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FREE-FLOW ELECTROPHORETIC APPARATUS FOR SEPARATION AND CONCENTRATION OF PROTEINS

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SUMMARY

Protein separation in the free-flow electrophoretic apparatus with 48 channels at both the inlet and outlet using "artificial" pH gradients was investigated. The separation was carried out in borate–mannitol pH gradients and in pH gradients created by the concentration gradient of boric acid in the solutions of borax and mannitol. The separation pattern depended on the ratio of the rate of sample injection to the flow-rate of solutions in the separation chamber and did not change when the protein concentration in the sample was changed. The protein separation in the pH gradient was better than in case of conventional free-flow electrophoresis. The free-flow electrophoretic apparatus with multi-channel inlet of the separation chamber is suitable for concentration of biological materials, e.g. proteins and bacterial cells.

INTRODUCTION

A free-flow electrophoretic apparatus is used for both electrophoresis and isoelectric focusing (IEF) in synthetic carrier ampholytes. However, the latter method has not yet gained wide application. In our opinion, this is for the following reasons. Analytical free-flow IEF has no advantages in its resolving power and cost of the analysis over polyacrylamide gel IEF. Preparative IEF in the free-flow apparatus, compared with IEF in density-gradient electrophoretic columns, requires relatively large amounts of synthetic carrier ampholytes. Fawcett concluded, as early as 1976 [1], that the use of synthetic carrier ampholytes in the

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free-flow apparatus to purify a protein is reasonable, only cost is not a consideration. The author suggested that, for IEF in such apparatus, other types of pH gradients, e.g. the borate–polyol pH gradients, could be more suitable.

The borate–polyol pH gradients, first described by Troitsky et al. [2], have been well studied [3]. The gradients are generated by the concentration gradient of polyhydroxy compounds in borate buffer in such a way that the greater the concentration of the compounds, the lower the pH. The main disadvantage is that such gradients change under the influence of the electric field. This problem is of extreme importance and it is being tackled now by optimizing the chemical composition of borate–polyol pH gradients. The procedure of optimization is highly complex and consists of the empirical selection of polyhydroxy compounds, borate buffer (e.g. sodium borate, Tris borate, etc.) and also of the concentrations of all the components in solutions. Consequently, for each particular electrophoretic apparatus and the sample to be fractionated, the corresponding composition of the pH gradient is often described. The known borate–polyol pH gradients widely vary in composition of components [3]. They are mainly employed for IEF of proteins in density-gradient electrophoretic columns.

The aim of the present work was to demonstrate the potential of the free-flow electrophoretic apparatus for the separation and concentration of proteins in "artificial" pH gradients. The following three types of pH gradient were investigated.

(1) The borate–mannitol pH gradients with an initially constant ratio of the analytical concentration of mannitol to that of borate ions along the pH gradient. We shall call them type 1 pH gradients. The gradients were described by us previously [4]. Their stability is increased by the creation of a constant ratio of the concentrations of polyol and borate ions. Such pH gradients covering 0.6–1 pH unit can be obtained in the pH range 9.2–4.5.

(2) The pH gradients formed by the concentration gradient of boric acid in solutions of borax and mannitol. We shall call them type 2 pH gradients. They were first described by us in 1985 [5]. Type 2 pH gradients are not borate–polyol pH gradients in a true meaning of the term, because this term applies to pH gradients generated by the concentration gradient of polyhydroxy compound (e.g. mannitol) in borate buffer (in particular, in borax solution). The range, shape and location of such gradients on the pH scale is determined mainly by the concentration gradient of the polyhydroxy compound. In contrast, the range and shape of type 2 pH gradients are dependent on the concentration gradient of boric acid, and mannitol ensures a shift of the pH gradient to a more acidic region of the pH scale. Such pH gradients, covering 1–2 pH units, can be obtained in the pH range 9.2–3.5. In type 2 pH gradients, as in type 1, the analytical concentrations of polyol and borate ions are initially maintained constant.

(3) The step pH gradients combined with the conductivity gradients. We shall call them type 3 pH gradients. In contrast to type 1 and type 2 pH gradients, they were used by us not for separation but for concentration of proteins [6]. The method of free-flow field step focusing [7] is similar to the concentration in type 3 pH gradients.

EXPERIMENTAL

The free-flow apparatus has been described previously [4]. Obligatory elements of its design are a vertical separation chamber with one side cooling and 48 channels at both inlet and outlet, electrode compartments with dialysis or cation-exchanging membranes (e.g. of MK-40 type, Azot, Shtchekino, U.S.S.R.) between the electrodes and separation chamber, vessels for electrode solutions equipped with centrifugal pumps, a cooling unit, a power supply, a 48-channel peristaltic pump to pump solutions through the separation chamber, fraction collector, and, when working with type 1 and type 2 pH gradients, a separate peristaltic pump for sample injection at a lower rate than that of the liquid flow in the chamber. To improve the separation it is desirable that chamber walls have a coating reducing the zeta potential. To coat a glass (cooled) chamber wall we used triethylsilane.

