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NEW EXPERIMENTAL APPROACHES TO THE ISOELECTRIC FRACTIONATION OF CELLS

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SUMMARY

Several parameters that could affect the survival rate and the resolution of cells in continuous-flow isoelectric focusing have been investigated. Cell survival at progressively lower pHs was found to be a function of the osmolarity of the medium: at pH 4 there was an absolute requirement for a 300 mOsm environment. A pH-dependent interaction of the carrier ampholytes with the cell surface has been demonstrated. The binding was very strong at pH 4, weak at pH 5 and totally abolished above pH 5. The same interaction was obtained with pentaethylenehexamine, the polyamino backbone of carrier ampholytes, stripped of carboxyl groups. A model was derived showing how at pH 4 the oligocationic carrier ampholytes, via their nitrogen groups, would bind to the polyanionic cell surface.

The Hannig apparatus was adapted for cell separation in a continuously flowing curtain of isoelectric ampholytes. The medium osmolarity was maintained with glycine (ΔpK 7.2), taurine (ΔpK 8.5) and trimethylaminopropionyl sulphonate (TMAPS, ΔpK 11). With increasing ΔpK, the pH range that can be created in the presence of these compounds progressively widens, from a pH 4.3–7.5 range with glycine, to pH 3.5–7.5 with taurine up to pH 3.5–9.5 with TMAPS. The 48 fractions collected were routinely assayed for conductivity, osmolarity and pH.

INTRODUCTION

Isoelectric focusing (IEF) of cells was first introduced by Sherbet and co-workers[1–3] in 1972, by using stationary, linear gradients of sucrose (10–55%), glycerol (10–70%) or Ficoll (5–30%), containing 1% Ampholine, in a standard LKB 110-ml column. In these early systems, the cells were subjected to the IEF process for as long as 30 h. Subsequently, Just and co-workers[4–8] introduced the method of continuous-flow IEF separation of cells, in a modified Hannig apparatus. In this system, the residence time of the cells in the apparatus was only 7–10 min.

Cell focusing has also been described in several other reports. Leise and

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LeSane[9] reported the application of IEF to the separation of peripheral lymphocytes of human and rabbit origin in a gradient of dextran 40; Hirsch and Gray[10] described a similar separation of rat peripheral lymphocytes in dextran gradients in isotonic sucrose; Boltz *et al.*[11] reported the IEF separation of Chinese hamster fibroblasts in a linear Ficoll density gradient made isotonic throughout by sucrose and glucose; Manske *et al.*[12] have described the IEF analysis of Ehrlich–Lettré mouse ascites tumor cells, as well as rat hepatocytes, in Ficoll–sucrose gradients; even spermatozoa from boars have been isolated by IEF in a Ficoll gradient by Moore and Hibbitt[13]. In the field of prokaryotic cells, the IEF of bacterial cells has been reported by the Sherbet group[14] and by Langton *et al.*[15]. According to Talbot[16], viruses also seem to be amenable to isoelectric fractionation.

Notwithstanding this large body of experimental data, isoelectric focusing of cells is still not established. For instance, Catsimpoolas and Griffith[17], upon focusing mouse spleen lymphocytes in a Ficoll–sucrose gradient, have reported that as soon as the cells are focused they start to defocus and are finally lysed. They hypothesize that, in the p*I* region, changes in the membrane occur that could alter the p*I* towards a more acidic value so that the cells have to seek a new p*I* position. This may occur repetitively until extensive damage to the membrane causes lysis of the cells. It is a fact that, even though many workers[10,12] have claimed high cell viabilities (up to 90%) after isoelectric focusing, as measured by dye exclusion tests, in reality viabilities in terms of plating efficiencies are usually low (10–20%)[11], which has led many workers to suspect that carrier ampholytes might have some degree of cytotoxicity. Therefore, the question of whether membrane stability and hence cell viability can be maintained under electrofocusing conditions should be further critically analysed. There also remains the question of whether the measured cell p*I*s are "true" isoelectric points, representing a balance between positive and negative groups in the cell surface. For instance, the p*I* of *E. coli* is given as 5.6 (ref. 14), the same as the p*I* of red blood cells (RBCs)[4]. However, in *E. coli*, the ratio of negative to positive charges is 2:1 ($2.889 \cdot 10^{13}$/cm^2 carboxyl groups *versus* $1.4334 \cdot 10^{13}$/cm^2 amino groups)[14], while in RBCs, the ratio of negative to positive groups is 25:1 (ref. 18), *i.e.*, enormously higher. How these two cell types can exhibit the same p*I* remains a mystery.

Additionally, three other parameters that further increase the uncertainty of the data should be considered: (a) at the steady state, focused carrier ampholytes represent a medium of unknown and very low "ionic strength"; (b) ampholytes for IEF are capable of chelating doubly positively charged metal ions[19]; (c) they have been demonstrated to form complexes with polyanions, such as nucleic acids[20] and sulphated and carboxylated polysaccharides[21–23], including polyglutamate and polyaspartate[22].

