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Isoelectric Fractionation, Analysis, and Characterization of Ampholytes in Natural pH Gradients. II. Buffering Capacity and Conductance of Isoionic Ampholytes

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For a complete separation of proteins in a natural pH gradient, the presence of low-molecular ampholytes with buffering capacity and conductance in the isoelectric state (carrier ampholytes) is necessary. The concentrations of cations and anions in solutions of simple ampholytes are deduced as functions of pH. In the isoionic state, these concentrations are the same, and a simple expression for the degree of ionization is derived. The buffering capacity is deduced as the derivative with respect to pH of the concentration of cations minus the concentration of anions. In the isoionic state, the expression becomes particularly simple and equals that for the degree of ionization but for a numerical factor. Since the conductance is proportional to the concentration of ions, it may be concluded that the conditons for a good buffering capacity and for an appreciable conductance are the same. Both properties rise as the distance pI-pK_1 between the isoionic point and the nearest pK value decreases. Consequently, good carrier ampholytes are those which are isoelectric between two closely spaced pK values.

Conductance measurements are reported for solutions of some ampholytes which can be used as carriers. They are compared with theoretical conductances deduced from the degree of ionization and from estimated, reasonable mobility data of the ions of the ampholytes. These measurements form a semi-quantitative verification of the theory, substantiating the importance of the quantity pI-pK_1.

It is pointed out that the crucial difficulty in stationary electrolysis of proteins is the scarcity of carrier ampholytes isoelectric between pH 4 and 7. Successful separations of proteins isoelectric in this region cannot be expected until useful carrier ampholytes have been synthesized or supplied by other means.

In the preceding article [1] it was proved that two ampholytes can be completely separated by stationary electrolysis only if there is a third ampholyte with an intermediate isoelectric point, or if the two ampholytes are isoelectric on either side of the pH of the pure solvent. For example, histidine may be

completely separated from glutamic acid because a region of pure water may develop between them, but in a run with histidine and lysine there will necessarily be a zone in which the two amino acids remain mixed even in the steady state obtained on prolonged electrolysis.

The above rule also applies to proteins. It is thus impossible to obtain a complete separation between two proteins isoelectric below pH 7 by electrolysis in the absence of other ampholytes. Only a certain degree of purification is possible, which is of course better the greater the difference between the two isoelectric points.

A complete separation of proteins isoelectric on the same side of the neutral point thus requires the presence of amino acids or other low-molecular ampholytes, which may be called *carrier ampholytes*. They will then determine the pH course in the steady state. Because of the very sharp isoelectric points exhibited by proteins, isoelectric separation can be expected to be very selective. Of course each protein is first obtained contaminated by carrier ampholytes, but they can easily be removed by dialysis.

Since even the carrier ampholytes condense in isoelectric zones, the stability of the pH gradient depends on their buffering capacities in and near the isoelectric state. On the other hand, in order to avoid excessive field strengths due to rarefaction of ions, especially in the neutral region, it is also desirable to have access to low-molecular ampholytes with appreciable conductances in the isoelectric state. This article will be devoted to an analysis of the properties of an ampholyte which determine its buffering capacity and conductance in the isoelectric state.

THE ION CONCENTRATIONS OF AN AMPHOLYTE IN THE ISOELECTRIC RANGE

The isoelectric point is defined as the pH at which the ampholyte is immobile in an electric field. At this point, the concentrations of the cationic and anionic subspecies are not necessarily the same. First, the ampholyte may form complexes with other ions than H^+, and second, the mobilities of the cationic and anionic forms need not be the same. A calculation of the state of ionization at the isoelectric point is therefore too complicated and of little practical value. Instead it will be carried out for the isoionic state, in which cations and anions have the same concentration. Experimentally, the isoionic point may be defined as the pH of a solution which does not change its acidity on addition of a small quantity of the ampholyte.