The experiments with type 1 and type 2 pH gradients were carried out in the apparatus with a $38 \times 5 \times 0.06$ cm separation chamber and 25 cm long electrodes. For type 3 pH gradients, a $36.5 \times 5 \times 0.05$ cm separation chamber and 28 cm long electrodes were used.

Formation of type 1 pH gradients

The anode buffer was prepared as a 2.5 m*M* borax solution containing D-mannitol. The concentration of the latter was adjusted so that the pH value of the buffer would become equal to that of the solution of the acidic end of the pH gradient in which the protein separation was attempted. The precise value of the polyol concentration was determined empirically. The anode buffer was diluted step by step with distilled water to yield a set of solutions with linearly varying pH values. The pH gradient was generated by the formation of the concentration gradient of polyol in borate buffer. Besides, the concentration gradient of borax resulted in the generation of a linear conductivity gradient, the conductivity increasing towards the acidic end of the pH gradient. Fig. 1 shows the distribution of concentrations of components in the pH gradient. The formation of the type 1 gradients has been described in detail previously [4].

On employment of the pH gradients of the type 1, the cathode buffer was 4 times less concentrated than the anode one, and did not differ in composition of components from that of the solution of the alkaline end of the pH gradient.

Formation of type 2 pH gradients

The pH gradients were generated in 2.5 m*M* borax solutions containing D-mannitol. The polyol concentration was adjusted so that the pH of the solution would become equal to that of the alkaline end of the pH gradient in which the protein separation was attempted. A logarithmic concentration gradient of boric acid in the range 0–45 m*M* was formed in this solution. Thus a linear pH gradient covering 1 pH unit was obtained, which was shifted towards more acidic values in proportion to the amount of mannitol added. Such pH gradients lack a conductivity gradient. The formation of type 2 gradients has been described in detail

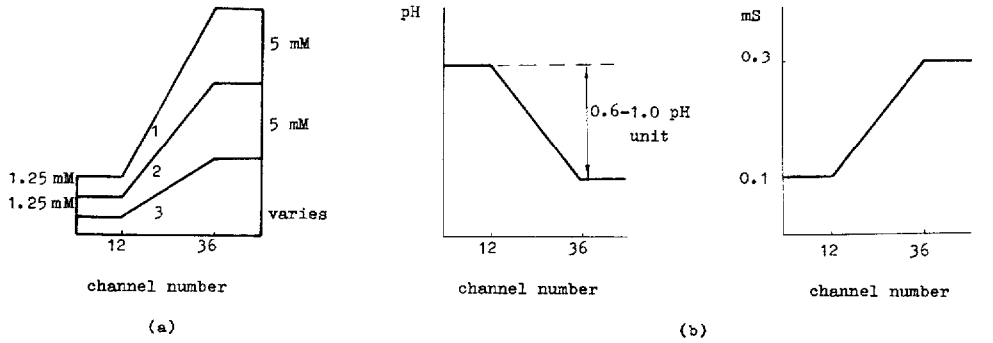

Fig. 1. Borate-mannitol pH gradients with an initially constant ratio of the analytical concentration of mannitol to that of borate ions (type 1 pH gradient). (a) Distribution of the concentrations of the components of solutions at the inlet of the separation chamber: (1) boric acid; (2) borate ions; (3) D-mannitol (the concentration is greater in the more acidic region the pH gradient is generated). (b) Variation of the pH and the conductivity of solutions at the inlet of the separation chamber.

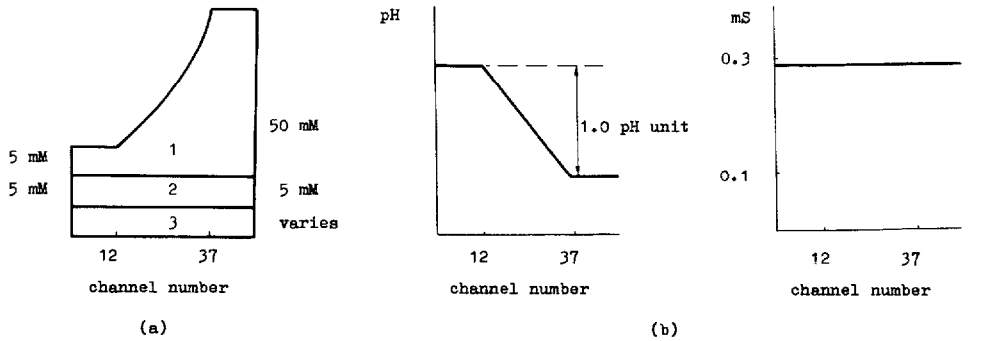

Fig. 2. pH gradients obtained by formation of the concentration gradient of boric acid in solutions of borax and mannitol (type 2 pH gradient). (a) Variation of the concentrations of components of solutions at the inlet of the separation chamber: (1) boric acid; (2) borate ions; (3) D-mannitol (the concentration is greater in the more acidic region the pH gradient is formed). (b) Variation of the pH and the conductivity of solutions at the inlet of the separation chamber.

earlier [5]. Fig. 2 shows the distribution of concentrations of components in these pH gradients.

