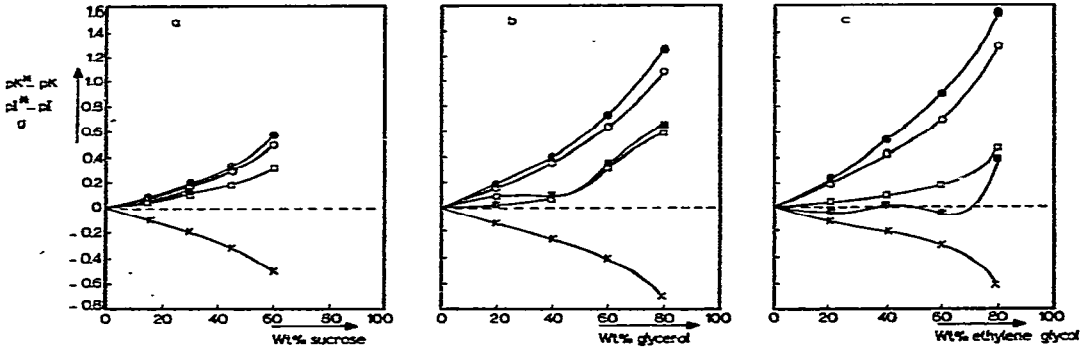

Fig. 3. Values at 25° of $pK^* - pK$ for propionic acid (●) and propylammonium ion (■), $pI^* - pI$ for Ampholines, $pI = 4.34$ (○) and $pI = 9.86$ (□) and δ (×) in (a) sucrose–water, (b) glycerol–water and (c) ethylene glycol–water mixtures.

upon the isoelectric point) and the error $\delta = pI_{app} - pI^*$ (the effect of the solvent composition upon the diffusion potential and the standard potential of the glass electrode in the pH measuring cell).

As stated above, $pI_{app} - pI$ values for Ampholines can be used in correcting pI_{app} values for proteins. This is carried out most conveniently as follows. From the curves in Fig. 1, or analogous curves for the other solvent systems or at 4°, the correction terms $pI_{app} - pI$ can be read at the value of pI_{app} found experimentally for the protein in question at rounded values of the solvent composition. From a plot of this correction term as a function of the solvent composition, the value of $pI_{app} - pI$ can then be found by interpolation, for the solvent composition of the focused protein fraction.

This procedure was used for the correction of the values of pI_{app} determined for bovine serum albumin (Table IV). In Fig. 4 three curves are given for the values of pI_{app} found experimentally in sucrose, glycerol and ethylene glycol gradients. The correction terms, $pI_{app} - pI$, pertaining to the solvent compositions found experimentally (Table IV), are read from these curves. They also are compiled in Table IV, together with the estimates obtained for the isoelectric point in water. The mean value at 25° and its standard deviation from these three estimates appears to be 4.75 ± 0.02. This can be compared with the following literature values at 25°: 4.85 and 4.70 (on Sephadex G-75 gel[9]); 4.86, 4.82 and 4.78 (on polyacrylamide gel[10]); and 5.28 and 4.75 (in a sucrose gradient[11]).

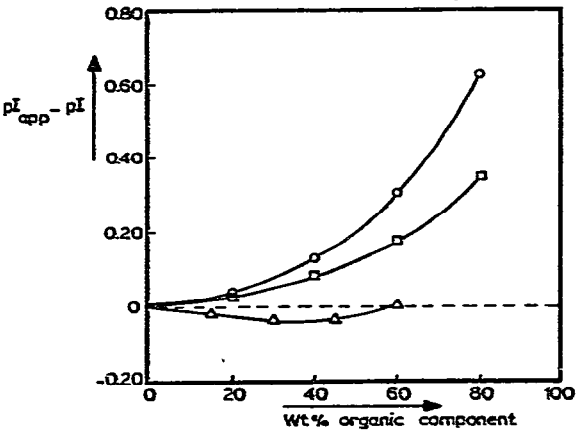

Fig. 4. Plot of $pI_{app} - pI$ at 25° *versus* the concentration of the organic component at constant values of pI_{app} in sucrose–water mixtures ($pI_{app} = 4.67$) (△), glycerol–water mixtures ($pI_{app} = 4.93$) (□) and ethylene glycol–water mixtures ($pI_{app} = 5.12$) (○).

The primary medium effect upon the dissociation constant of weak acids has been exploited by Troitsky *et al.*[12], who succeeded in forming stable pH gradients by using simple buffers, such as acetate, citrate and phosphate buffers, in gradients of organic solvents, such as ethanol, dioxane and glycerol. With a 0.001 *M* acetate buffer in a glycerol gradient, for example, a stable pH gradient covering 0.8–1.0 pH unit was obtained.

When these gradients are used for the determination of the isoelectric points

of proteins, the same interpretation errors, $pI_{app} - pI$, depending on the pI value of the protein and the solvent composition, occur. This contrasts with the opinion of Troitsky *et al.*[12] that the pK values of (protolytic groups in) proteins (and thus the pI values of proteins) are not influenced by the addition of organic solvents. This opinion was based on the erroneous application of the Born[13] equation on protolytic dissociation equilibria. It is wellknown[14,15] that this equation does not describe adequately the variation of the dissociation constant with the dielectric constant of the medium even for equilibria as simple as the dissociation of acetic acid. This is not surprising, as the Born equation is derived from a simple electrostatic model, whereas the variation of the free enthalpy of the hydrogen ion is not of a purely electrostatic nature.

