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MICROPREPARATIVE VERSION OF HIGH-PERFORMANCE ELECTROPHORESIS

THE ELECTROPHORETIC COUNTERPART OF NARROW-BORE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

High-performance liquid chromatography (HPLC) permits rapid separations with high resolution, on-line (*i.e.*, fast) monitoring of the effluent and the collection of interesting fractions for further investigations. High-performance electrophoresis (HPE) was developed in an attempt to create an electrophoresis method with characteristics similar to those of HPLC. The application range of the first version of HPE was limited to analytical runs. However, a new version of HPE has been introduced that mimics HPLC also in the sense that it permits both micropreparative and analytical separations. A series of experiments performed with this version is described and discussed.

INTRODUCTION

There are many high-resolution methods for analyses of biological materials (such as proteins and nucleic acids) that require only minute amounts of sample, for instance, isoelectric focusing and electrophoresis using agarose and polyacrylamide gels, immunoelectrophoretic techniques, two-dimensional electrophoresis, free zone electrophoresis and high-performance liquid chromatography (HPLC). Of these methods, only HPLC and free zone electrophoresis[1,2] can be used without modification even for preparative fractionations on a micro-scale. With the aim of adapting the above analytical techniques for micropreparative applications we have developed several methods for recovering proteins and nucleic acids from gels in high yields and in concentrated form[3–8] (see also ref. 9).

The recently introduced high-performance electrophoresis (HPE), the electrophoretic counterpart of HPLC, was originally only an analytical tool[10,11]. However, in an effort to give HPE another attractive feature of HPLC, we have modified it so

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that it can also be used for micropreparative purposes[12,13], which is the subject of this paper.

EXPERIMENTAL

Phycoerythrin, a red, acidic protein (molecular weight 290 000), was extracted from the red alga *Ceramium rubrum* and purified as described previously[14]. Adenine, cytidine, guanosine, uridine and β-amylase from hog pancreas were bought from Sigma (St. Louis, MO, U.S.A.) and thymidine from Schwarz Bio-Research (New York, NY, U.S.A.). Enolase was a gift from Dr. G. Petterson at this Institute. Pharmalyte (pH 2.5–5) was obtained from Pharmacia (Uppsala, Sweden). The activity of β-amylase was determined as described by Bernfeld[15].

The pump (HPLC 2150) and the variable-wavelength detector (2151), equipped with a 10-μl flow cuvette, were obtained from LKB (Bromma, Sweden), as also was the photodiode array spectrophotometer (LKB 2140 rapid spectral detector), which was connected to an IBM personal computer and a Canon A-1210 ink-jet colour printer.

High-performance electrophoresis apparatus

A characteristic of HPE is that the experiments are performed in very narrow (I.D. 0.05–0.3 mm), thin-walled (about 0.1 mm) and, preferably, actively cooled glass tubes for rapid dissipation of the Joule heat in order to obtain high resolutions and short run times. For detection of the substances one can use either an on-tube technique[10,11,16] or the elution technique employed in this study, *i.e.*, when the solutes migrate down the electrophoresis column and emerge from it they are transferred by a flow of buffer to the cuvette of a detector and finally to the test-tubes of a fraction collector[12,13]. This technique differs from that previously used for large-scale preparative columns in that the volume of the flow cuvette (1–10 μl) is considerably larger than the volume of the zones (around 0.1 μl) in the electrophoresis tube. This elution technique is discussed in refs. 12 and 13. The pump and detector were taken from an HPLC apparatus.

RESULTS

All of the electrophoresis experiments described here were performed in glass tubes of I.D. 0.2 mm and with a wall thickness of 0.1 mm. The samples were electrophoresed into the tube (for instance, for 10 sec at 1000 V) or applied by layering. The samples were dissolved in (or dialysed against) the buffer used for the electrophoresis experiment diluted 1:5 with water. In this way, a narrow starting zone is guaranteed[17]. The amount of sample used is often in the range 0.01–1 μg.

HPE of a phycoerythrin solution

The electrophoresis tube, which had a length of 110 mm, was treated with methylcellulose as described previously to suppress electroendosmosis[1] and possible adsorption of solutes on the tube wall. The experiment was conducted at room temperature in buffer alone (0.05 *M* sodium borate, pH 8.2) and without active cooling of the tube. The pump speed was adjusted to 0.2 ml/min and the detection was carried out at 220 nm. At 1000 V (40 μA) the run was completed within 10 min (Fig. 1).