The cathode buffer for type 2 gradients was 2.5 m*M* borax solution containing D-mannitol. (The concentration of polyol was the same as that in the solution of the alkaline end of the pH gradient.) The anode buffer, in contrast to the cathode one, also contained 45 m*M* boric acid.

Formation of type 3 pH gradients

We used pH gradients formed by solutions of borax and boric acid. A 2.5 m*M* solution of borax (pH 9.2, conductivity 0.5 mS) was injected into the separation chamber via the channels adjacent to the cathode and a 0.45 *M* solution of boric acid (pH 4.3, conductivity 0.025 mS) was introduced via the remaining channels. In consequence, a step pH gradient combined with a conductivity gradient was generated in the separation chamber. Fig. 3 shows the distribution of concentrations of components in the pH gradient.

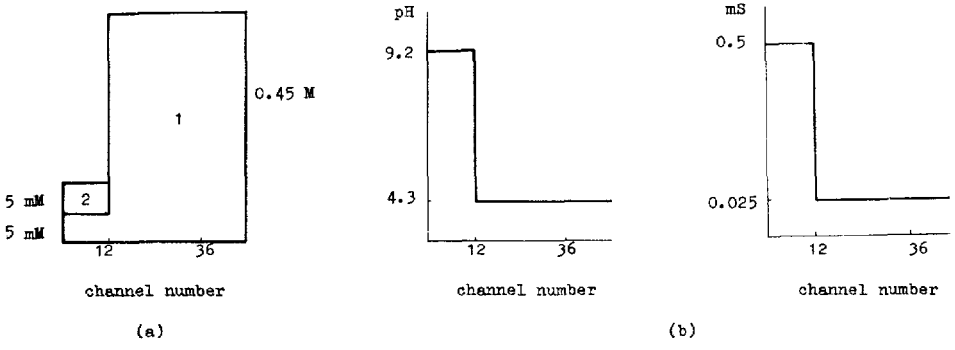

Fig. 3. Step pH and conductivity gradients obtained in the borax-boric acid system. (a) Variation of the concentrations of components of solutions at the inlet of the separation chamber: (1) boric acid; (2) borate ions. (b) Variation of the pH and the conductivity of solutions at the inlet of the separation chamber.

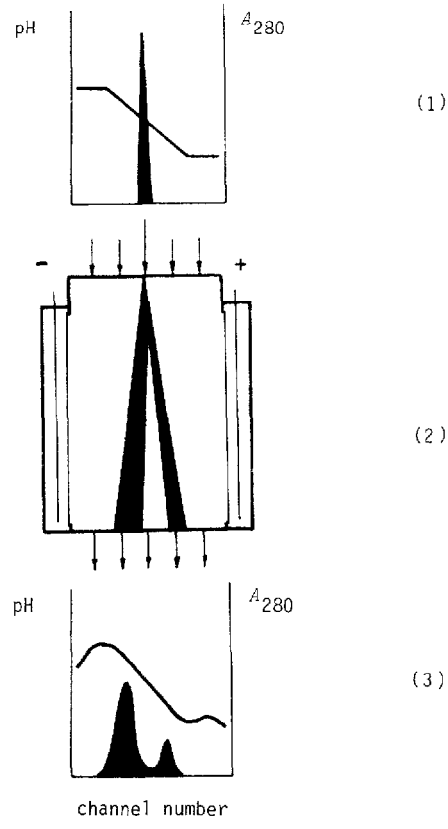

Fig. 4. Principle of protein separation in type 1 and type 2 pH gradients. (1) pH gradient and protein distribution at the inlet of the separation chamber; (2) separation chamber (section); (3) pH gradient and protein distribution at the outlet of the separation chamber.

Protein separation in type 1 and type 2 pH gradients

Fig. 4 illustrates the principle of protein separation in our experiments. The prepared pH gradient represented a set of solutions with subsequently varying pH values. Each solution was put into a separate vessel from which it was introduced via a separate channel into the separation chamber. According to our data [4,5], pronounced changes of pH and conductivity occur under the influence of the electric field in the regions near the membrane, therefore it is unreasonable to introduce pH gradients into these regions. Rather the electrode solutions were introduced here and the pH gradient was introduced via the central channels. The sample was injected into the chamber via a separate channel. The rate of sample injection was usually 2–3 ml/h. The operating conditions are described in detail in refs. 4 and 5. The fractions were collected at the outlet of the chamber after passing through the electric field. To obtain the information on the pH gradients and protein separation, the pH and optical density at 280 nm were determined in each fraction.

The process of protein separation in the free-flow apparatus using both types of pH gradient is similar in many features, but there are differences. In particular, the separation pattern in type 1 pH gradients as distinct from that in type 2 pH gradients depends on the point of injection of the protein into the pH gradient; this is probably associated with existence of a conductivity gradient in type 1 pH gradients [4,5].