All of these considerations have led us to explore further cell IEF and to propose some explanations for a few observed phenomena and some alternative experimental approaches.

EXPERIMENTAL

Materials

Ampholine carrier ampholytes were obtained from LKB (Bromma, Sweden), glycine and taurine from Serva (Heidelberg, G.F.R.), sucrose, analytical-reagent

grade, from Merck (Darmstadt, G.F.R.) and pentaethylenehexamine (PEHA) from Hoechst-Italia (Milan, Italy). Trimethylaminopropane sulphonate (TMAPS) was a kind gift from Drs. L. M. Hjelmeland and A. Chrambach, N.I.H., Bethesda, MD, U.S.A.

Continuous-flow IEF

The Desaga FF48 free-flow electrophoresis apparatus designed by Hannig was used for the IEF experiments. This apparatus has a 0.15 × 70 × 480 mm separation chamber with 410-mm long electrodes housed on each side of the chamber. Ion-exchange membranes isolate the electrode chambers from the separation chamber. Each electrode has a rinse pump and reservoir. A pump at the base of the separation chamber draws the fluid through the chamber into 48 fractions, collected in cooled tubes. The separation chamber is cooled from front and back by a separate circuit of fluid. The anolyte was 5% acetic acid and the catholyte 1.5% ethanolamine[4]. In two experiments EDTA, either 1 or 5 m*M*, was added to the cathode rinse. The chamber fluid was a 1% Ampholine solution, pH 3.5–10, with 0.1 m*M* EDTA. To this standard solution was added 250 m*M* sucrose, 300 m*M* glycine, 300 m*M* taurine or 300 m*M* TMAPS, depending on the experiment.

The pre-focusing procedure involved applying a 100 V/cm field gradient to the chamber filled with fluid, with cooling and electrode rinsing. A three-fold decrease in current followed by stability at that level for a minimum of 10 min was used as the criterion for focusing. The chamber pump then drew additional fluid through at the rate of 50 ml/h or approximately 1 ml per fraction per h.

Osmolarities were checked with an Osmette A apparatus from Precision Systems (Sudbury, MA, U.S.A.), using potassium chloride standards.

Conductivities were measured with a Model 31 conductivity bridge from Yellow Springs Instrument Co. (Yellow Springs, OH, U.S.A.) pH was determined with a PHM64 Research pH meter from Radiometer (Copenhagen, Denmark).

Cell survival studies

These studies were performed by incubating the cells (usually RBCs freshly drawn from healthy adults) in 1% Ampholine solution, between pH 4 and 7, of different osmolarities ranging from 150 to 300 mOsm. A given osmolarity was obtained by dissolving in the Ampholine buffer either sodium chloride (2 mOsm ≈ 1 m*M*), glycine, taurine, TMAPS or sucrose (1 mOsm ≈ 1 m*M*). The various pHs of incubation were obtained by using narrow-range Ampholine, adjusted to a given pH, if needed, by mixing with a more acidic or a more alkaline pH range. Those pH and osmolarity values of the medium which did not produce more than 10% cell lysis over a period of 2 h at 4 °C, as determined by measuring the adsorption of the released free hemoglobin at 550 nm in a Beckman Acta CII spectrophotometer, were considered adequate for cell survival.

Microscope cell electrophoresis

Cell mobility measurements were performed with a Rank I microelectrophoresis apparatus from Rank Brothers (Cambridge, Great Britain), with the chamber thermostated at 25°C. The standard buffer was 0.15 *M* sodium chloride solution titrated to pH 6.95 with sodium hydrogen carbonate. When electrophoresis

was performed at lower pH values the sodium chloride solution with 0.1 m*M* EDTA was titrated to a given pH with citric acid (control runs) or with 1% Ampholine or 0.5% PEHA (with the polyamine, hydrochloric acid was added to the desired pH value).

RESULTS

As cells are driven to equilibrium in a pH gradient and are supposed to have an acidic p*I*, owing to their characteristic polyanionic surface, we first studied their survival as a function of the prevailing pH of the medium and of its osmolarity. It is well known that at pH 7 the cells can withstand a broad range of osmolarities, from 150 to 300 mOsm, without suffering any damage or irreversible modification. In Fig. 1, the shaded region represents pH and osmolarity conditions of the medium compatible with cell viability. Below pH 6, the osmolarity requirements narrow rapidly, until at pH 4 there is an absolute requirement for a 300 mOsm environment. Below pH 4, the cells rapidly lyse. It appears, therefore, that in the presence of carrier ampholytes, under conditions which strictly mimic those obtainable during