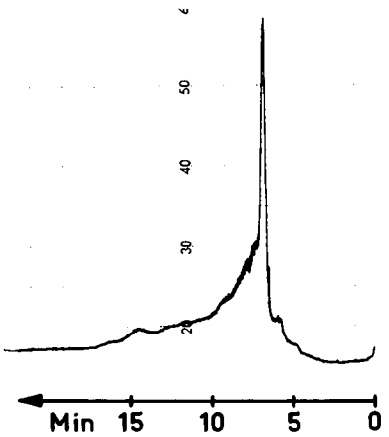


Fig. 1. High-performance electrophoresis of an extract of phycoerythrin. The run was performed in free solution in an electrophoresis tube treated with methylcellulose to eliminate electroendosmosis and possible adsorption of the protein to the glass wall.

HPE of a mixture of pH indicators

Another example of a separation performed in free solution is shown in Fig. 2a. The sample was an artificial mixture of the pH indicators naphthol green, phenol red, bromthymol blue and methyl orange. The same methylcellulose-treated tube, the

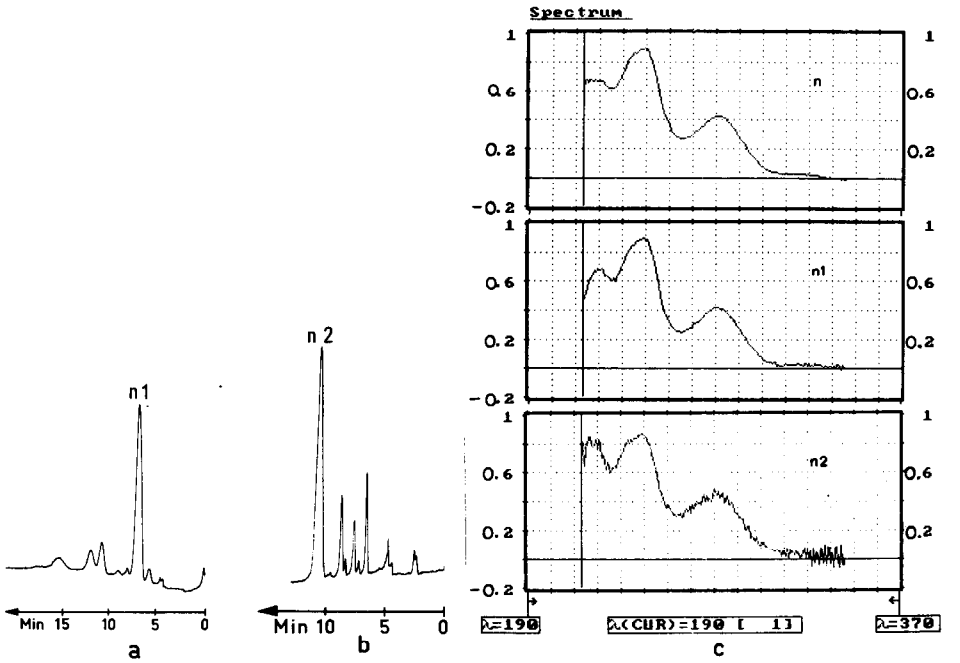


Fig. 2. High-performance electrophoresis of an artificial mixture of naphthol green, phenol red, bromthymol blue and methyl orange. The runs were performed (a) in free solution and (b) in a polyacrylamide gel. (c) Instantaneous spectra of naphthol green (n) and material corresponding to peaks n1 (a) and n2 (b).

same buffer and the same detection wavelength were used as in the experiment shown in Fig. 1. However, the voltage was higher (1500 V, giving a current of 60 μA). The analysis took about 15 min.

The electropherogram in Fig. 2b was obtained when the pH indicators were separated in a glass tube containing a gel in addition to buffer. The gel had the composition $T = 6\%$ and $C = 3\%$ (for the definition of T and C, see ref. 18). Other experimental conditions were similar to those in the experiment corresponding to Fig. 2a, with the difference that the length of the electrophoresis tube was 14 cm, the voltage 2000 V (60 μA) and the tube was cooled with water (of temperature 20°C).