Protein concentration in type 3 pH gradients

Fig. 5 illustrates the principle of concentration of proteins in our experiments. The proteins were introduced into the chamber as a wide zone, together with the solution of boric acid. The borax solution was injected in the region adjacent to the cathode (25% of the chamber width). The flow-rate via each of the channels was 11.5 ml/h. The 2.5 m*M* borax solution served as an electrode buffer.

RESULTS

Type 1 pH gradients

Some opportunities for protein separation using type 1 pH gradients have been demonstrated previously [4]. Using bovine haemoglobin (Reanal, Budapest, Hungary) as a model, it was established that IEF was not achieved, and the results of separation depend on the field strength, residence time (i.e. the time during which the proteins move along the electrodes) and also on the point of sample injection in the pH gradient [4].

The quality of protein separation in the pH gradients investigated also depends on the ratio of the rate of sample injection (V_1) to the flow-rate of solutions through the separation chamber (V_2). If the $V_1 : V_2 = 1:1$, fractionation of haemoglobin is not observed. If $V_1 : V_2 = 1:2$ (or more) several protein fractions are registered at the outlet of the chamber (Fig. 6). These results can be explained by the influence of disturbing factors, such as the electroosmotic velocity profile and the Poisseuille velocity profile of the liquid flow in the gap of the separation chamber. It is known [8] that, in case of conventional free-flow electrophoresis,

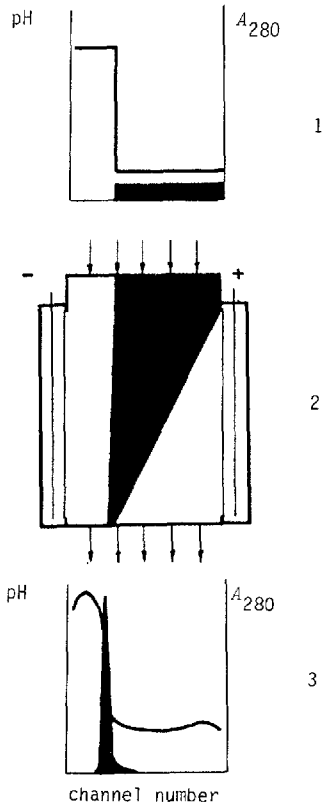

Fig. 5. Principle of protein concentration in type 3 pH gradients. (1) pH gradient and protein distribution at the inlet of the separation chamber; (2) separation chamber (section); (3) pH gradient and protein distribution at the outlet of the separation chamber.

bands of the proteins separated would be significantly distorted under the influence of the electric field if the sample jet completely fills the gap of the chamber touching its walls. In consequence, at the outlet of the chamber, an overlapping of adjacent protein fractions will be registered that can appear to be so significant that there would appear to be a complete lack of separation. This seems to occur in our case when $V_1 : V_2 = 1:1$. If the sample jet gets to the middle of the chamber gap where the influence of disturbing factors is insignificant, the distortions of the protein bands will be slight [8], their overlapping at the outlet will be limited and each fraction will be registered. When type 1 pH gradients are used in our apparatus, this is achieved when $V_1 : V_2 = 1:2$. Fig. 6 shows that changing the $V_1 : V_2$ ratio from 1:3 to 1:6 does not affect the degree of fraction overlapping. Therefore, the rate of sample injection relative to the flow-rate of solutions in the separation chamber should not be reduced drastically. This does not improve the separation pattern, whereas the throughput of the free-flow apparatus (sample volume with time) decreases.

An attempt to improve the separation quality by conversion of a free-flow apparatus in a cyclic mode of operation was not a success. The experiments were carried out as follows. The sample was injected first into the separation chamber

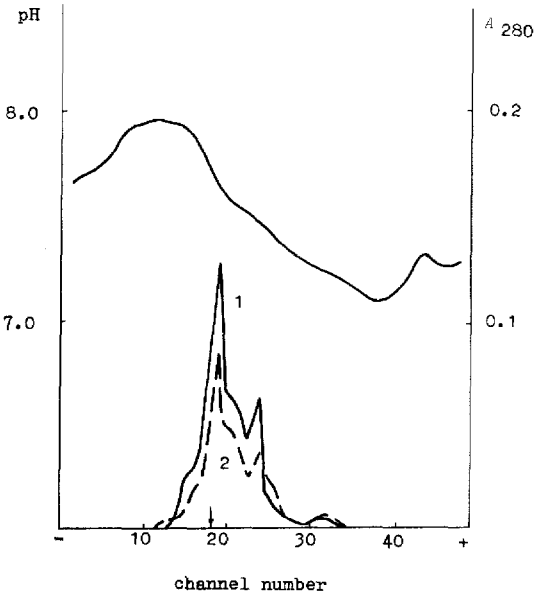

Fig. 6. Separation of bovine haemoglobin in type 1 pH gradient at different ratios of the rate of sample injection (V_1) to the rate of solution flows in the separation chamber (V_2): (1) $V_1 : V_2 = 1:3$; (2) $V_1 : V_2 = 1:6$. Voltage, 750 V; residence time, 70 s; protein concentration in the sample, 1 mg/ml.