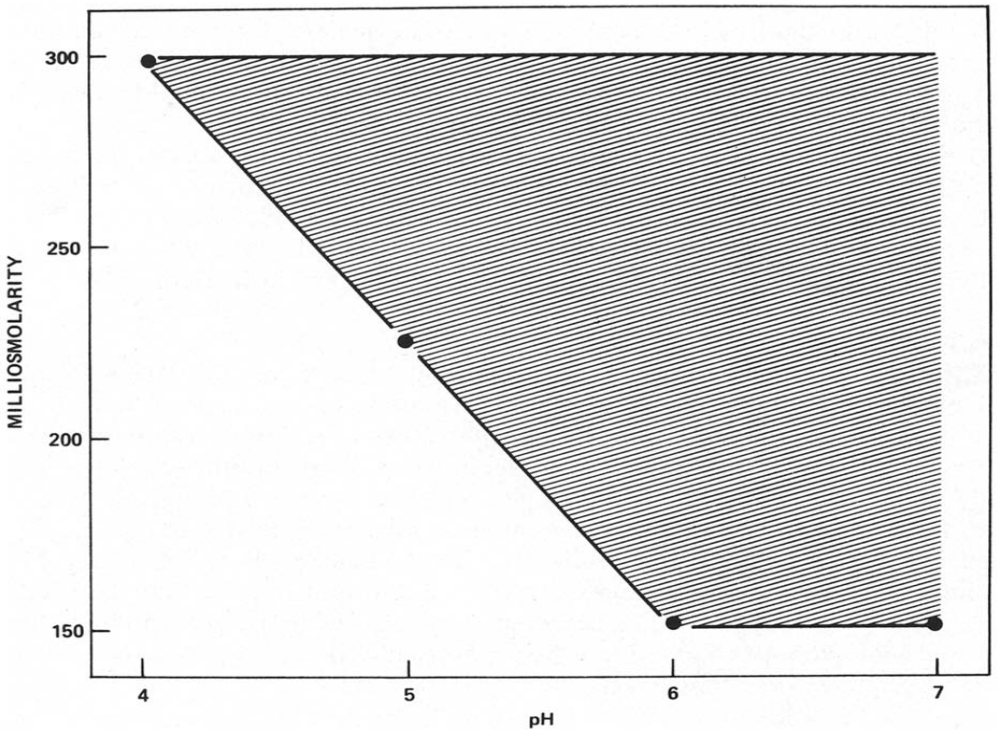

Fig. 1. Cell survival as a function of pH and osmolarity conditions of the medium. The shaded area represents a region of stability of the cells, enveloping those pH and osmolarity values which allow no more than 10% cell lysis upon incubation for 2 h at 4°C. Lysis was measured as the appearance of free hemoglobin in the supernatant of centrifuged red blood cells. The various pH values were obtained by using narrow-range 1% Ampholine. The medium osmolarity was given by sucrose, glycine, taurine or TMAPS.

an IEF experiment, intact red blood cells can be obtained even down to pH 4, provided that the proper osmolarity is maintained. The presence of Ampholine, *per se*, does not appear to alter the survival rate markedly, as essentially the same data are obtained when the pH of the solution is dictated by other buffering ions, such as citric acid (not shown). The data of Fig. 1 are in general agreement with similar data reported by Seaman (see Fig. 5 in ref. 44) on the electrokinetic behavior of erythrocytes as a function of bulk pH. In this last case, a region of metastability was observed between pH 3 and 4.5.

We next investigated the possibility of whether or not the carrier ampholytes could bind to the cell surface, thus generating artefactual p*I* values and possibly contributing to the suspected cytotoxicity. Just and Werner[7,8] have recently hypothesized a similar mechanism. We utilized electrokinetic measurements by microscope electrophoresis, as this experiment does not seem feasible by equilibrium IEF. As shown in Fig. 2A and B, when RBCs migrate electrophoretically in standard buffers (either hydrogen carbonate or citrate) in the pH range 4–7, their mobilities are essentially constant, indicating that there are no titratable groups within the surface of shear in this pH range. These data are in excellent agreement with the RBC titration curves of Seaman (Fig. 9 in ref. 24), which indicate a sharp drop in mobility below pH 4, with an extrapolated isoelectric point of 1.7. However, when the same mobility data are measured in presence of 1% Ampholine, it can be seen that at pH 5 and below there is a sharp drop in anodic mobility, as though negative charges were disappearing or positive charges were appearing on the cell surface.

