Journal of Chromatography, 190 (1980) 275–282
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CHROM. 12,521

MOLARITY AND IONIC STRENGTH OF FOCUSED CARRIER AMPHOLYTES IN ISOELECTRIC FOCUSING

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(Received November 19th, 1979)

SUMMARY

Focused 1% carrier ampholytes in the pH range 3.5–10 have a molarity of 9–10 m*M*, as determined by osmolarity measurements of fractions focused in free water. From electrophoretic and isoelectric focusing data of red blood cells, it has been demonstrated that the corresponding ionic strength is 0.5 mg-ions/l. Also, theoretical considerations and conductivity measurements point to a value in the range of 0.5–1.0 mg-ions/l. The following equations for ionic strength (I) calculations have been derived:

$$I = 1/20\,\bar{C}_{amph} + C_H$$

in the pH range 2.5–7 and

$$I = 1/20\,\bar{C}_{amph} + C_{OH}$$

in the pH range 7–11.

INTRODUCTION

Usually, when performing electrophoresis, the physico-chemical parameters that define the buffer medium, *i.e.*, its pH, molarity and ionic strength (I), are known by the experimenter. This is very important, as it enables one to reproduce the same data at any given time and place. With the advent of isoelectric focusing (IEF), things have considerably changed. The only parameter that can be measured with certainty after an IEF experiment is the pH, whose course is easily followed both in sucrose density gradients and in gels[1]. The exact molarity and ionic strength of focused carrier ampholytes are impossible to establish. This has created much confusion, especially when performing IEF of cells, as their measured isoelectric point (pI) is a function of the environmental ionic strength[2]. Moreover, if the molarity and ionic strength of focused carrier ampholytes are unknown, any comparison between electrophoretic and IEF data is unrealistic. Notwithstanding the fact that IEF has been available for about 14 years, no-one has been able to solve this problem, not even the theoreticians who described the basic equations of present day IEF[3,4].

While working on a totally different project (space research at the NASA Marshall Space Flight Center), I found out that, partly from the data we had obtained and partly from literature data, we had in fact an answer to such problems.

This paper presents these data, which I regard not as the final answer to the problem, but as a first approach to it, with the hope that other workers will be stimulated to study this topic further.

RESULTS AND DISCUSSION

Molarity of focused carrier ampholytes

Fig. 1 shows the pH gradient, conductivity and milliosmolarity profiles of focused 1% carrier ampholytes in the pH range 3.5–10. As these data were obtained in a free liquid curtain, in the Hannig apparatus, the osmolarity contribution to each eluted fraction must be due solely to the presence of focused Ampholine species. As each carrier ampholyte molecule, upon dissolution in water, does not dissociate into several, independent ionic species, but gives rise to a single, polyprotic species, the osmolarity measured must be identical with the molarity of the Ampholine solution. Thus, a 1% focused carrier ampholyte solution has a molarity of 9–10 m*M*. Notice that the molarity profile throughout the gradient (except at the two extremes, where contributions from anolyte and catholyte solutions must be taken into account) is very smooth, with variations of only 10%, suggesting that the various carrier ampholyte species (according to a recent investigation[6,7] there are about 600 different amphoteric species buffering in the pH range 2.5–11) are present in a remarkably

