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ISOELECTRIC FOCUSING AS A METHOD FOR THE CHARACTERIZATION OF AMPHOLYTES

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SUMMARY

The potential properties of isoelectric focusing as a method for the characterization of ampholytes are critically analyzed in a discussion and an evaluation of the effects of temperature and solvent composition on the isoelectric point of an ampholyte and of the effect of the solvent composition on the difference between the measured pH and the negative logarithm of the hydrogen-ion activity.

INTRODUCTION

As Haglund[1] stated in 1971, "isoelectric focusing has rapidly become an important tool of many researchers, mainly in biochemistry and related sciences". Since 1971, its importance has further increased, in particular as a result of methodical contributions by Righetti and Drysdale[2], Radola[3] and Rilbe and Petterson[4].

Also, according to Haglund, "the two main applications of isoelectric focusing are: (1) analytical or preparative separation of ampholytes, especially proteins, according to their isoelectric points, and (2) characterization of ampholytes, especially proteins, by determining their isoelectric points. This can be done simply and exactly in a single experiment".

Of these two applications, however, the former is far more often encountered. The latter application, *viz.*, characterization of an ampholyte by determination of its isoelectric point, is not frequently reported in the literature. In fact, this determination is not as simple and exact as might be understood from the statement quoted above.

The main reason for this lies in the following three effects: (1) The isoelectric point of an ampholyte depends on the temperature. (2) The isoelectric point of an ampholyte depends on the solvent composition. (3) The pH, as it it is usually measured in a solution containing a focused ampholyte, differs from $-\log a_H^*$ by an amount that depends on the solvent composition in that solution*.

Consequently, characterization of an ampholyte by measurement of its iso-

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* An asterisk (*) is used to denote that the quantity under consideration (here the activity of H^+) 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).

electric point requires either standardization of the temperature during the final stage of the separation, of the solvent composition in the focused fraction and of the carrier ampholytes, or correction for differences in both the temperature and the solvent composition occurring between different measurements.

In the following section it will be shown that standardization of temperature is not generally feasible and that standardization of solvent composition is obtained only in some variants of isoelectric focusing. Moreover, as will be seen in subsequent sections, correction for differences in temperature and solvent composition cannot be made owing to the lack of appropriate data.

The result of this situation is that all stated isoelectric points are beset with error. The magnitude of these errors, resulting from the three effects mentioned above, will be estimated separately.

STANDARDIZATION OF TEMPERATURE, SOLVENT COMPOSITION AND CARRIER AMPHOLYTES

The temperature prevailing during the final stage of a separation by isoelectric focusing results from the heat balance in the apparatus. It thus depends, on the one hand, upon the final voltage, the dimensions of the apparatus, and the type and concentration of carrier ampholytes and stabilizing additives, and, on the other hand, upon the cooling efficiency of the experimental set-up.

Clearly, rigorous control of these factors could result in standardization of the final focusing temperature. However, as the specific conductance of carrier ampholytes in the stationary state depends upon their isoelectric point, the final temperature is not constant over the entire pH gradient. Thus the final temperature of a focused zone depends upon the isoelectric point of the ampholyte focused in that zone; *i.e.*, strict standardization to the same standard temperature for all ampholytes in one electrofocusing experiment is impossible and some correction of measured pI values will generally be necessary. Evidently, a comparison for identification purposes of pI values determined by different isoelectric focusing techniques also requires correction for temperature differences.