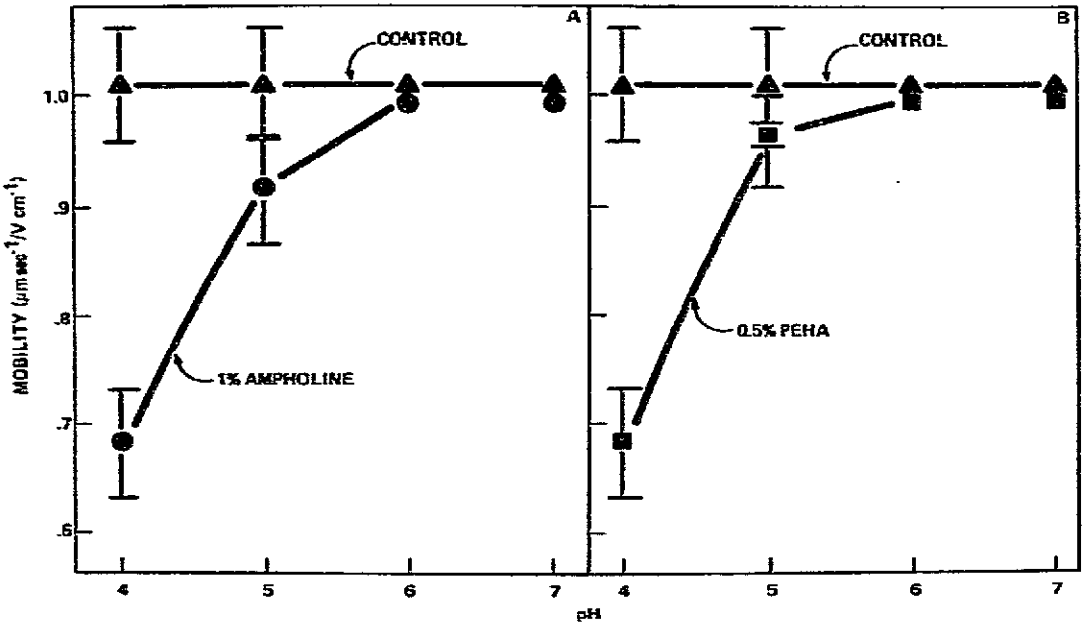

Fig. 2. Microscope electrophoresis of RBC incubated either in 1% Ampholine (A) or in 0.5% PEHA (B) at different pH values ranging from 4 to 7. The pH in the controls was obtained either with hydrogen carbonate (pH 7) or citrate (pH 4, 5 and 6). In all instances the electrophoresis buffer contained 150 m*M* sodium chloride. The vertical bars represent the standard deviation. Each point represents mobility readings of 50 different cells in opposite directions.

That these changes are brought about by direct interaction with carrier ampholytes has been demonstrated by performing the same experiment in the presence of PEHA, the polyamino backbone of carrier ampholytes, stripped free of carboxyl groups. As shown in Fig. 2B, there is a strikingly similar decrease in electrophoretic mobility, indicating this to be due to loss of negative charges from the cell surface, neutralized by the bound polyamine.

Fig. 3 depicts a hypothetical model of this interaction. A segment of a carrier ampholyte molecule is shown, exhibiting a stretch of four positively charged nitrogens (at pH 4) bound to four negative charges on the cell surface. It might be argued that because an acidic carrier ampholyte would probably have an equivalent number of carboxyl groups, there should not be a decrement in cell mobility, as the negative charges lost on the cell surface, upon binding to the polyamino stretch, would still be available as free carboxyls on the Ampholine molecule (here depicted oriented away from the cell surface). Actually, the electrokinetic behavior of RBCs seems to be due mostly to carboxyl groups of sialic acids (pK 2.6) and α-carboxyls of proteins (pK 2.35). On the other hand, the carboxyls of carrier ampholytes, being mostly β- and γ-groups[25], have a considerably higher pK (average p$K = 4$). By replacing strong carboxyls on the cell surface with the weak carboxyls of carrier ampholytes, the negative charge density is lowered because, at pH ≈ 4, about 50% of the carboxyls on the Ampholine molecule are protonated.

We subsequently investigated the possibility of performing the IEF of cells in media whose osmolarity is not due to the presence of sucrose, dextran or Ficoll,