The electropherogram in Fig. 2a differs from that in Fig. 2b, particularly regarding the position of the main peaks n1 and n2 (n1 appears early and n2 late in the electropherogram). To investigate whether these two peaks corresponded to the same component, the spectra of the effluent zones were recorded, on-line, by means of a photodiode array spectrophotometer (see Fig. 2c).

HPE of nucleosides

In order to render all nucleosides negatively charged we performed the experiment in a 0.1 *M* sodium borate solution of pH 10 (borate forms complexes with *cis*-diols and hence with ribonucleosides). The electrophoresis tube (length 140 mm) contained polyacrylamide gel of composition $T = 6\%$ and $C = 3\%$. The experiment was run at 3000 V (180 μA) and at a pump speed of 0.32 ml/min. The electropherogram is shown in Fig. 3.

HPE of carrier ampholytes for isoelectric focusing

The sample was a Pharmalyte solution (pH 2.5–5) and the analysis was performed in an electrophoresis tube with a length of 140 mm containing a polyacrylamide gel of composition $T = 6\%$ and $C = 3\%$. As the buffer we chose 0.05 *M* sodium borate (pH 8.2). The temperature of the cooling water was around 30°C. Detection was effected at 230 nm and at a pump speed of 0.1 ml/min. The electropherogram in Fig. 4 shows that at least some of the constituents of Pharmalyte have a high UV absorption at 230 nm. Caution is therefore advised when this wavelength is used for the detection of proteins in isoelectric focusing experiments.

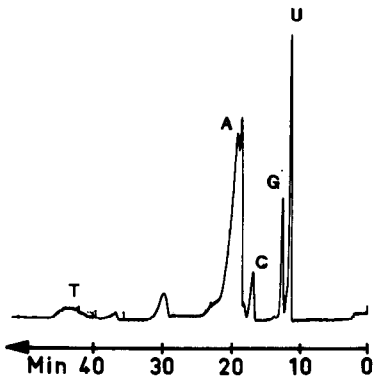


Fig. 3. High-performance electrophoresis of nucleosides. Sample: (U) uridine, (G) guanosine, (C) cytidine, (A) adenosine and (T) thymidine. The run was performed in a polyacrylamide gel.

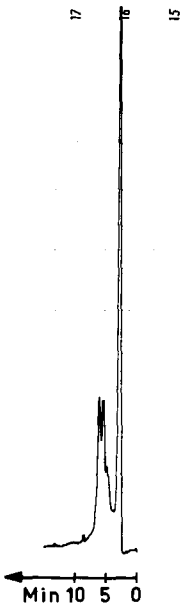
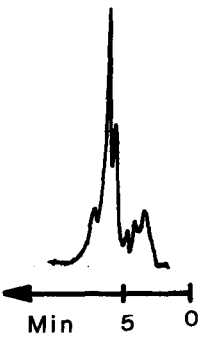


Fig. 4. High-performance electrophoresis of Pharmalyte, pH 2.5–5. Pharmalyte, pH 2.5–5, is the trade name of a carrier ampholyte for isoelectric focusing. The run was carried out in a polyacrylamide gel.



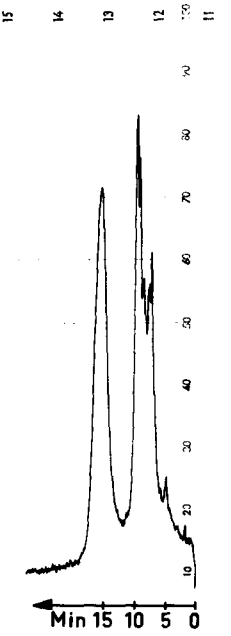


Fig. 5. High-performance electrophoresis of an enolase preparation. The run was carried out in a polyacrylamide gel.

Fig. 6. High-performance electrophoresis of a β-amylase preparation. Only material corresponding to the last peak had enzyme activity. The run was carried out in a polyacrylamide gel.