via one of the channels, and the separation was performed as described in Experimental. After fraction collection the solutions that had passed through the near-membrane regions of the separation chamber were exchanged for fresh ones. The latter, and the protein-containing fractions, were placed at the inlet of the separation chamber and the run was repeated. Fig. 7 shows the results obtained for bovine haemoglobin. The degree of overlapping of protein fractions does not decrease when the solutions are passed through the separation chamber again. This can be explained by the influence of disturbing factors at $V_1 : V_2 = 1:1$ and by increase of changes of the pH gradient. Thus it is hardly reasonable to separate proteins in type 1 pH gradients with a cyclic mode of operation. This does not improve the separation quality, but the experiments take longer and the throughput is less.

Type 2 pH gradients

With type 2 pH gradients IEF was not achieved as with type 1 pH gradients [5]. However, the quality of protein separation in the pH gradients investigated was higher than that obtained with conventional electrophoresis. For instance, human serum albumin is not separated by free-flow electrophoresis [9], whereas in type 2 pH gradients the separation pattern obtained was similar to the isoelectric spectrum [5].

Another example is the result of the purification of alkaline phosphatase from *Escherichia coli* C-90 strain. Bacterial cells were destroyed by ultrasonic disintegration, the cell fragments were removed by ultracentrifugation, and the supernatant containing the enzyme was fractionated in the free-flow apparatus. The

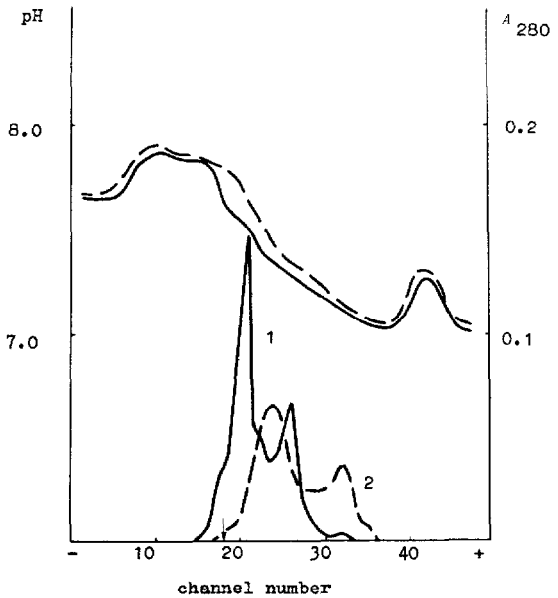

Fig. 7. Separation of bovine haemoglobin in type 1 pH gradient resulting from single (1) and double (2) passage of solutions through the separation chamber. Voltage, 750 V; residence time, 70 s per run; protein concentration in the starting solution, 1 mg/ml.

proteins were determined at the outlet of the separation chamber according to the Bradford method [10] and alkaline phosphatase activity was estimated by *p*-nitrophenylphosphatase reaction [11]. Conventional free-flow electrophoresis was carried out in 0.01 *M* Tris–HCl buffer (pH 7.4, conductivity 1.3 mS) containing 0.041 m*M* zinc chloride, 0.1 m*M* sodium dihydrogenphosphate, 3 m*M* sodium nitrate and 1 m*M* magnesium chloride. The voltage drop was 100 V/cm and the residence time was 80 s. The results are presented in Fig. 8a.

The separation in a type 2 pH gradient was carried out at 40 mA constant current and residence time of 70 s. The sample (protein concentration 0.09 mg/ml) was injected near the isoelectric point of the enzyme. The results are presented in Fig. 8b. Larger amounts of ballast proteins are removed in type 2 pH gradients than in the case of conventional free-flow electrophoresis. Moreover, after fractionation in type 2 pH gradients, several peaks of enzyme activity are recorded at the outlet of the separation chamber. It is not excluded that this is a result of the separation of multiple forms of alkaline phosphatase, which are known to exist in *E. coli* cells [12], but we did not perform a detailed experimental check of this hypothesis.