It was pointed out in the previous article [1] that, for the great majority of low-molecular ampholytes, only two pK values exert a measurable influence on the ionization around the isoionic point. One then has the following simple relation between the isoionic point pI and these two pK values:

$$2\ \mathrm{pI} = \mathrm{p}K_1 + \mathrm{p}K_2 \qquad (1)$$

where pK_1 is defined to be smaller than pK_2. If the cation concentration is denoted by C_+, the anion concentration by C_-, and the added concentrations

of zwitterionic and undissociated ampholyte by C_o, then the mass-action law gives the following equations, activity factors being left without consideration:

$$C_+ = C_o \times 10^{pK_1 - pH} \quad (2)$$

$$C_- = C_o \times 10^{pH - pK_2} \quad (3)$$

Further, if C is the total concentration, one has the relation:

$$C = C_+ + C_- + C_o \quad (4)$$

Elimination of C_o gives, after rearrangement:

$$C/C_+ = 1 + 10^{pH - pK_1} + 10^{2(pH - pI)} \quad (5)$$

$$C/C_- = 1 + 10^{pK_2 - pH} + 10^{2(pI - pH)} \quad (6)$$

The degree of ionization must be defined as

$$\alpha = (C_+ + C_-)/C \quad (7)$$

In the isoionic state, one gets the simple expression:

$$\alpha = 2/(2 + 10^{pI - pK_1}) \quad (8)$$

Since pI is never smaller than pK_1, the second term in the denominator is always bigger than unity, and consequently there is an upper limit of $\alpha = 2/3$ for the fraction of ions in an isoionic ampholyte *.

BUFFERING CAPACITY

The mean electric charge of the ampholyte expressed in units of the elementary proton charge is given by the equation:

$$Q = \frac{C_+ - C_-}{C} \quad (9)$$

A plot of Q *versus* pH is identical with the titration curve of the ampholyte; hence the buffering capacity is given by the derivative:

$$-\frac{dQ}{d(pH)} = \frac{d(C_-/C)}{d(pH)} - \frac{d(C_+/C)}{d(pH)} \quad (10)$$

Calculation with the aid of eqns. (5) and (6) gives for the isoionic state, pH = pI:

$$-\frac{dQ}{d(pH)} = \frac{2 \ln 10}{2 + 10^{pI - pK_1}} \quad (11)$$

The buffering capacity as a function of $pI - pK_1$ is shown in Fig. 1. First it falls off linearly, and for $pI - pK_1 = 1$ pH unit as much as 1/4 of the limiting capacity is still retained. The curve then assumes an exponential decline, and

* In another connection [2], the author erroneously stated, without calculation, that α would equal 1/2 for a hypothetical ampholyte with $pK_1 = pK_2$, and that the fractions of zwitterionic and undissociated subspecies were both = 1/4. The relative abundance of the latter two subspecies cannot at all be calculated from titration data alone, but can be derived from other physicochemical measurements together with certain plausible assumptions (Green and Tong [3]; Lumme [4,5]).

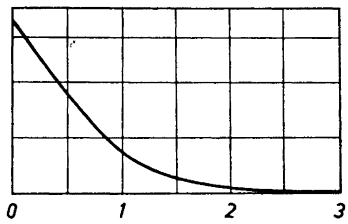

Fig. 1. The buffering capacity of an isoionic ampholyte as a function of pI—pK_1.

for pI—pK_1 = 2 units only 1/34 remains. It can thus be concluded that *carrier ampholytes should have the property of being isoelectric between two closely spaced* pK *values*. Eqn. (11) is also useful as a guide for making up formulas for mixtures of carrier ampholytes. The better the buffering capacity, the smaller amounts of the ampholyte can be taken in order to get a uniform pH gradient.

CONDUCTANCE

The conductance of a solution can be calculated by using one of the well-known equations:

$$\varkappa = F\sum_i c_i z_i u_i = \sum_i c_i z_i \Lambda_i \qquad (12)$$

Here F denotes the Faraday constant = 96 500 coulombs per equivalent, c_i concentrations in moles per cm^3 (always positive), z_i valences with sign according to charge, u_i mobilities in cm^2/V sec. (with positive sign for cathodic, negative for anodic migration), and Λ_i equivalent conductances in cm^2/ohm equiv. (with the same sign as u_i). The subscript i generally refers to the ion constituents (MacInnes [6]) of the solution, and eqns. (12) should be used in that way whenever possible.

For ampholytes in the isoionic state, however, the cation and the anion belong to the same ion constituent, *viz.* the organic radical of the ampholyte. In the isoelectric state, no current is transported by this ion constituent; the whole current is actually transported by the hydrogen ion constituent (Svensson [2]; Van Os and Möller [7]). Since the mobility of the latter cannot be measured in electrolytic systems of this type, the only remaining possibility is to apply eqns. (12) to the free ions actually present and to disregard from the concept of ion constituent. There are three such ions, the cation and the anion of the ampholyte, and in addition the hydrogen ion for acidic and the hydroxyl ion for basic ampholytes. The contribution of the solvent ions is big for strongly acidic and strongly basic ampholytes, but negligible for neutral ones.