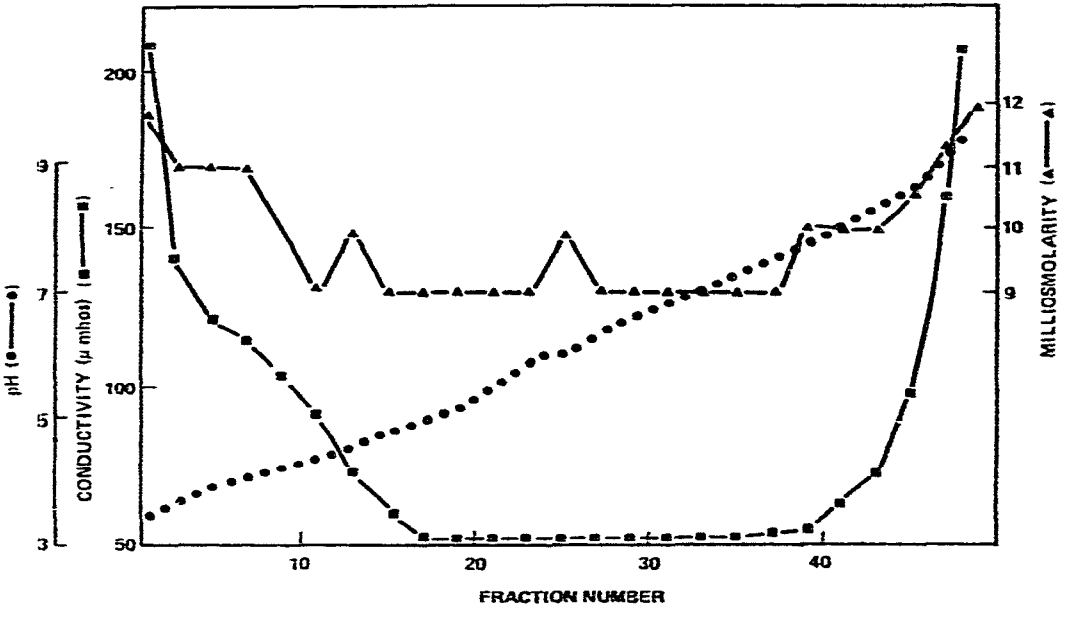

Fig. 1. pH gradient (●), conductivity (■) and osmolarity (▲) profiles of focused 1% LKB Ampholine, pH range 3.5–10, in the Hannig apparatus (Desaga FF48 free-flow electrophoresis cell). Conditions: anolyte, 5% acetic acid; catholyte, 1.5% ethanolamine; field strength (at equilibrium), 100 V/cm; elution rate, 1 ml/fraction/h. The osmolarity was measured with an Osmette Apparatus from Precision Systems, Sudbury, Mass., U.S.A. and the conductivity with a Model 31 conductivity bridge from Yellow Springs Instruments (Yellow Springs, Ohio, U.S.A.) (unpublished experiments reported by McGuire *et al.*[5]).

constant concentration over the whole pH range investigated. Moreover, at least within our resolution limits there do not appear to be any major gaps among adjacent species of focused Ampholine, in agreement with theoretical considerations[8,9] which predict strong interdigitation among the (Gaussian or quasi-Gaussian) distribution of focused carrier ampholytes. As we are eluting 48 fractions from a separation distance in the cell of 7 cm, this represents intervals of 1.46 mm from one zone to the next. Whether or not discontinuities exist over much shorter increments, in the micrometre range, remains to be seen. In any event, it is clear that what we are seeing does not represent the distribution profile of a single, focused Ampholine molecule, but of clusters thereof. On average, it can be stated that, given the number of carrier ampholytes (about 500 in the pH range 3.5–10) and assuming a fairly even distribution along the pH gradient, no less than ten individual amphoteric species should be present in each collected fraction.

How valid is the value of 9–10 m*M* calculated for focused 1% Ampholine? There is other evidence that suggests that this value is accurate. Thus, Gelsema *et al.*[10], on the basis of an average molecular weight for Ampholine of 700 daltons, as determined by gel filtration and osmotic measurements[11], have calculated a molarity for a 1% solution of 15 m*M*. This figure, even though more than 50% higher than that given above, is nevertheless of the same order of magnitude. That the value of 15 m*M* cannot be the correct one is implicit in the fact that the molecular weight is an average estimate, accounting for the presence of species of even higher molecular weight (up to 4000 and above[12]) and for the uneven distribution of molecular weights along the pH gradient. Both Gelsema *et al.*[13] and we[14] have recently demonstrated that acidic carrier ampholytes have an average molecular weight considerably higher than basic ampholytes.

Other evidence comes from Sherbet's book[2]. In a footnote to Table 38 on p. 178 he talks in terms of 9 m*M* Ampholine, which corresponds to the figure given above. However, as he refers to a substantial amount of unpublished work, it is difficult to establish how he measured this value. Moreover, in the footnote to Table 62 on p. 227, he gives the ionic strength of focused carrier ampholytes as 9–10 m*M*, which suggests that he might have confused molarity with ionic strength, two parameters which, in isoelectric focusing, are unrelated (see below).

More data can be extracted from the literature if we consider the buffer capacities of 1% focused Ampholine as given by Davies[15] and Fredriksson[16]. In the pH range 5–8 both authors find a constant minimum of buffering power, centred around 6 mequiv./l (ref. 15) or 6–7 mequiv./l (ref. 16) (incidentally, the same minimum in the same pH range can be seen in Fig. 1 in this paper). As these values were obtained by titrating only for 1 pH unit around the p*I* values of the focused carrier ampholytes, this probably gives no more than 70% of the total amount of buffering ion. If they are increased by 30%, we obtain values of 9–10 mequiv./l. In the present work the value obtained is 9–10 milliosmoles/l (*i.e.*, 9–10 m*M*), the two sets of data could agree if we assume that within the pH range 5–8 each carrier ampholyte focuses close to only one buffering p*K* in its molecule. At pH 4 and below and at pH 9 and above the buffering capacity becomes 2–3 times greater, while the osmolarity remains constant at 9–10 milliosmoles. This could mean that at these pHs each Ampholine molecule possesses 2–3 (or more) buffering groups close to its p*I*. This can be easily verified for acidic carrier ampholytes[14], owing to the presence of several

carboxyl groups in the molecule; it is less obvious for the alkaline species, unless it is assumed that their polyamino backbone is considerably longer than 6 nitrogen atoms (perhaps 10 or more), thus allowing for closely spaced pK values in the pH range 9–11.