As a result of the observations of Vesterberg and Svensson[5], one could imagine that standardization of the focusing temperature is of minor importance. In experiments on myoglobins at different focusing temperatures and different temperatures of the subsequent pH measurements, these authors found that isoelectric points pertaining to 25° (the standard temperature for pK measurements) are obtainable by simply making the pH measurements at 25°, irrespective of the zone focusing temperature (the differences in pI values found when changing the temperature of the pH determination from 25 to 4° were about 0.36 pH unit). A possible explanation, given by Vesterberg and Svensson, is that the acid–base balance of both the carrier ampholytes used (Ampholines, LKB, Stockholm, Sweden) and the proteins is governed by the same type of protolytic groups, *viz.*, carboxylic and amino groups. If this explanation is correct, the use of this rule with the recently introduced new types of carrier ampholytes, *viz.*, Servalytes (Serva, Heidelberg, G.F.R.) and Biolytes (Bio-Rad Labs, Richmond, Calif., U.S.A.), could possibly give rise to errors caused by the temperature effect, as these ampholytes contain, in addition to the above protolytic groups, sulphonic acid and phosphonic acid groups.

In spite of this optimistic view on the unimportance of temperature standardization, most authors[6–8] prefer to perform the pH measurements at the focusing temperature, which, in most instances, is taken to be equal to the temperature of the cooling liquid. However, as a result of the temperature difference between the focused zone and the cooling liquid, this practice leads to pI values that are in error by an amount depending on this temperature difference, and thus on the isoelectric point of the ampholyte and on the difference between the temperature coefficients of the pI values of the carrier ampholytes and those investigated.

The solvent composition of a focused zone in a separation by isoelectric focusing varies considerably with the experimental technique used. On the one hand, there is the isoelectric focusing in a stabilizing density gradient of a non-electrolyte (sucrose, glycerol or ethylene glycol); on the other hand, the so-called zone convection isoelectric focusing, in which stabilization of the pH gradient is achieved by the very design of the apparatus and the solvent used is essentially water. But also in some variants of thin-layer gel isoelectric focusing, the use of relatively high but constant concentrations of non-electrolyte additives (*i.e.*, 12% sucrose) is recommended[9].

In fact, standardization of the solvent composition is automatically achieved with zone convection and with gel- and thin-layer isoelectric focusing. With isoelectric focusing in a density gradient, however, this standardization is not obtained, as it is inherent in the method that the solvent composition of a focused zone varies with the pH value and therefore with the pI value of the ampholyte focused in that zone, *i.e.*, strict standardization to the same standard solvent composition for all ampholytes in one electrofocusing experiment is impossible and some correction of measured pI values will generally be necessary. Evidently, a comparison for identification purposes of pI values of the same ampholyte determined by different isoelectric focusing techniques also, in many instances, demands correction for the solvent effect.

CORRECTION FOR THE EFFECT OF TEMPERATURE UPON THE ISOELECTRIC POINT

Correction for differences in temperature requires knowledge of the final focusing temperatures and of the temperature coefficients of the isoelectric points of the focused ampholytes. While the former data can, in principle, be determined, the latter are generally unknown.

In order to estimate the errors made in the inevitable omission of any temperature correction we collected in Fig. 1 values for the temperature coefficient (at 25°) of the dissociation constant of protolytic groups of several acids, bases, amino acids and dipeptides, calculated from literature values of the standard heat of ionization (at 25°), together with data on the mean temperature coefficient (for the temperature interval 4–25°) of the isoelectric point of some proteins.

The dashed lines in Fig. 1 enclose 95% of the data on carboxylic acids, amines, amino acids and pyridines. It can be concluded that the distribution of the data on $(\mathrm{d}pK_a/\mathrm{d}T)pK_a$ has a standard deviation of about 0.005 K^{-1}. It can be assumed that the distribution of the data on $(\Delta pI/\Delta T)pI$ for ampholytes containing these protolytic groups has the same standard deviation.