The concentration of free ions is given by eqn. (8). Since this expression differs from the buffering capacity, eqn. (11), by a numerical factor only, it is understood that a good conductance and a good buffering capacity are given by the same type of ampholytes, those which are isoelectric between two closely spaced pK values.

The mobilities of ions of ampholytes. Eqn. (12) in conjunction with eqn. (8) is useful for calculation of the conductance of isoionic ampholytes if the mobi-

lities of their ions are known. However, no such data seem to be available in the literature; those for glycine, alanine, and glycyl-glycine reported by Svensson, Benjamisson and Brattsten [8] are without interest in this connection since these ampholytes have practically no conductance in the isoionic state.

For ions of unit charge, the mobility varies inversely with the linear dimensions of the hydrated ions, *i.e.* for spherical ions roughly with the cubic root of the kinetic molecular volume. Consequently a good approximation of the mobility of an ampholytic ion can be obtained by taking the value of an ordinary ion of similar size and structure, and by applying a suitable correction for the difference in molecular volume. The empirical relations between mobilities and molecular volumes presented by Edward [9,10] can be used for this purpose. The procedure will be demonstrated below for a number of easily available ampholytes which can be expected to possess an appreciable conductance in the isoionic state.

The anions of the nicotinic, isonicotinic, and picolinic acids must be expected to be about as mobile as the benzoate ion. In Conway's [11] book, one finds the figure −32.3 for the equivalent conductance of the benzoate ion at infinite dilution and at 25°C. According to Edward, the kinetic molecular volume of the benzoate ion may be estimated to 167.8 Å^3, which corresponds to a sphere radius of 3.42 Å. If this is introduced into Edward's equation for monovalent ions:

$$\Lambda = \pm k/r \qquad (13)$$

the constant k gets the value 110.5. The pyridine-carboxylic acids contain one aromatically bound nitrogen atom instead of one aromatic CH group, which reduces the molecular volume to 165.3 Å^3 and the corresponding radius of a sphere to 3.40 Å. The latter value introduced into (13) gives the figure −32.5 for the equivalent conductance of the pyridine-carboxylic acids. A similar calculation for the cations gives + 37.3 cm^2/ohm equiv.

The amino-benzoic acids are of about the same size and shape as chlorobenzoic acids, for which Saxton and Meier [12] have reported the figures −30.3 (*o*-acid) and −31.0 (*m*-acid). Edward's table gives the molecular volume 184.0 Å^3 for the chlorobenzoate ion and 174.0 Å^3 for an amino-benzoate ion. With a k value of 107.9, one gets an equivalent conductance of −31.2 cm^2/ohm equiv. for the anion of the ampholyte. A similar calculation for the cation gives + 35.7.

In the same way, the probable mobilities of the ions of arsanilic acid, aspartic acid, and glutamic acid have been calulated. For aspartic acid comparison has been made with succinic acid monoamide, for glutamic acid with glutaric acid monoamide, the mobilities of which have been measured by Jeffrey and Vogel [13].

Among the ions for which mobility data are available, the heptanoate ion resembles the lysine anion most closely. Its mobility has been measured by Dippy [14] and can be used to calculate a probable mobility of lysine ions. Similarly, the octanoate ion, also measured by Dippy, is the best one to use for calculating mobilities of arginine ions. Finally, for histidine comparison may be made with phenylpropionic acid, the mobility of which has been measured

Table 1. Probable equivalent conductances in cm^2/ohm equiv. of ions of some ampholytes at 25°C and infinite dilution

Ampholyte	Λ_+	$-\Lambda_-$
Pyridine-carboxylic acids	37.3	32.5
Aminobenzoic acids	35.7	31.2
Arsanilic acids	31.7	29.8
Aspartic acid	36.4	28.1
Glutamic acid	34.2	27.0
Histidine	31.3	31.3
Lysine	29.0	29.0
Arginine	29.3	29.3

by Dippy and Lewis [15]. The probable mobilities thus calculated for ions of ampholytes are collected in Table 1.