Ionic strength of focused carrier ampholytes

In theory, the ionic strength of isoelectric ampholytes should be zero, because their transferance number at pH = pI is zero[17], and therefore their conductivity also should be zero, as well as their mobility, according to the equation

$$\kappa = F \Sigma c_i z_i u_i$$

where κ is the conductivity, F the Faraday constant, c_i the individual molar concentrations of the ion constituents, z_i their valencies and u_i their mobilities[18]. This can be easily demonstrated. For instance, if we add to a solution of 1% carrier ampholyte, at pH $\approx$ 6, up to 0.5 M glycine, taurine or trimethylaminopropane sulphonate, the conductivity increments measured with a conductimeter are almost zero. Moreover, even when running an IEF experiment in the presence of the same amounts of these three amphoteric species, in the appropriate pH ranges, the milliampere readings in the power supply are the same as in the presence of Ampholine alone[5]. On the other hand, experimentally the ionic strength of focused Ampholine, albeit vanishing small, is a finite quantity and as such should be amenable to measurement. It is reasonable to hypothesize that the ionic strength of the medium should be due to that fraction of Ampholine molecules found instantaneously outside their steady-state focused positions, a hypothesis which has also been suggested by Just *et al.*[19]. The direct and indirect evidence that has allowed this vanishing ionic strength to be calculated is considered below.

Cell focusing and ionic strength measurements. It is well known, from electrophoretic mobility data, that the "apparent" isoelectric point of red blood cells is strongly dependent on the ionic strength of the medium. Thus, while at 145 mg-ions/l the pI is 1.7 at 2 mg-ions/l the pI is 4.5 (see Table IV, p. 1192, in ref. 20). Fig. 2 shows plots of four of these pI data obtained by electrophoresis at different ionic strengths. The fifth value was measured by isoelectric focusing in 1% Ampholine by Just *et al.*[19] and by us[5]. In this last instance, we only know the measured pI of red blood cells but not the experimental ionic strength. By extrapolating the pI *versus* ionic strength curve to meet the IEF pI value, a corresponding I value can be read on the abscissa, corresponding to $I = 0.5$ mg-ions/l. Thus, it would appear that the ionic strength of focused carrier Ampholine is a measurable quantity, and would correspond to *ca.* one twentieth of its molarity value.

Theoretical considerations. Fig. 3A depicts a hypothetical concentration profile of a single carrier ampholyte in a focusing column along a pH gradient. Most (90–95%) of the molecules will exist as a "true" isoelectric species and therefore in this region (shaded area), the net charge being zero, the total mass of the focused species will contribute solely to the molarity or osmolarity of the medium, and not at all to its ionic strength. Having "zero electrophoretic mobilities", these molecules can diffuse away from their pI, because no force will counteract the diffusion process. However, as they diffuse along the pH gradient, at one point they will acquire a

fractional excess of positive or negative charge, so that they will move electrophoretically back toward their p*I* positions. These two regions, on the extreme sides of the Gaussian curve (dotted areas), will be regions of ionic strength, owing to the fractional excess of positive or negative charge on the amphoteric ion. These areas, under a steady state, will represent equilibrium conditions created by the balancing of the diffusional force and the electrophoretic mobility in the voltage gradient applied, *i.e.*, at equilibrium, the number of molecules entering the dotted zones by diffusion will be equal to the number leaving them and being forced back by the applied electric field into the molarity (osmolarity) region. Assuming that, at any given time, the number of molecules in the two dotted areas is never more than 5–10% of the total, we can derive for 1% focused Ampholine an ionic strength of 0.5–1.0 mg-ions/l. This ionic strength can only be valid between pH 4 and 10, because it neglects the contributions of H^+ and OH^-, which are present at a given level (dictated by pH) within each focused Ampholine zone. Within this pH range it can be neglected but, for instance, at pH 3, the concentration of H^+ (1 mg-ion/l) would be the same as or higher than the ionic strength in this zone due to focused carrier ampholytes. The situation shown in Fig. 3A is unreal, as it would allow large conductivity or ionic strength gaps to develop along a focusing column. In reality, as we deal with a multitude of carrier ampholytes, under a steady state their distribution will considerably overlap[8,9], giving rise to a continuous (and uniform) ionic strength background throughout the separation column (Fig. 3B).