The throughput of the free-flow apparatus (amount of the protein fractionated with time) when working with the pH gradients under study can be raised by increasing the concentration of the protein in the sample. In this case the separation pattern does not change (Fig. 9) if the proteins do not precipitate and the density of the sample solution is not too high. The results presented in Fig. 9 were obtained in the chamber with a 0.05-cm gap. Haemoglobin separation deteriorates in this case compared with that achieved in the chamber with the 0.06-cm

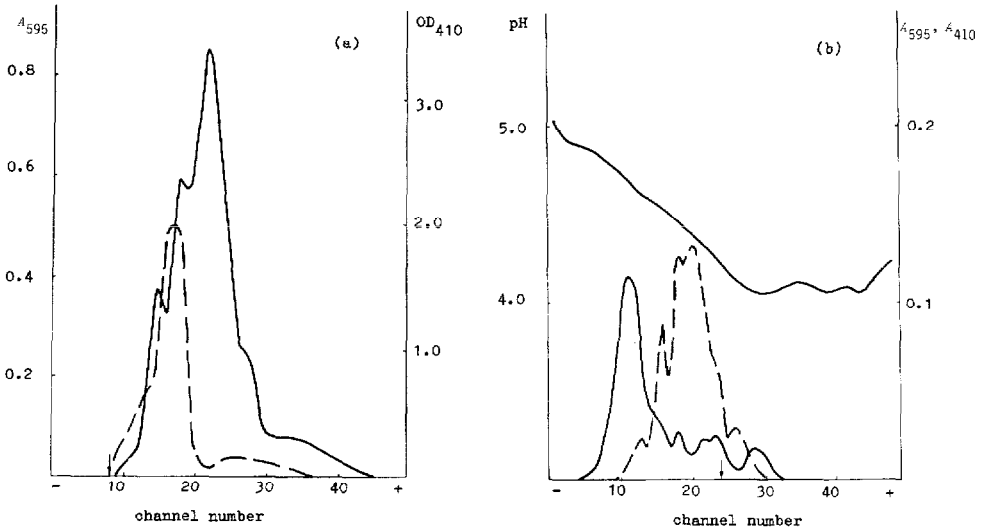

Fig. 8. Purification of alkaline phosphatase from *E. coli* by free-flow electrophoresis at pH 7.4 (a) and in type 2 pH gradient (b). The broken line indicates the variation of enzyme activity at the outlet of the separation chamber. The arrows designate the points of sample injection in the separation chamber. For details see the text.

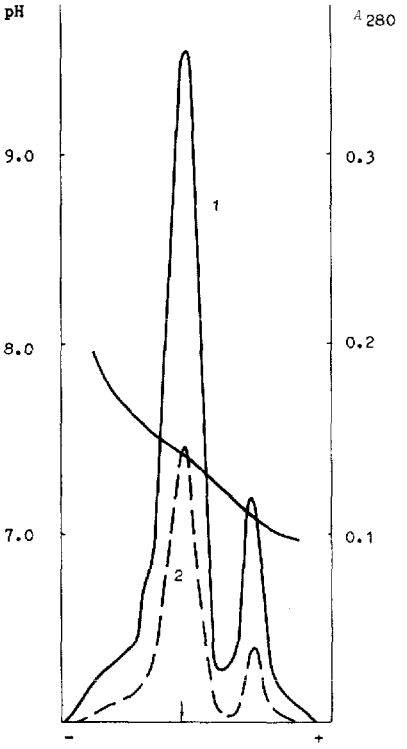

Fig. 9. Separation of bovine haemoglobin in type 2 pH gradient at different protein concentrations in the sample: (1) 5 mg/ml; (2) 2 mg/ml. Constant current, 30 mA; residence time, 140 s.

gap (see Fig. 6). It is possible that alteration of the separation pattern with reduction of the gap is caused by disturbing factors (the electroosmotic velocity profile and the Poisseuille velocity profile of the liquid flow in the separation chamber), as is observed when the $V_1 : V_2$ ratio is changed from 1:2 to 1:1 (see above).

Type 3 pH gradients

Fig. 10 shows the results of concentration of the mixture of bovine haemoglobin and human serum albumin in the free-flow apparatus using a type 3 pH gradient obtained in the borax–boric acid system. Haemoglobin is concentrated at the isoelectric point at the boundary between solutions of borax and boric acid; albumin is not concentrated and leaves the separation chamber as a wide zone. Thus, simultaneously with the concentration of a neutral protein (haemoglobin), removal of an acidic protein (albumin) occurs. The protein concentration in the starting solution does not affect the results of the experiments. In the experiment described, a partial precipitation of haemoglobin was observed at the isoelectric point. If the protein concentration is decreased in the starting solution, the precipitation would not occur, but the results of concentration would be the same as shown in Fig. 10 [6]. In contrast to ultrafiltration, the employment of free-flow apparatus permits concentration of proteins excluding their adsorption and inactivation on the membranes. The borax–boric acid system is not useful for concentration of acidic proteins in free-flow apparatus. These proteins should be concentrated from solutions with low conductivities and high pH values.