HPE of enolase

The experiment was performed in 0.05 *M* sodium borate (pH 8.2). The electrophoresis tube, which had a length of 70 mm, contained a polyacrylamide gel of composition $T = 6\%$ and $C = 3\%$. The cooling water had a temperature of 20°C. The effluent was monitored at 220 nm at a pump speed of 0.2 ml/min. A voltage of 2000 V gave a current of 60 μA. The first peaks in the electropherogram (Fig. 5) correspond to very rapidly migrating components. These are therefore probably low-molecular-weight components. The other peaks represent proteins. Electrophoresis of the enolase preparation in the same buffer and in a gel of the same composition, but with the more conventional dimensions 1.5 × 140 × 140 mm, gave a pattern similar to that shown in Fig. 5 (following staining with Coomasssie Brilliant Blue), with the difference that the first peaks were missing. When the HPE experiment was repeated at 0°C instead of 20°C, the run time was longer (the main peak emerged after about 13 min instead of 7 min).

HPE of β-amylase

The experimental conditions were similar to those given above for the HPE run with enolase. Fractions were collected at 1-min intervals and analysed for β-amylase activity. The electropherogram is shown in Fig. 6. Only fractions corresponding to the last peak showed enzymatic activity.

DISCUSSION

In the carrier-free electrophoresis experiments described herein (Figs. 1 and 2a) the capillary tube was coated with methylcellulose to eliminate disturbances caused by electroendosmosis[1] (without this treatment the zones were distorted); an alternative is to perform the runs in a buffer containing methylcellulose[19].

High-performance electrophoresis can be performed both in carrier-free solution (Figs. 1 and 2a) and in a supporting medium (Figs. 2b and 3–6). However, we often prefer to conduct a free electrophoresis experiment in the equipment particularly designed for such experiments[1], as it permits repeated scanning of the revolving electrophoresis tube and thereby permits control and observation of a run from start to finish. In addition, with this free zone electrophoresis apparatus one can determine mobilities with much greater accuracy than is possible with the equipment for HPE. Free zone electrophoresis also has the great advantage of being applicable to interaction studies, *e.g.*, interactions between different proteins and between proteins and cells[20]. The possibility of scanning the electrophoresis tube automatically at any desired time makes the free zone electrophoresis apparatus also very suitable for isoelectric focusing and displacement electrophoresis (isotachophoresis), as one can easily see when the steady state has been attained[21]. Free zone electrophoresis with its scanning system can even be used to detect uncharged solutes (see the comments on the nucleoside experiment below), as well as cationic and anionic solutes in the same run.

We have previously shown that the nucleosides cytidine, adenosine, guanosine and uridine can be rapidly separated within 10 min and analysed at pH 2.85 by free zone electrophoresis[1]. The same separation can be achieved at this pH by HPE. However, as uridine is uncharged at pH 2.85 and the monitoring system in HPE is

designed to detect only electrophoretically migrating components, uridine will not be recorded in the electropherogram[12]. An analogous situation occurs in chromatography when a substance is strongly adsorbed on the support. However, in both electrophoresis and chromatography, the problem can be circumvented by changing the experimental conditions such that all components in the sample migrate. Therefore, the separation shown in Fig. 3 was performed in a borate buffer of pH 10, in which borate forms negatively charged complexes with ribonucleosides (but not with the deoxyribonucleoside thymidine); in fact, uridine is the most rapidly migrating nucleoside in this system, as Fig. 3 illustrates. A separation of nucleosides can also be obtained by HPLC on dihydroxyboryl-agarose by using the same kind of complex formation[22].

If extremely short run times are required, one should not only work at high field strengths, but also at elevated temperature, as the mobility increases when the viscosity decreases. The effect of temperature was investigated briefly in connection with the enolase experiment (Fig. 5). When the temperature of the cooling water was decreased from 20 to 0°C, the migration rate of enolase was approximately halved. Most of the observed decrease in the migration rate can probably be ascribed to the increase in viscosity of water from 1.0 cP at 20°C to 1.8 cP at 0°C. As the duration of any HPE run is short, the risk of thermal denaturation of the sample is small, even if the temperature is increased considerably to decrease the run time. The experiment shown in Fig. 4 was deliberately carried out at elevated temperature (30°C) in order to obtain a very short analysis time.