To obtain an estimate of the error associated with the temperature effect, this standard deviation must be multiplied by the difference between the temperature of

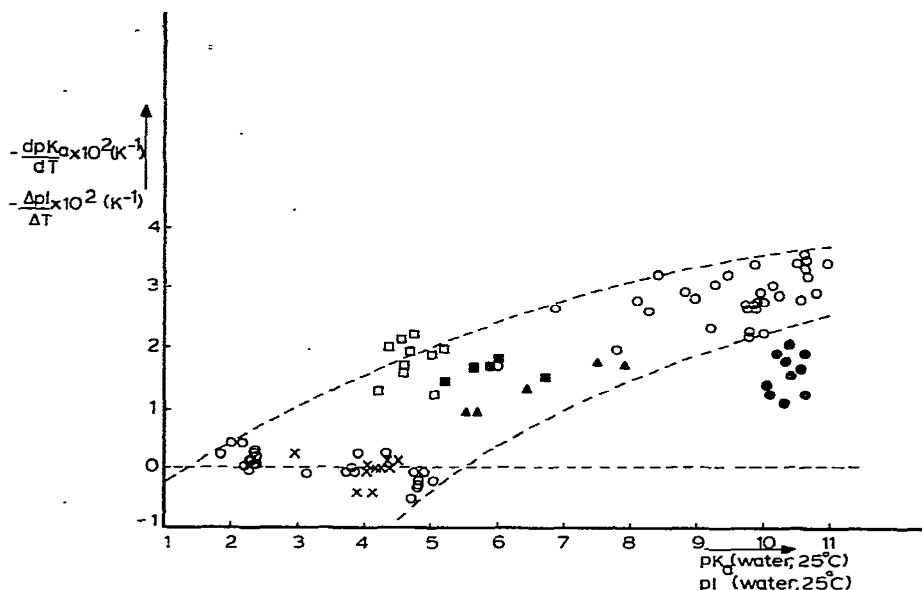

Fig. 1. Values of dpK_a/dT at 25° in water of the protolytic groups of several aliphatic carboxylic acids[10], hydroxycarboxylic acids[11], amines[12–14], hydroxyamines[15–17], amino acids and dipeptides[11,18] (○), aromatic carboxylic acids[19] (×), aniline, toluidines and xylidines[20] (□), phenol, cresols and xylenols[21,22] (●) and pyridine, picolines and lutidine[23] (■). Values of $\Delta pI/\Delta T$ for the temperature interval 4–25° of some proteins[7] (▲).

the focused zone and that of the cooling liquid*. The latter is strongly[25] technique and apparatus dependent; it is probably less in thin-layer isoelectric focusing[26], in which cooling is efficient owing to the high ratio of cooling surface to total volume. However, even with this technique, the temperature difference can amount to 8° (ref. 27) or even 16° (ref. 28). Thus, the standard deviation due to the temperature effect can amount to 0.04 or even 0.08 pI units, even under such relatively favourable conditions.

It can also be concluded from Fig. 1 that the temperature effect can give rise to far larger errors if either the carrier ampholytes or those investigated contain groups other than carboxylic, amino or pyridine groups.

CORRECTION FOR THE EFFECT OF SOLVENT COMPOSITION UPON THE ISOELECTRIC POINT

Correction for differences in the solvent composition requires knowledge of the solvent compositions in the focused fractions and of the solvent effect upon the isoelectric point of the ampholytes. The former can, in principle, be determined, but the latter is generally unknown.

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* Evidently, dpK/dT at the mean temperature should be used instead of that at 25°, as pointed out by Fredriksson[24]. From known values of Δc_p for many of the dissociation equilibria, however, it can be demonstrated that, on the average, values of (dpK/dT) at 0° are only slightly different from those at 25° $[(dpK/dT)_0 \approx 1.10\ (dpK/dT)_{25} - 0.003]$. This results in an increase of only 10% in the standard deviation mentioned above.