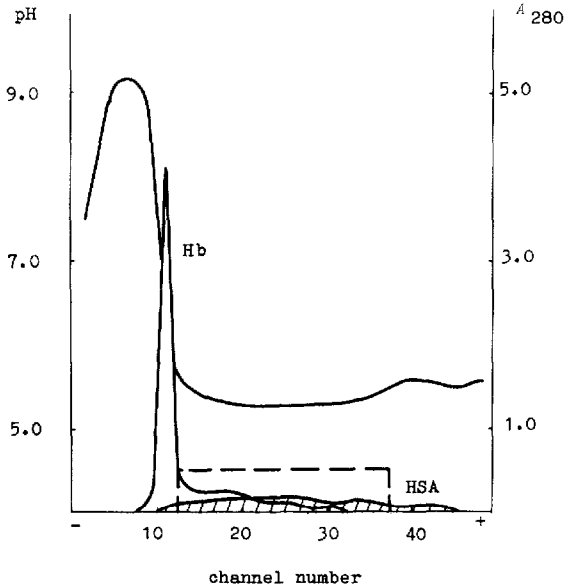

Fig. 10. Concentration of proteins in type 3 pH gradient (borax–boric acid system). The broken line indicates the distribution of the protein at the inlet of the separation chamber. Hb, bovine haemoglobin; HSA, human serum albumin; initial concentration of each protein, 0.25 mg/ml; constant current, 30 mA; residence time, 45 s.

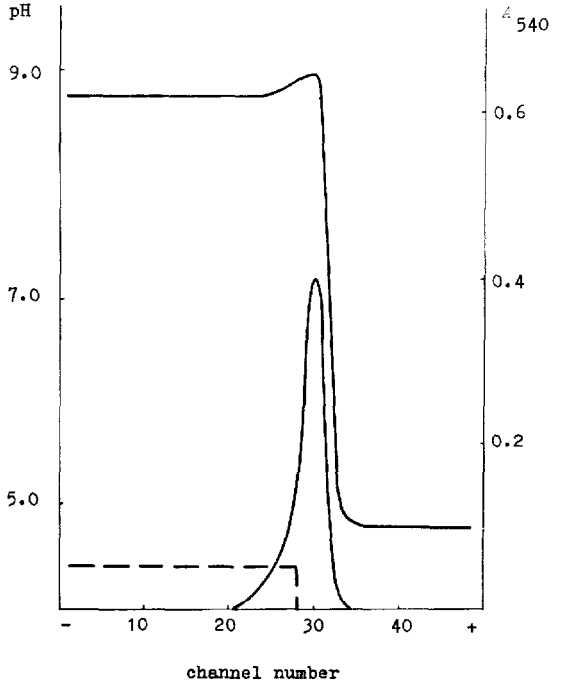

Fig. 11. Concentration of *E. coli* C-600 cells in type 3 pH gradient (Tris–glycine Tris–phosphate buffer system). The broken line indicates the distribution of cells at the inlet of the separation chamber. Starting concentration of cells, $5 \cdot 10^6$ ml^{-1}; constant current, 100 mA; residence time, 45 s.

When type 3 pH gradients are used other biological materials as well as proteins can be concentrated in the free-flow apparatus. The results of concentration of *E. coli* C-600 cells using the system Tris–glycine buffer (pH 8.5, conductivity 0.46 mS) and Tris–phosphate buffer (pH 5.3, conductivity 1.25 mS) are exemplified in Fig. 11. The concentration of the cells was carried out from the Tris–glycine buffer. It would seem that free-flow apparatus may be useful for rapid concentration of low-molecular-mass biologically active materials, including peptides, in the step pH and conductivity gradients.

DISCUSSION

The separation in type 1 and type 2 pH gradients results from electrophoresis not from IEF. Comparison of our results with the data for haemoglobin obtained by Hannig [13] shows that the resolving power of such separation is lower than that of analytical IEF using synthetic carrier ampholytes. Nevertheless, type 1 and type 2 pH gradients can replace synthetic carrier ampholytes in preparative fractionation of proteins in the free-flow apparatus. These pH gradients can appear to be particularly useful for purification of peptides whose relative molecular masses are close to those of synthetic carrier ampholites.

Using type 1 and type 2 pH gradients in the free-flow apparatus, it is possible to achieve better protein separation than with conventional electrophoresis. This

is probably because the band-broadening resulting from the Poisseuille velocity profile of liquids is smaller in the pH gradients than that occurring at constant pH in the separation chamber. It can be deduced from the data of Hannig et al. [14] that during electrophoresis at constant pH the displacement of charged particles (including protein molecules) along the electric field in the separation chamber can be described by the following equation:

$$X-X_0=\frac{2}{3}\left(1+\frac{1}{2}\frac{U_w}{U_e}\right)U_eE\bar{t}+\frac{2}{3}U_eE\bar{t}\left(1-\frac{U_w}{U_e}\right)\frac{y^2/h^2}{1-y^2/h^2} \quad (1)$$

where U_e is the electrophoretic mobility of protein molecules, U_w is the electroosmotic mobility, E is the field strength; $\bar{t}$ is the average residence time, X_0 is the coordinate of the point of sample injection into the separation chamber, X is the coordinate along the electric field, y is the coordinate across the chamber gap ($-h\leqslant y\leqslant +h$) and $2h$ is the chamber gap width.