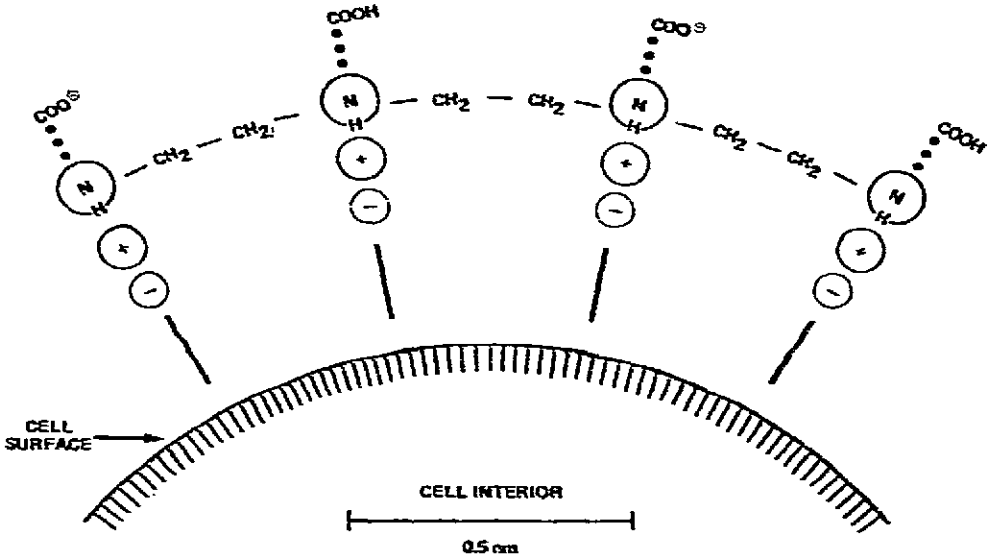

Fig. 3. Hypothetical model of the interaction of one Ampholine species with the cell surface. A segment of an Ampholine molecule, represented by a stretch of four protonated nitrogens, is depicted bound to four negative charges (possibly sialic acid residues) on the cell surface. The carboxyls in the carrier ampholyte are drawn facing away from the plasma membrane. At pH 4, these weak carboxyls (average p$K = 4$) would be 50% protonated. The curvature of the cell surface, over a distance of 1 nm, has been grossly exaggerated for easy visualization. In reality, over such a short distance, it should be almost planar.

compounds which usually have a high viscosity and would thus slow down the migration of charged particles toward their p*I*. We first ran a free curtain of 1% Ampholine alone, in the Hannig apparatus, in order to find the osmolarity contribution of focused carrier ampholytes to the medium osmolarity. Fig. 4 shows the pH gradient and conductivity and osmolarity profiles of focused 1% Ampholine, pH 3.5–10. It can be seen that the osmolarity profile is smooth and uniform throughout the pH gradient, and amounts to a low and constant value of 9–10 mOsm. This is an interesting finding, as it allows the calculation of the molarity of focused carrier ampholytes, a figure which has not previously been available in the literature. In fact, as each carrier ampholyte molecule, upon dissolution in water, does not dissociate into several independent ionic species, but gives rise to single polyprotic species, the osmolarity measured here must be coincident with the molarity of the Ampholine solution.

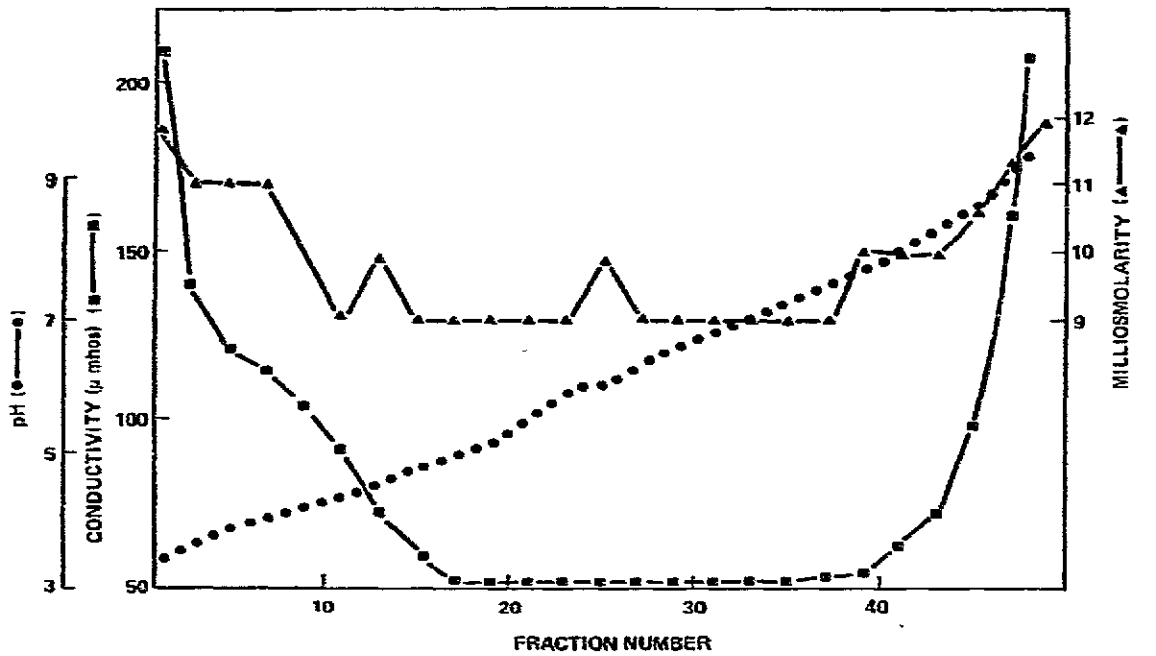

Fig. 4. pH gradient and conductivity and osmolarity profiles of focused 1% Ampholine, pH 3.5–10, in the Hannig apparatus. The carrier ampholyte curtain, diluted in water, was focused at 100 V/cm and eluted at a speed of 1 ml per tube per h. About 3 ml were collected in each tube. Even though the focusing was run at 2°C, pH and conductivity measurements were made at 22°C.