From theoretical considerations[3,4] and experimental data[5], the minimal fractional excess of positive or negative charge needed to move electrophoretically an amphoteric molecule back towards its p*I* position ca n be calculated. For example, when focusing cells in presence of 300 m*M* glycine[5] in the pH range 3–10, no glycine could be found below pH 4 or above pH 8. At pH 4, the carboxyl group of glycine will be 5% protonated, leaving a 5% excess of positive charge on the molecule, which then moves back electrophoretically towards its p*I*. The same applies at pH 8, this time via deprotonation of the amino group.

Conductivity measurements. Is it possible to extrapolate ionic strength data from conductivity measurements? According to Rilbe (personal communication), as the ionic strength in IEF is too small, an IEF system should be defined only through conductivity, rather than I values. It is well known that the conductivity is proportional to the degree of ionization, α, defined as

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

where C_+ and C_- are the concentration of cationic and anionic forms, respectively, and C is the total concentration, $C_+ + C_- + C_0$ (undissociated or zwitterionic or both forms). It can also be demonstrated that α_i is correlated to the buffering capacity, b_i, by the equation[18]

$$\alpha_i = b_i/4$$

In our treatment, as we have assumed that the conductivity region is coincident with the ionic strength region, owing to the fractional excess of positive or negative charges in the amphotere*, then the α_i value should give directly the ionic

* "Amphotere" = Amphoteric molecule.

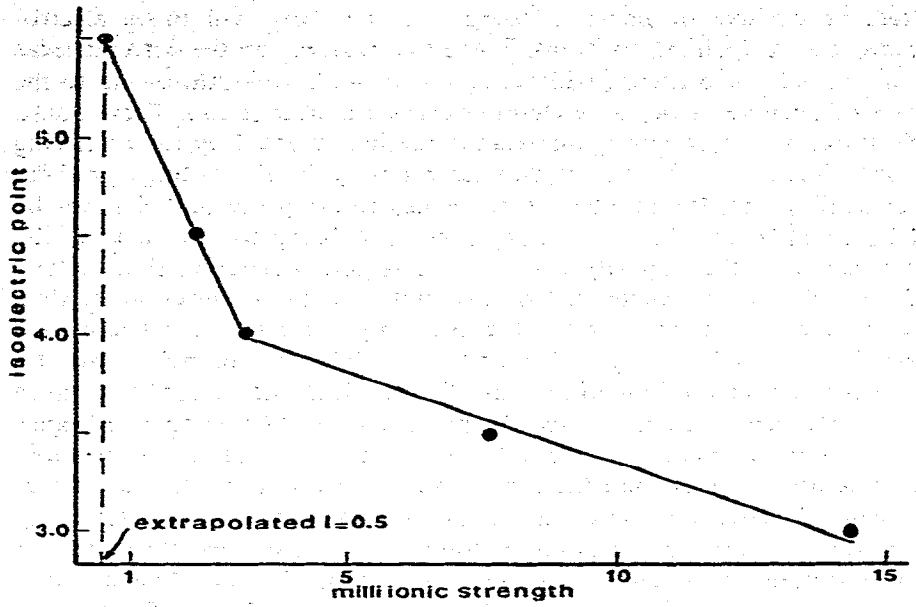

Fig. 2. Dependence of the isoelectric point of red blood cells on the ionic strength of the medium. The first point on the left was obtained from the IEF experiments of Just *et al.*[19] and McGuire *et al.*[5], and the other four points are from electrophoretic data tabulated by Seaman[20]. The ionic strength in IEF is the missing coordinate value of the first point of the curve.