Using eqn. 1 with $U_w=0$, the band-broadening at the outlet of the chamber gains the shape:

$$\Delta B=\frac{2}{3}U_eE\bar{t}\frac{1}{(h^2/y^2-1)} \quad (2)$$

It follows from eqn. 2 that band-broadening is a function of the field strength and the residence time, and depends also on the distance between molecules and the chamber wall. When $y\rightarrow\pm h$, $\Delta B\rightarrow\infty$. This is the main cause of the relatively low resolution of free-flow electrophoresis, since the fractions separated can overlap strongly because of broadening and can be mixed at the outlet of the chamber.

According to our data [15], in the case of protein electrophoresis in the linear pH gradient near the isoelectric point (this occurred in the experiments described above) the migration of molecules at $U_w=0$ can be described by eqn. 3, and the broadening by eqn. 4:

$$X-X_i=(X_0-X_i)\exp\left\{-\frac{2U_0(pH_b-pH_a)E\bar{t}}{3(1-y^2/h^2)(pH_0-pI)l}\right\} \quad (3)$$

$$\Delta B=(X_0-X_i)\exp\left\{-\frac{2U_0(pH_b-pH_a)E\bar{t}}{3(pH_0-pI)l}\right\} \quad (4)$$

where U_0 and pH_0 are the electrophoretic mobility of molecules and the pH of the solution at the point of sample injection, respectively, pH_b and pH_a are the pH values of the solutions of the alkaline and acidic ends of the pH gradient, respectively, pI is the isoelectric point of the protein, X_i is the coordinate of the isoelectric point and l is the distance between the alkaline and acidic ends of the pH gradient in the chamber.

It follows from eqn. 4 that the band-broadening at the outlet of the chamber decreases exponentially with increasing field strength and residence time and does not depend on the distance between the molecules and chamber wall. Thus, the band-broadening in the linear pH gradient and the overlapping of fractions at the outlet of the chamber should be smaller than that observed with conven-

tional electrophoresis and hence the quality of separation in the pH gradient must be higher than that achieved with conventional free-flow electrophoresis.

The type 1 and type 2 pH gradients are reasonable to use in the free-flow apparatus for purification of proteins from mixtures without attention to the separation of the latter. To achieve a successful purification, the following separation conditions should be optimized.

(1) The field strength and the residence time [4]: their values must permit the purification of the proteins prior to significant alterations of the pH gradient. It should be emphasized that for any design of the free-flow apparatus distinct from ours, the optimal voltage and residence time can differ from those described in Experimental.

(2) The ratio of the rate of sample injection to that of the solution flow in the separation chamber: the sample jet is to be introduced into the middle of the chamber gap, not touching the walls.

(3) The point of sample injection into the pH gradient: since the residence time is limited in the use of type 1 and type 2 pH gradients in the free-flow apparatus, the point of injection should be chosen so that mixtures would migrate from the protein to be purified under the influence of the electric field as quickly as possible. It should be noted that, with the same point of injection, the separation patterns in the pH gradients of both types can be different (Fig. 12).

It is possible that our design of the apparatus is not the best for work with type

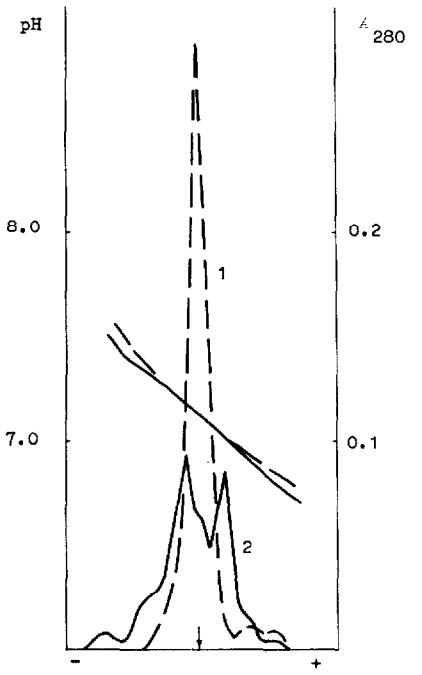

Fig. 12. Separation of bovine haemoglobin in type 1 pH gradient (1) and in type 2 pH gradient (2). The sample was injected at pH 7.12; the point of injection into the separation chamber is indicated by the arrow. Voltage, 750 V; residence time, 70 s; protein concentration in the sample, 1 mg/ml.

1 and type 2 pH gradients. The optimum dimensions of the separation chamber have not yet been determined and it is likely that the most suitable membranes to separate the electrodes from the chamber have not been used.

As shown above, the free-flow electrophoretic apparatus is suitable for the rapid concentration of different biological materials in the step pH and conductivity gradients. In our experiments the apparatus used was not designed especially for such concentration. It seems highly probable that free-flow apparatus of simplified designs can be successfully used for concentration in type 3 pH gradients. It is not obligatory to use the two vessels with centrifugal pumps (one vessel is sufficient), the separate peristaltic pump for sample injection and the 48-channel inlet of the separation chamber (e.g. a 4-channel inlet would suffice). Simplification of the design of the free-flow apparatus would increase its availability and that would promote increased use of the method.

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