The contribution to conductance of the ions of the solvent. The equivalent conductances of the H^+ and OH^- ions are 350 and −192 cm^2/ohm equiv., respectively, at 25°C and infinite dilution. At pH 7, both these ions have a concentration of 10^{-10} moles per cm^3. By the use of eqn. (12), one thus finds the following contribution to conductance of the water ions at 25° and zero ionic strength:

$$\varkappa_w(7) = 0.0542 \times 10^{-6} \text{ ohm}^{-1} \text{ cm}^{-1} \qquad (14)$$

This contribution is too small to require any consideration in the measurements to be described.

Already at pH 6, the conductance contribution of the OH^- ions can be neglected. For that and lower pH values, therefore, one arrives at the following general formula:

$$\varkappa_w(\text{pH}) = 0.35 \times 10^{-\text{pH}} \qquad (\text{pH} < 6) \qquad (15)$$

On the other hand, above pH 8.5, the contribution of the H^+ ions is negligible, and one finds:

$$\varkappa_w(\text{pH}) = 1.92 \times 10^{\text{pH}-15} \qquad (\text{pH} > 8.5) \qquad (16)$$

The equations still refer to zero ionic strength, although this is in itself a contradiction at low and high pH values.

Conductance measurements. In order to test at least semiquantitatively the validity of the theoretical predictions presented above, some conductance measurements have been carried out. A fully quantitative verification would require a more elaborate theory and extensive and careful measurements at varying concentrations on well purified preparations. For the present purpose of finding suitable carrier ampholytes for isoelectric separation of proteins, a semi-quantitative check on the equations is adequate.

Consequently, commercially available ampholytes of analytical grade were in general used without further purification. However, commercial lysine and histidine were found to be partially decomposed. Instead their monohydrochlorides were used, and their solutions were deprived of HCl by passing them through a column of an ion exchanger.

Table 2. The conductance of solutions of pure ampholytes at 25°C.

No.	Ampholyte	Concentration, μmoles/ml	Conductance, μmhos/cm	pH
1	Nicotinic acid	154	527	3.50
2	Isonicotinic acid	16.34	131	3.51
3	Picolinic acid	100	227	3.34
4	*o*-Aminobenzoic acid	54.7	183	3.61
5	*m*-Aminobenzoic acid	54.6	446	3.88
6	*p*-Aminobenzoic acid	36.0	209	3.69
7	*o*-Aminophenylarsonic acid	9.22	432	3.02
8	*p*-Aminophenylarsonic acid	13.04	311	3.17
9	Aspartic acid	56.3	653	2.95
10	Glutamic acid	100	595	3.34
11	Histidine	88.55	172	7.81
12	Lysine	358	2 146	9.96
13	Arginine	100	200	10.84

If possible, the ampholytes were dissolved in distilled water to a concentration of 0.1 M. Slightly soluble ampholytes were dissolved to saturation, and the concentrations were measured by weighing 2 ml samples before and after evaporation of the solvent. The lysine and histidine solutions emerging from the ion exchange column were measured directly, and their concentrations were determined in the same way. The conductances were measured at 25°C on an LKB precision Wheatstone bridge. The pH of each solution was also measured. The results of these measurements are presented in Table 2.

Calculation of conductances. Using the mobility data in Table 1 and the ion concentrations according to eqn. (8), one can now derive theoretical conductances of isoionic ampholytes. The calculation will be explained below with reference to Table 3. For space reasons, the first column of that table contains only a number; the corresponding name of the ampholyte can be found in Table 2. The second column gives the concentration of the solution subjected to conductance measurement. In the third column, the distance from the isoionic point to the nearest pK value is given. The fourth column contains the degree of ionization in the isoionic state, calculated with the aid of eqn. (8). The product of total concentration, degree of ionization, and mean equivalent conductance of cation and anion (Table 1) is the conductance contribution of the ampholytic ions, column 5. From the measured pH data given in Table 2, the contribution of the solvent ions can be calculated by using eqns. (15) or (16), column 6. The figures in column 7 are the sum of those in columns 5 and 6 and represent the conductances of the isoionic solutions that would be expected if the mobility data for infinite dilution could be used at the prevailing concentrations. This is of course not the case, so a correction for the depression of mobilities due to a finite ionic strength must be carried out.