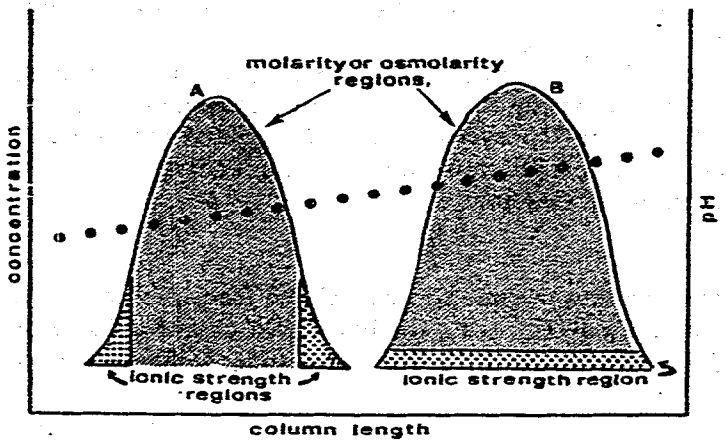

Fig. 3. Hypothetical distribution of a single amphoteric species in (A) the absence and (B) the presence of a series of carrier ampholytes in a pH gradient generated in a focusing column. The amphotere concentration profile is divided into two zones: a molarity or osmolarity region (shaded area, 90–95% of the total) of zero ionic strength, and a region of ionic strength (dotted area, 5–10% of the total) due to molecules diffusing away from the p*I* zone. In B the ionic strength zone is continuous, due to interdigitating Ampholine species, whereas in A there are two discontinuous zones on the side of the Gaussian. The slanted line of black dots is the pH gradient.

strength of the solution. From the data in refs. 15 and 16 ($b_t = 6$ mequiv./l), we can thus calculate an ionic strength of 1.5 mg-ions/l, which is close to the 0.5–1.0 I estimated in this paper.

From the above, it is clear that the classical definition of ionic strength (I) of Lewis and Randall[21]:

$$I = [\Sigma c_i z_i^2]^{\frac{1}{2}}$$

is no longer applicable to the conditions found in isoelectric focusing. The following equation was given by Gelsema *et al.*[10]:

$$I \leqslant 1/3\ \bar{C}_{\text{amph}} + 1/2\ C_{\text{H}}$$

which was stated to have been taken from Rilbe[18]. However, in fact there appears to be no trace of this equation in Rilbe's chapter in Catsimpoolas' book. In any event, as Gelsema *et al.*[10] obtained from this equation an ionic strength of 5 mg-ions/l, it is clear that this value is too high by a factor of 5–10. I propose the following equations:

$$I = 1/20\ \bar{C}_{\text{amph}} + C_{\text{H}}$$

in the pH range 2.5–7 and

$$I = 1/20\ \bar{C}_{\text{amph}} + C_{\text{OH}}$$

in the pH range 7–11,

where $\bar{C}_{\text{amph}}$ is the molarity of the focused carrier ampholytes and C_{H} and C_{OH} the molarities of protons and hydroxyl ions, respectively, at a given pH.

However, the following factors have been neglected here:

(a) dipole moments, which might generate a fractional charge difference even in purely isoelectric regions (shaded areas in Fig. 3A and B);

(b) distance of the charges in the amphoteric (or polyprotic) molecules; according to Rilbe (personal communication), if the two charges in the amphotere are close (*e.g.*, glycine) at pH = pI their contribution to conductivity and ionic strength is zero, but if they are far apart in the molecule, they might behave as partially independent charges, thus contributing to some extent to conductivity and ionic strength;

(c) "poor" versus "good" carrier ampholytes. According to Bjellqvist (personal communication), the model in Fig. 3 applies only with "poor" carrier ampholytes (pI—pK>2 or ΔpK=6 or greater), while "good" species (pI—pK<1.5 or ΔpK<3) should contribute to conductivity even at their pIs. However, this may not be correct, and the difference in behaviour between "good" and "poor" amphoteres may lie mostly in the width of their gaussians about the pI value. "Poor" amphoteres will have very wide Gaussian distributions, while "good" ones will exhibit very narrow distributions, but only that fraction of the molecules that acquires an excess of fractional charge, owing to diffusional movement away from the pI zone (dotted regions in Fig. 3A and B) will, in both instances, contribute to ionic strength.

In conclusion, it has been demonstrated that 1% focused carrier ampholytes

in the pH range 3.5–10 have a molarity (osmolarity) of 9–10 m*M*. Their total ionic strength, however, unlike the electrophoresis, is only 5% of the total molarity, being 0.5 mg-ions/l. Theoretical considerations and conductivity measurements also indicate a value in the range 0.5–1.0 mg-ions/l.

ACKNOWLEDGEMENTS

I am deeply indebted to Mrs. Janice McGuire for performing the experiments illustrated in Fig. 1. Drs. H. Rilbe, B. Bjellqvist, H. Davies, A. Winters, H. Haglund, K. Ek and E. Gianazza have greatly contributed to this paper through stimulating discussions and criticism. This work was supported in part by grants 78.02249.04 and 78.01533.06 from Consiglio Nazionale delle Ricerche (CNR, Rome, Italy).

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