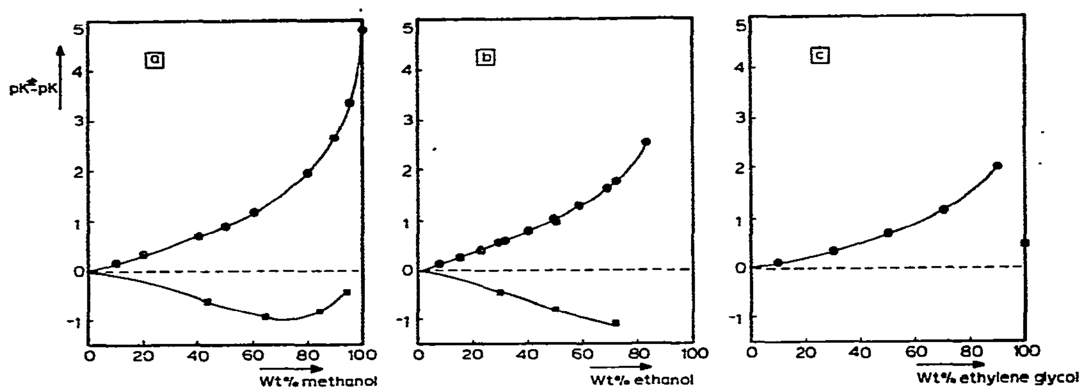

Fig. 2. Literature values of pK^* — pK (at 25°) for acetic acid (●) and methylammonium ion (■) in methanol–water (a)[29–31], ethanol–water (b)[32,33] and ethylene glycol–water (c)[34,35] mixtures.

In order to estimate the errors caused by the omission of a solvent-effect correction, we present in Figs. 2a, b and c the available literature values of pK^* — pK of acetic acid and methylammonium ion in some methanol–water, ethanol–water and ethylene glycol–water mixtures, and in Fig. 3 the available literature data on pK^* — pK in 71.89 wt.% ethanol for carboxylic acids, amines and phenols (unfortunately, no such data exist in the solvents most utilized, *viz.*, sucrose–water and glycerol–water mixtures).

As can be seen in Figs. 2 and 3, the magnitude of the solvent effect upon the dissociation constant varies not only with the solvent composition, but also with the nature of the acid. This implies, that the magnitude of the solvent effect upon the isoelectric point of an ampholyte also depends upon the nature of the protolytic groups that determine its isoelectric point. At present, no correction is possible,

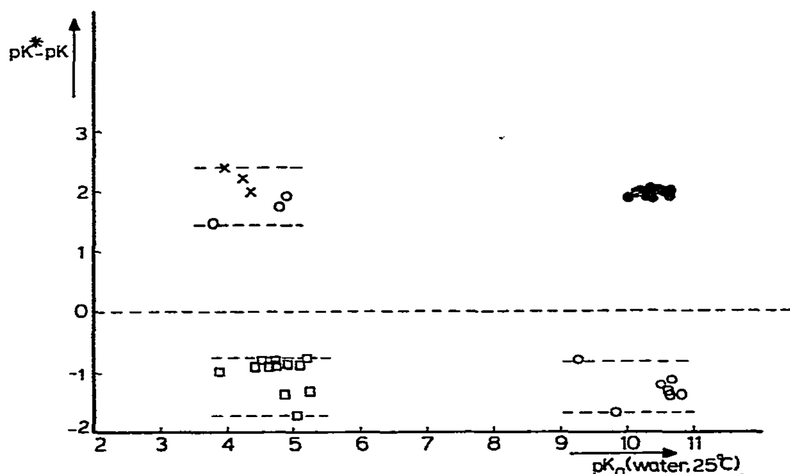

Fig. 3. Literature values[32] of pK^* — pK (at 25°) in 71.89 wt.% ethanol for several aliphatic carboxylic acids and amines (○), aromatic carboxylic acids (×), phenol, cresols and xylenols (●), and aniline, toluidines and xylidines (□).

which may lead to considerable errors. Consider for example an ampholyte (a protein), the isoelectric point of which is determined by protolytic groups with a solvent effect resembling that of the carboxylic group of acetic acid (this can be supposed to be true for proteins with pI values in water of 4.5–5.0). If one compares pI values of such a protein, obtained by isoelectric focusing in a gel containing no non-electrolyte additives and by density-gradient isoelectric focusing in 80% ethylene glycol, a correction for the solvent effect upon the isoelectric point in the order of — 1.5 pH units should be accounted for.