A theoretical correction for the ionic strength effect by Onsager's equation has not been attempted. Instead, the table on pp. 141—42 in Conway's [11] book over the concentration dependence of equivalent conductances of various salts has been studied. Among the mono-mono-valent salts listed there, the two

Table 3. Calculation of theoretical conductances for ampholytes from known concentrations, calculated ionic concentrations, and probable mobilities.

Ampholyte No.	Concentration, μmoles per ml	pI-pK_1	α	$\varkappa_{amph.}$	$\varkappa_w$	$\varkappa$	Ionic strength	Factor	$\varkappa_{corr.}$ μmhos per cm
				μmhos/cm					
1	154	1.37	0.0786	422	111	533	0.00620	0.93	496
2	16.34	1.51	0.0681	39	108	147	0.00071	0.976	143
3	100	2.15	0.0140	49	160	209	0.00093	0.972	203
4	54.7	1.47	0.0635	116	86	202	0.00186	0.96	194
5	54.6	0.81	0.2367	432	46	478	0.00653	0.93	445
6	36.0	1.30	0.0911	110	72	182	0.00174	0.96	175
7	9.22	0.77	0.2532	72	334	406	0.00164	0.962	391
8	13.04	0.92	0.1938	78	236	314	0.00160	0.962	302
9	56.3	0.89	0.2049	372	393	765	0.00632	0.93	711
10	100	1.03	0.1574	482	160	642	0.00810	0.923	592
11	88.55	1.50	0.0595	165	0	165	0.00217	0.956	158
12	358	0.79	0.2448	2 542	17	2 559	0.04385	0.845	2 162
13	100	1.72	0.0367	108	133	241	0.00218	0.956	230

organic salts sodium acetate and sodium butyrate show the strongest concentration dependence. It is therefore possible that the big organic ions in question here have mobilities that are still more concentration-dependent; this would also be in agreement with Onsager's equation. On the other hand, a considerable part of the total conductance is due to H^+ or OH^- ions, which have a small concentration dependence. It is therefore probable that the use of ionic strength factors valid for sodium butyrate will yield conductance corrections of the right order of magnitude; most corrections are also quite small, of the order of 5 %.

The ionic strength of a solutions of a pure ampholyte is identical with the concentration of anions for an acidic and with the concentration of cations for a basic ampholyte. It is obtained by adding the hydrogen or hydroxyl ion concentration to the total concentration of ampholytic ions in the isoionic state, and dividing the sum by 2. These data are given in column 8, and the mobility depression factors pertaining to these ionic strengths are listed in column 9. Column 10, finally, gives the theoretical conductances corrected for the ionic strength effect. They are to be compared with the experimental data in column 4 of Table 2.

RESULTS AND DISCUSSION

As can be seen in Table 3, the degree of ionization varied from 1.40 to 25.32 % among the ampholytes subjected to conductance measurements. The total conductance varied between the limits 143 and 2 162 μmhos/cm. The relative contribution of the solvent ions varied from 0 for histidine to 82.3 % for *o*-aminophenylarsonic acid. In spite of these more than 10-fold variations in the properties of the ampholytes, the calculated conductances lie, with two exceptions, within ± 10 % of the measured ones. In view of the uncertainty

Table 4. Possible carrier ampholytes arranged in the order of increasing isoionic points.

Ampholyte	pI	pI-pK_1
Aspartic acid	2.77	0.89
Glutathion	2.82	0.70
Aspartyl-tyrosine	2.85	0.72
o-Aminophenylarsonic acid	3.00(?)	0.77(?)
Aspartyl-aspartic acid	3.04	0.34
p-Aminophenylarsonic acid	3.15(?)	0.92(?)
Picolinic acid	3.16	2.15
L-Glutamic acid	3.22	1.03
β-Hydroxyglutamic acid	3.29	0.96
Aspartyl-glycine	3.31	1.21
Isonicotinic acid	3.35	1.51
Nicotinic acid	3.44	1.37
Anthranilic acid	3.51	1.47
p-Aminobenzoic acid	3.62	1.30
Glycyl-aspartic acid	3.63	0.82
m-Aminobenzoic acid	3.93	0.81
Diiodotyrosine	4.29	2.17
Cystinyl-diglycine	4.74	1.62
a-Hydroxyasparagine	4.74	2.43
α-Aspartyl-histidine	4.92	1.90
β-Aspartyl-histidine	4.94	2.00
Cysteinyl-cysteine	4.96	2.31
Pentaglycine	5.32	2.27
Tetraglycine	5.40	2.35
Triglycine	5.59	2.33
Tyrosyl-tyrosine	5.60	2.08
Isoglutamine	5.85	2.04
Lysyl-glutamic acid	6.10	1.65
Histidyl-glycine	6.81	1.00
Histidyl-histidine	7.30	0.50
Histidine	7.47	1.50
L-Methylhistidine	7.67	1.19
Carnosine	8.17	1.34
α,β-Diaminopropionic acid	8.20	1.40
Anserine	8.27	1.23
Tyrosyl-arginine	8.38(?)	1.00(?)
	8.68(?)	1.13(?)
L-Ornithine	9.70	1.05
Lysine	9.74	0.79
Lysyl-lysine	10.04	0.59
Arginine	10.76	1.72