Even if the pI_{app} values of proteins focused in these buffer gradients are properly corrected for with our $pI_{app} - pI$ values, a value for the isoelectric point in water might result that differs from the value obtained by isoelectric focusing in carrier ampholyte solution. This occurs in those instances where the protein strongly binds negative ions. Bovine serum albumin is an example of such a protein, which is clearly demonstrated by the much smaller value[16,17] of the isoelectric point in aqueous solutions, determined by moving-boundary electrophoresis. To check this, we also focused bovine serum albumin in a 0.02 *M* acetate buffer in a 0–80% (w/w) glycerol gradient according to Troitsky *et al.*[12]. We found the protein focused in 52.3% (w/w) glycerol at $pI_{app} = 4.56$, which yields, after correction, $pI = 4.39$. This value is much lower than that obtained in Ampholine gradients, but is in reasonable accord with the moving-boundary value. The latter is 4.72 at 0° in 0.02 *M* acetate[17], which corresponds to about 4.50 at 25°, taking into account the mean temperature coefficient resulting from Fig. 2 at this pH.

CONCLUSIONS

(1) $pI_{app} - pI$ values, the errors made in the measurement of isoelectric points by the conventional execution of density-gradient isoelectric focusing, depend on the acidity/basicity and the chemical type of the ampholyte and upon the solvent composition.

(2) These errors are within ± 0.1 pH unit for Ampholines (and presumably proteins) with $3 < pI < 9$ in sucrose gradients from 0 to 60% (w/w) of sucrose.

(3) The temperature coefficient of the pI values of Ampholines (and presumably proteins) is in good accordance with that of the pK values of carboxyl and amine groups.

(4) The primary medium effect ($pI^* - pI$) of sucrose, glycerol and ethylene glycol on the pI values of Ampholines is in good accordance with that ($pK^* - pK$) on the pK values of carboxylic acids and alkyl-substituted ammonium ions.

(5) $pI_{app} - pI$ values that hold for Ampholines can be used for the correction of the pI_{app} values of proteins determined by density-gradient isoelectric focusing.

(6) The isoelectric point in water at 25° of bovine serum albumin is 4.75 ± 0.02.

APPENDIX

As has been demonstrated theoretically by Svensson[18] (see also the work of Rilbe[19]), the pa_H value of an aqueous solution of a pure biprotic ampholyte differs from its isoelectric point* by an amount that increases with decreasing concentration of the ampholyte, with increasing acidity for acidic ampholytes and increasing basicity for basic ampholytes and with increasing difference between the pK values determining the isoelectric point of the ampholyte.

In the more complicated (mixture of) carrier ampholytes, the effective value of the latter factor is unknown. Therefore, we checked experimentally the influence of the concentration upon the pH value for the most acidic and most basic carrier ampholytes used in this study. The results, which are in agreement with those of Salaman and Williamson[10], are given in Fig. 5.

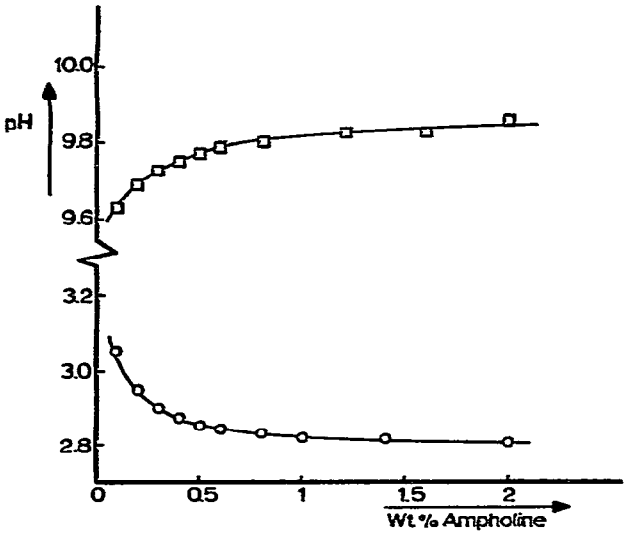

Fig. 5. pH values at 25° of aqueous Ampholine solutions, pH range 2.5–4 (○) and 9–11 (□), as a function of the Ampholine concentration.

A general requirement for the validity of eqn. 2, and consequently for the pH values in Fig. 5 to be interpretable in terms of pa_H values, is that the ionic strength of the carrier ampholyte solution should be of the same order of magnitude or less than that of the (NBS) standard buffer solutions used, *i.e.* $\leqslant 0.05$ M. It can be argued that this condition is fulfilled for Ampholine solutions at a concentration of 2% (w/v). The average molecular weight of Ampholines, as determined by gel filtration on Sephadex G-50 and G-75 and from osmotic measurements, is about 700[20]. Thus, the mean concentration in a 2% (w/v) solution ($\bar{c}_{amph}$) is about 0.03 M, giving an ionic strength[19] $\leqslant \frac{1}{3}\bar{c}_{amph} + \frac{1}{2}c_H \approx 0.01$.

* For simplicity we neglect here the difference between isoprotic and isoelectric point.

It can be concluded from these arguments and Fig. 5 that measured pH values of Ampholine solutions at a concentration of 2% (w/v) are very close to the (mean) isoelectric points.

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