It follows from an argument analogous to that given in conjunction with Fig. 1 that, even if data similar to those presented in Fig. 3 were available for all solvent mixtures employed in isoelectric focusing (so that the average solvent effect could be accounted for), the standard deviation due to the solvent effect would still amount to several tenths of a pI unit, if the solvent effect on the isoelectric points of the carrier and investigated ampholytes is governed by the same type of protolytic groups.

CORRECTION FOR THE EFFECT OF SOLVENT COMPOSITION UPON THE DIFFERENCE BETWEEN THE MEASURED pH AND LOG — a^{*}_{H}

The determination of the pH of a focused fraction, whether it contains a non-electrolyte additive or not, is usually performed electrometrically with a glass electrode and a calomel electrode. This pH determination is based upon the measurement of the electromotive forces E_S and E_X of the galvanic cells:

Glass electrode | stand. buffer soln. S | satd. KCl (aq.) | calomel electrode (I)
Glass electrode | "unknown" soln. X | satd. KCl (aq.) | calomel electrode (II)

and the use of the operational pH definition:[36]

$$\mathrm{pH}_X = \mathrm{pH}_S + \frac{E_X - E_S}{(RT \ln 10/F)} \tag{1}$$

where R represents the molar gas constant, F the Faraday and T the absolute temperature.

In common practice, this determination is achieved by calibrating a pH meter with the aid of a standard buffer solution S of known pH_S (at the temperature T) and reading the pH_X value (pertaining to the temperature T) of the "unknown" solution.

In order to obtain pH_X readings that can be identified very closely with the conventional values of $\mathrm{p}a^{*}_{\mathrm{H},X}$, one must use standard buffer solutions that have the same solvent composition as the "unknowns". Standard pH^{*}_S values, approaching very nearly to $\mathrm{p}a^{*}_{\mathrm{H},S}$, have been published for several buffers in water[36] (at several temperatures) and, among others, for some buffer solutions in methanol, ethanol and some of their mixtures with water (at 25°)[32,37]. Unfortunately, no such standard buffers are known in aqueous solutions of sucrose, glycerol or ethylene glycol.

If one employs (as is usual) an aqueous standard buffer solution in the calibration procedure, the meter readings obtained for pH_X of non-aqueous or partially aqueous solutions can no longer be identified with $\mathrm{p}a^{*}_{\mathrm{H},X}$, but differ from it by an amount δ. In this instance, E_S and E_X (at 25°) are equal to:

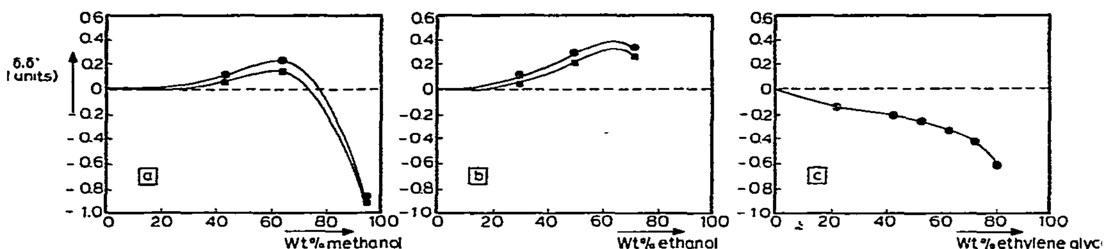

Fig. 4. Literature values (at 25°) of δ (●) and δ' (■) in methanol–water (a)[41], ethanol–water (b)[41] and ethylene glycol–water (c)[43] mixtures.