Fig. 5 shows the possibility of running a free curtain containing an even distribution of the following species: glycine (Fig. 5A), taurine (Fig. 5B), TMAPS (Fig. 5C) and sucrose (Fig. 5D). In all instances, a fairly constant value of osmolarity is obtained centered around 290–300 mOsm.

With the zwitterionic compounds in Fig. 5A–C, we have taken advantage of their unwanted quality of being "poor" carrier ampholytes, in Rilbe's terms[26]. Thus, glycine (pK_1 2.35, pK_2 9.6, ΔpK 7.2) exhibits a flat plateau between pH 4.5 and 7.7;

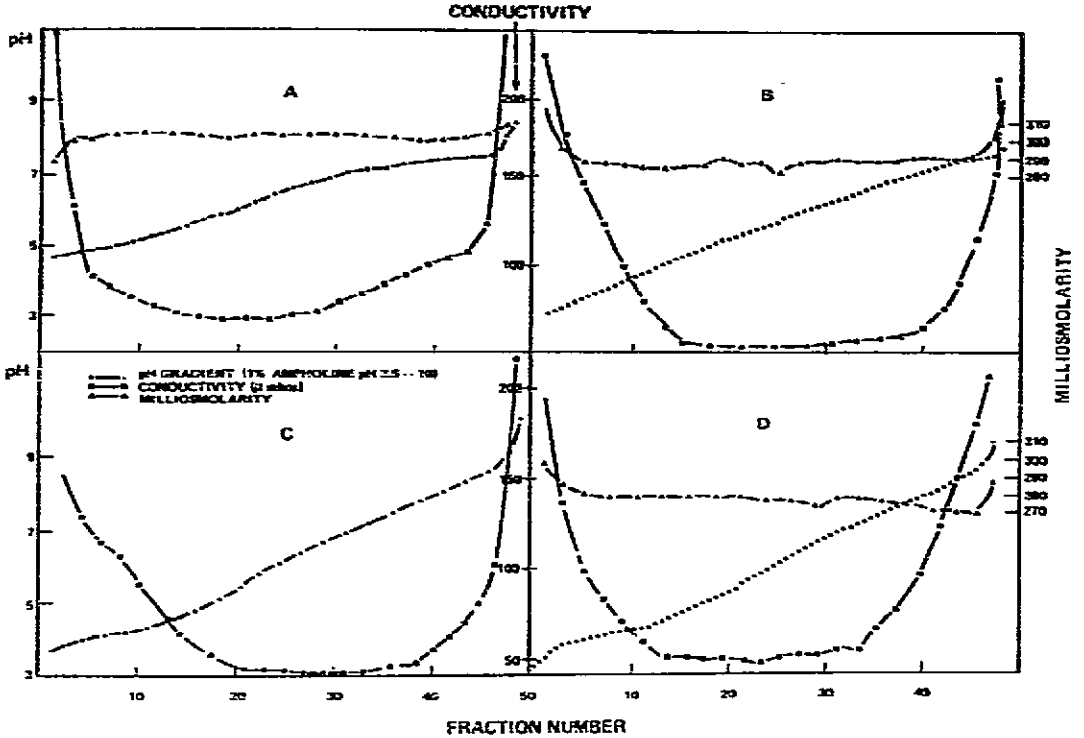

Fig. 5. pH gradient and conductivity and osmolarity profiles of focused 1% Ampholine, pH 3.5–10, in the presence of glycine (A), taurine (B), TMAPS (C) or sucrose (D). All other conditions as in Fig. 4. There is no osmolarity profile for TMAPS, as too little of this material was available. However, a few osmolarity measurements made at different pH intervals by pooling several fractions gave the expected value of 300 mOsm.

taurine (pK_1 1.1, pK_2 9.6, ΔpK 8.5) is evenly distributed between pH 3.0 and 7.7; TMAPS (pK_1 1.1, $pK_2 \approx 12$, ΔpK 11) presents a uniform plateau over the pH range 3–10. All of these systems appear to be suitable for cell focusing.

DISCUSSION

Our preliminary data indicate the following aspects of cell focusing.

Interaction between oligocationic carrier ampholytes and polyanionic cell surface