of the mobility values, the simplified theory and calculation, and the possibility of impure preparations, the discrepancies are not surprising. Although one cannot speak about a quantitative verification, the measurements nevertheless show that the quantity pI-pK_1 is of a decisive importance for the conductance of isoionic ampholytes. The fact that the same quantity appears in the expression for the buffering capacity, eqn. (11), leads to the conclusion that this quantity, beside the solubility, is the only property that requires consideration in the choice of carrier ampholytes in stationary electrolysis. The smaller the value of pI-pK_1, the more effective is the ampholyte as a carrier.

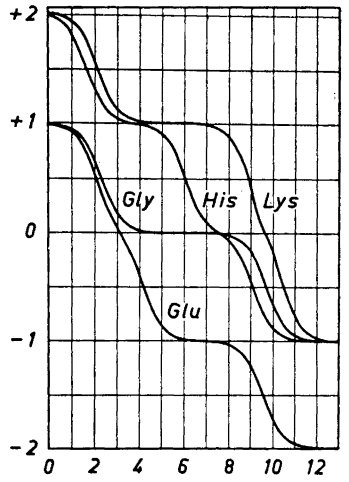

Fig. 2. Titration curves of glutamic acid, glycine, histidine, and lysine. The three amino acids with sharp isoionic points are useful as carrier ampholytes, while glycine, with its extended horizontal part of the curve, is useless.

From pK data available in the monographs of Cohn and Edsall[16], Conway[11], Sahyun[17] Kortüm, *et al.*[18] and in other sources[3–5], a list of possible carrier ampholytes, arranged in the order of increasing isoionic points, has been prepared in Table 4. Ampholytes with pI-pK_1 values bigger than 2.5 units have been excluded as completely useless; to this category belongs the whole group of neutral amino acids. Compounds with pI-pK_1 values between 1.5 and 2.5 units are included, but must be regarded as poor carriers.

The usefulness of an ampholyte as a carrier can also be judged directly from its titration curve. In Fig. 2 the titration curves of glutamic acid, glycine, histidine, and lysine are presented. Good carriers are those with sharp isoionic points. Useless is glycine, which is isoionic over a pH region covering almost four units.

A closer inspection of Table 4 reveals the crucial difficulty in the creation of natural pH gradients suitable for work with proteins. Between pH 3.9 and 7.5 there is only the almost insoluble diiodotyrosine and a number of peptides, either very expensive or not at all on the market. Unfortunately, this is the most important pH range for many proteins. It is thus necessary for continued work along this line to synthesize or to supply by other means new low-molecular ampholytes isoelectric between pH 4 and 7 and with the desired buffering and conductive capacities. The appearence of a number of peptides containing histidine in this region of the table gives a hint of possible ways to solve this problem. Partial hydrolysis of a histidine-rich protein (hemoglobin or globin) can be expected to give a mixture of various peptides containing this amino acid. Experimental work in this direction has been started. The results seem promising and will be dealt with in a forthcoming article.

The data in Table 1 raise some interesting physico-chemical problems. The absence of mobility data for ampholytes in the chemical literature is puzzling, and it appears as very desirable to design moving boundary systems allowing accurate measurements. In particular, it would be most interesting to see if

the big difference in mobility between cation and anion, predicted in Table 1 for many ampholytes, is real. This difference originates in the heavy hydration of carboxylate groups assumed by Edward in order to account for the comparatively low mobilities observed for carboxylate ions. Such a mobility difference, if substantiated by experiments, would results in a marked difference between isoionic and isoelectric points for the ampholytes in question. Such a difference is well known for proteins, but depends on complex formation with other ions than protons.

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