$$E_S = E_{cal} - E^{\circ}_{glass} - 0.05916 \log a_{H,S} + E_{j,S} \quad (2)$$

and

$$E_X = E_{cal} - E^{\circ *}_{glass} - 0.05916 \log a^{*}_{H,X} + E^{*}_{j,X} \quad (3)$$

where E_{cal}, E°_{glass} and E_j represent the potential of the calomel electrode, the standard potential of the glass electrode and the liquid junction potential, respectively. By combining eqns. 2 and 3 with the operational definition (1), one can easily derive for δ:

$$\delta \equiv pH_X - pa^{*}_{H,X} = \frac{(E^{*}_{j,X} - E_{j,S}) - (E^{\circ *}_{glass} - E^{\circ}_{glass})}{0.05916} \quad (4)$$

where pH_S has been assumed to be equal to $pa_{H,S}$.

Thus δ is composed of two terms reflecting the influence of the solvent composition on the liquid junction potential and on the standard potential of the glass electrode. It has been provéd that in methanol–water and ethanol–water mixtures, $E^{*}_{j,S} - E_{j,S}$ is nearly independent of the nature of the buffering solutes[38–40] and of the type of device forming the liquid junction of the calomel electrode[41]. However, it was found[42] that $E^{\circ *}_{glass} - E^{\circ}_{glass}$ in these solvents depends on the method of fabrication and pre-conditioning in the laboratory (rather than on the composition of the glass). This means that in these media a correction term $\delta' = (E^{*}_{j,X} - E_{j,S})/0.05916$ can be applied to measured pH_X values, but that correction for the influence of the solvent composition on the standard potential of the glass electrode is not feasible. Fortunately, the error made in assuming that $E^{\circ *}_{glass} - E^{\circ}_{glass} = 0$ is not large (in methanol–water and ethanol–water mixtures, $+0.01 \pm 0.04$ pH unit).

δ and δ' values have been published for methanol–water and ethanol–water mixtures[42] and δ values are available for ethylene glycol–water mixtures[43]; they are represented in Figs. 4a, b and c. Unfortunately, no such values are known in sucrose–water and glycerol–water mixtures.

As can be seen in Fig. 4, the magnitude of the solvent effect upon δ, *i.e.*, upon the difference $pH_X - pa^{*}_{H,X}$, depends upon the nature and concentration of the non-aqueous component. The errors in measured pI values, made in omitting a correction for this solvent effect, appear to be important; in the example depicted at the end of the preceding section, a correction for this solvent effect of about $+0.6$ pH unit should be accounted for, which gives, in combination with the correction of about -1.5 pH unit for the effect of the solvent composition upon the isoelectric point, a correction term for the total solvent effect of -0.9 pH units.

DISCUSSION

In spite of the scarcity of data relevant to the most utilized solvents, some general comments can be given on the potential properties of isoelectric focusing as a method for the characterization of ampholytes.

Even if it were possible to keep the difference between the final focusing temperature and the temperature of the pH determination as small as 2°, the corresponding standard deviation of the p*I* values would be 0.01 (this value holds for the class of ampholytes the isoelectric points of which are determined only by carboxylic and amino groups). Uncertainties of this order of magnitude, which corresponds to the resolution obtainable in isoelectric focusing, govern the potentiality of isoelectric focusing as a method of characterization only in those variants with which standardization of the solvent composition is obtained, *i.e.*, in zone convection and gel isoelectric focusing. In all other variants, the current practice of scrupulous notation of temperatures during focusing and pH measurement seems to be rather superfluous in view of the far greater solvent effects.

In those cases where standardization of the solvent composition is not obtained (*i.e.*, in density-gradient isoelectric focusing), the potentiality of the method as a characterization method is restrained mainly by the solvent effect upon the isoelectric point of an ampholyte. At present, a correction for the solvent effect is not possible, owing to the lack of data. This defect may cause errors of several p*I* units to occur. Even if sufficient data were available, variations in the solvent effect for different ampholytes would lead to a standard deviation of several tenths of a p*I* unit. However, even if it were possible, the use of such a correction would be meaningless if a correction for the effect of the solvent composition upon the difference pH —