The binding is typically pH dependent, being very strong at pH 4 (40% reduction in cell mobility), weak at pH 5 and abolished at pH 6. This could be due to the fact that at pH 4 Ampholine species would exhibit a stretch of 4–5 protonated nitrogens (each two methylene groups apart), while a large portion of their carboxyls (*ca.* 50%) will be protonated, thus allowing for strong, cooperative binding. At pH 6 or above, the net charge will be reversed and the anionic Ampholine will now be repelled by the polyanionic cell surface. This type of binding follows a characteristic pattern which we have demonstrated and fully explained not only for nucleic acids[20]

and polyanions[21–23] but even for acidic dyes[27,28]. It seems logical to conclude that the same type of mechanism is operating here. The bound Ampholine might change the surface charge characteristics, thus altering the cell stability and contributing to the decrease in cell viability. It is a fact that, even in early studies by the Sherbet group[1], all cells focusing around pH 5 (4.70–5.30) (Yoshida ascites, Ehrlich ascites, Py cells, HeLa cells, normal liver cells), where binding of Ampholine is still appreciable, were in fact all non-viable cells. It must be emphasized that the binding we have reported here must represent only a minimum value, which might be much higher and extend to higher pH values in the actual experimental conditions of IEF. In fact, our binding data refer to electrokinetic conditions characterized by high ionic strength (150 m*M* sodium chloride) while during IEF the medium ionic strength is vanishingly small, amounting to 0.5–1.0 mg-ion/l (ref. 29). Under IEF conditions, binding might be greatly enhanced, as the high ionic strength used in cell micro-electrophoresis would tend to disrupt interactions between the Ampholine and RBC surfaces that are due to hydrogen bonding, electrostatic interactions between opposite charges, as well as mosaics, charge fluctuation interactions and Van der Waals forces. While binding of oligo- or poly-cations to the RBC membrane is a well known phenomenon, an opposite type of interaction has been recently demonstrated. Pittz *et al.*[18] have in fact shown that also polyanions, iota carrageenans (but not oligoanions), can bind to the RBC surface and aggregate the cells in rouleaux and have proposed a mechanism by which bound carrageenans repel negatively charged and attract positively charged proteins. In fact, not only lateral diffusion[30] but even electrophoresis of proteins in the plane of the cell membrane[31] have been demonstrated.

Binding of carrier ampholytes to the cell surface could also explain the very high isoelectric points of most cells, reported to be in the range 6.0–6.8. On the other hand, when focusing is performed in citrate buffer[32] most of the cells investigated (rabbit spermatozoa, rat pituitary cells, rat mammary ascites tumor cells and M3-1F3 chinese hamster fibroblasts) appeared to have p*I* values in the pH range 3.5–4.7. This type of focusing should be further explored, as citrate allows higher medium ionic strengths, prevents cell aggregation and, being oligoanionic, should not bind to the cell surface[18].

It should be emphasized that, while we have considered as a probable hypothesis for the reduced cell mobility in the presence of carrier ampholytes an extensive binding of these compounds to ionic groups on the cell surface, thus producing an altered ionic cloud around the particles, other mechanisms could also operate here. Among these are (a) possible damage exerted on the membrane with exposure of more negative groups; (b) dissolution of the carrier ampholytes by the membrane; and (c) a combination of the previous mechanisms or other, at present unknown, biophysical processes operating at the level of the cell membrane.

Osmolarities via zwitterionic compounds

The results show that a proper osmolarity, adequate for cell survival, can be obtained in the absence of sugars or polysaccharides by using compounds with a high ΔpK value, such as glycine, taurine or TMAPS. As the ΔpK progressively increases, the pH range in which these zwitterions are isoelectric widens. A pH gradient between 4.5 and 7.7 can be obtained in presence of 300 m*M* glycine, between pH 3.0 and 7.7 with 300 m*M* taurine and between pH 3.0 and 10 with TMAPS. From the

point of view of cell separation, glycine or taurine should be adequate, as they are isoelectric in a pH range in which most cells should be isoelectric.

At the moment, the major problem we have with our system is aggregation and cell clumping. The cell clumps begin to sediment quickly in the focusing chamber and then cells are found smeared all over the collection tubes. Owing to the design of the Hannig chamber, it is impossible to focus in the presence of a constant background of 1–2 m*M* EDTA (these levels have been found not to disrupt the focusing process). This is due to the fact that the electrode chambers are limited by ion-exchange membranes. Therefore the EDTA added to the catholyte can leave the cathodic chamber but, as it approaches the anode, it is repelled by the negatively charged anodic membrane. This results in crystallization of EDTA close to the anodic extremity of the chamber and distortion of the pH gradient. In the near future, dialysis membranes to substitute for the ion-exchange membranes should be available from the manufacturer.

CONCLUSION

Our preliminary data confirm that the environment and conditions for isoelectric focusing will be critical for the successful fractionation of viable cells, especially if physico-chemical parameters, such as p*I* values, are to be measured. New systems, such as citrate pH gradients, should be further explored, perhaps in combination with "poor" zwitterions (glycine, taurine and TMAPS). Interestingly, cell IEF has been successful in other areas of research, as it has finally allowed a better understanding of the focusing process itself, by permitting measurements of molarity and ionic strength of focused carrier ampholytes[29].

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REFERENCES

1 G. V. Sherbet, M. S. Lakshmi and K. V. Rao, *Exp. Cell Res.*, 10 (1972) 113–123.
2 G. V. Sherbet and M. S. Lakshmi, *Biochim. Biophys. Acta*, 298 (1973) 50–58.
3 G. V. Sherbet and M. S. Lakshmi, in J. P. Arbuthnott and J. A. Beeley (Editors), *Isoelectric Focusing*, Butterworths, London, 1975, pp. 338–346.
4 W. W. Just, J. O. Leòn-V and G. Werner, in P. G. Righetti, (Editor), *Progress in Isoelectric Focusing and Isotachophoresis*, North-Holland, Amsterdam, 1975, pp. 265–280.
5 W. W. Just, J. O. Leòn-V and G. Werner, *Anal. Biochem.*, 67 (1975) 590–601.
6 W. W. Just and G. Werner, in B. J. Radola and D. Graesslin (Editors), *Electrofocusing and Isotachophoresis*, De Gruyter, Berlin, 1977, pp. 481–493.
7 W. W. Just and G. Werner, in H. Bloemendal (Editor), *Cell Separation Methods*, North-Holland, Amsterdam, 1977, pp. 131–142.
8 W. W. Just and G. Werner, in P. G. Righetti, C. J. Van Oss and J. W. Vanderhoff (Editors), *Electrokinetic Separation Methods*, North-Holland, Amsterdam, 1979, pp. 143–167.

9 E. M. Leise and F. LeSane, *Prep. Biochem.*, 4 (1974) 395–410.
10 R. L. Hirsch and I. Gray, *J. Cell Biol.*, 70 (1976) 358–365.
11 R. C. Boltz, Jr., P. Todd, R. H. Hammerstedt, W. C. Hymer, S. J. Thompson and J. Docherty, in H. Bloemendal (Editor) *Cell Separation Methods*, North-Holland, Amsterdam, 1977, pp. 145–155.
12 W. Manske, B. Bohn and R. Brossmer, in B. J. Radola and D. Graesslin (Editors), *Electrofocusing and Isotachophoresis*, De Gruyter, Berlin, 1977, pp. 497–502.
13 H. D. M. Moore and K. G. Hibbitt *J. Reprod. Fertil.*, 44 (1975) 329–332.
14 G. V. Sherbet, *The Biophysical Characterization of the Cell Surface*, Academic Press, New York, 1978.
15 R. W. Langton, J. S. Cole, IX, and P. F. Quinn, *Arch. Oral Biol.*, 20 (1975) 103–106.
16 P. Talbot, in J. P. Arbuthnott and J. A. Beeley (Editors), *Isoelectric Focusing*, Butterworths, London, 1975, pp. 270–274.
17 N. Catsimpoolas and A. L. Griffith, in B. J. Radola and D. Graesslin (Editors), *Electrofocusing and Isotachophoresis*, De Gruyter, Berlin, 1977, pp. 469–479.
18 E. P. Pittz, R. Jones, L. Goldberg and F. Coulston, *Biorheology*, 14 (1977) 33–42.
19 E. Galante, T. Caravaggio and P. G. Righetti, in P. G. Righetti (Editor), *Progress in Isoelectric Focusing and Isotachophoresis*, North-Holland, Amsterdam, 1975, pp. 3–12.
20 E. Galante, T. Caravaggio and P. G. Righetti, *Biochim. Biophys. Acta*, 442 (1976) 309–315.
21 P. G. Righetti and E. Gianazza, *Biochim. Biophys. Acta*, 532 (1978) 137–146.
22 E. Gianazza and P. G. Righetti, *Biochim. Biophys. Acta*, 540 (1978) 357–364.
23 P. G. Righetti, R. Brown and A. L. Stone, *Biochim. Biophys. Acta*, 542 (1978) 232–242.
24 G. V. F. Seaman, in D. Mac N. Surgenor (Editor), *The Red Blood Cell*, Vol. II, Academic Press, New York, 1975, pp. 1135–1229.
25 P. G. Righetti, L. Balzarini, E. Gianazza and O. Brenna, *J. Chromatogr.*, 134 (1977) 279–284.
26 H. Rilbe, in N. Catsimpoolas (Editor), *Isoelectric Focusing*, Academic Press, New York, 1976, pp. 14–52.
27 P. G. Righetti, E. Gianazza, O. Brenna and E. Galante, *J. Chromatogr.*, 137 (1977) 171–181.
28 E. Gianazza and P. G. Righetti, in B. J. Radola (Editor), *Electrophoresis '79*, De Gruyter, Berlin, in press.
29 P. G. Righetti, *J. Chromatogr.*, 190 (1980) 275–282.
30 M. Edidin, Y. Zagyansky and T. J. Lardner, *Science*, 191 (1976) 466–468.
31 M. M. Poo, J. W. Lam, N. Orida and A. W. Chao, *Biophys. J.*, 26 (1979) 1–21.
32 R. C. Boltz, Jr., and T. Y. Miller, in N. Catsimpoolas (Editor), *Electrophoresis '78*, Elsevier North-Holland, New York, 1978, pp. 345–355.