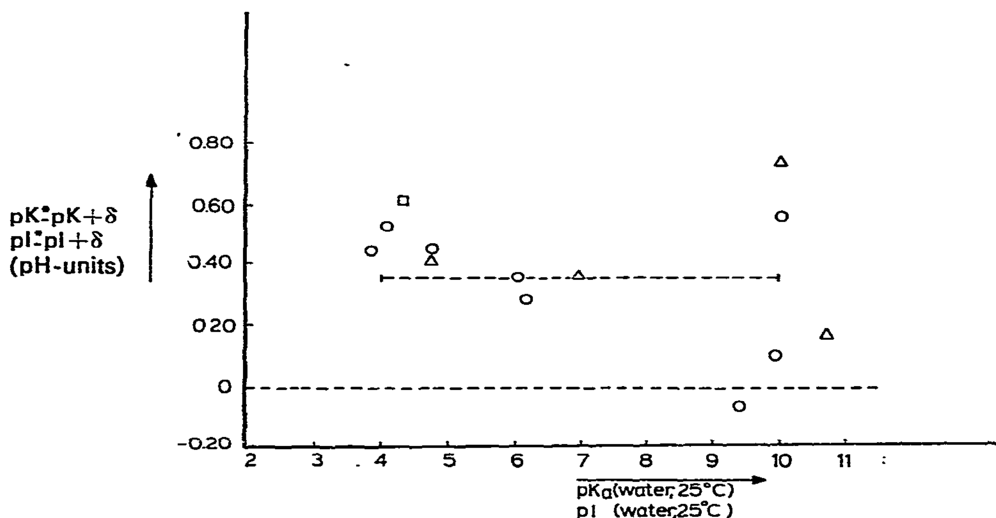

Fig. 5. Literature values of $pK^* - pK + \delta$ due to 5 *M* urea for protolytic groups of aspartic, benzoic, acetic and maleic acids, histamine, phenol and glycine at 30° (○)[45], bovine serum albumin at 25° (□)[46], acetic acid, imidazole, phenol and butylamine at 25° (△)[47]. Literature values of $pI^* - pI + \delta$ due to 5 *M* urea for LKB Ampholines (p*I*, 4–10) at 22° (—)[44].

pa_H^* is not also applied. The latter correction could be made if δ' values in the most utilized solvents were known; the inherent uncertainty would probably be of the order of 0.04 pH unit.

An overall correction term, accounting for the solvent effect of urea upon the isoelectric point of ampholytes ($pI^* - pI$) and for the accompanying changes in the liquid junction potential and the standard potential of the glass electrode (δ), has been used by Ui[44]. In his work on isoelectric focusing in sucrose density gradients 6 *M* in urea, he determined this correction term ($pI^* - pI + \delta$) due to urea in this concentration and showed it to have a value of 0.42 pH unit, irrespective of the p*I* value of the ampholytes studied (LKB Ampholines; p*I*, 4–10). This independence of the p*I* value is, however, scarcely possible in the light of the solvent effects upon p*K* values of carboxylic acids and amines (*cf.* Fig. 3). In Fig. 5 we collected values of $pK^* - pK + \delta$ due to 5 *M* urea, determined by other authors[45–47], together with that of $pI^* - pI + \delta$, determined by Ui. The distribution of $pK^* - pK + \delta$ values clearly bears a close qualitative resemblance to that of $pK^* - pK$ values in our Fig. 3 and suggest that the use of an unique correction term for all p*I* values is almost certainly incorrect.

CONCLUSIONS

Only those variants of isoelectric focusing in which the solvent composition of the focused zones is standardized (*e.g.*, zone convection, gel and thin-layer isoelectric focusing) are useful as a method for the characterization of ampholytes.

The difference between the final focusing temperature and the (standardized) temperature of the pH measurement should be kept within 2°, in order to keep the associated standard deviation of p*I* within 0.01.

The carrier ampholytes should be matched to those investigated with respect to their protolytic groups.

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