Electrophoresis in molecular-sieving gels often gives much higher resolution than does electrophoresis in buffer alone when the sample consists of biopolymers[23]. Therefore, most of the HPE runs with proteins were performed in gels of polyacrylamide. Low-molecular-weight compounds, on the other hand, should give similar electrophoretic patterns in free solution and in a polyacrylamide gel. It is therefore surprising that the electropherogram in Fig. 2b differs so markedly from that in Fig. 2a (the same sample and the same buffer were used in both experiments). The most striking difference is that the largest peak appears in the front part of the electropherogram in Fig. 2a (n1) and in the rear part of the electropherogram in Fig. 2b (n2). An on-line analysis by a photodiode array spectrophotometer showed that this peak corresponded to naphthol green. It is interesting that this compound is also strongly retarded in chromatography on columns of polyacrylamide[24], which may be ascribed to so-called "aromatic adsorption"[25]. This adsorption has been utilized to fractionate chromatographically low-molecular-weight aromatic substances[25]. If the parallel observed between electrophoresis and chromatography also applies to aromatic compounds other than naphthol green, the aromaticity of a solute can be an important separation factor in the HPE of low-molecular-weight compounds in polyacrylamide gels. The degree of interaction should then depend on the concentration of the polyacrylamide gel and can thus easily be varied to tailor-make a gel for optimal resolution.

As our studies of HPE are still in the developmental stage, we have so far used only relatively short electrophoresis tubes (length $\leqslant 14$ cm) to facilitate the methodological studies. One can expect higher resolution if longer tubes are used.

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REFERENCES

1 S. Hjertén, *Chromatogr. Rev.*, 9 (1967) 122–219.
2 S. Hjertén, *Methods Biochem. Anal.*, 18 (1970) 55–79.
3 S. Hjertén, *Biochim. Biochim. Biophys. Acta*, 237 (1971) 395–403.
4 S. Hjertén, in E. Reid (Editor), *Methodological Developments in Biochemistry. Preparative Techniques*, Vol. 2, Longman, London, 1973, pp. 39–48.
5 L.-G. Öfverstedt, G. Johansson, G. Fröman and S. Hjertén, *Electrophoresis*, 2 (1981) 168–173.
6 S. Hjertén, Z.-q. Liu and S.-l. Zhao, *J. Biochem. Biophys. Methods*, 7 (1983) 101–113.
7 S. Hjertén, Z.-q. Liu and S.-l. Zhao, in D. Stathakos (Editor), *Electrophoresis '82, Proceedings of the Second Meeting of the International Electrophoresis Society*, Walter de Gruyter, Berlin, 1983, pp. 183–187.
8 L.-G. Öfverstedt, K. Hammarström, N. Balgobin, S. Hjertén, U. Pettersson and J. Chattopadhyaya, *Biochim. Biophys. Acta*, 782 (1984) 120–126.
9 N. Y. Nguyen, J. DiFonzon and A. Chrambach, *Anal. Biochem.*, 106 (1980) 78–91.
10 S. Hjertén, *J. Chromatogr.*, 270 (1983) 1–6.
11 S. Hjertén, in H. Hirai (Editor), *Electrophoresis '83, Proceedings of the Third Meeting of the International Electrophoresis Society*, Walter de Gruyter, Berlin, 1983, pp. 71–79.
12 M.-d. Zhu and S. Hjertén, in V. Neuhoff (Editor), *Electrophoresis '84, Proceedings of the Fourth Meeting of the International Electrophoresis Society*, Verlag Chemie, Weinheim, 1984, p. 110.
13 S. Hjertén and M.-d. Zhu, *Pure Appl. Chem.*, in press.
14 A. Tiselius, S. Hjertén and Ö. Levin, *Arch. Biochem. Biophys.*, 65 (1956) 132–155.
15 P. Bernfeld, *Methods Enzymol.*, 1 (1955) 149.
16 J. W. Jorgenson and K. DeArman Lukacs, *J. Chromatogr.*, 218 (1981) 209–216.
17 S. Hjertén, S. Jerstedt and A. Tiselius, *Anal. Biochem.*, 11 (1965) 219–223.
18 S. Hjertén, *Arch. Biochem. Biophys.*, Suppl. 1 (1962) 147–151.
19 S. Hjertén, *Ark. Kemi*, 13 (1958) 151–152.
20 S. Hjertén, in H. Bloemendal (Editor), *Cell Separation Methods*, Elsevier/North-Holland Biomedical Press, Amsterdam, 1977, pp. 117–128.
21 S. Hjertén, in N. Catsimpoolas (Editor), *Methods of Protein Separation*, Vol. 2, Plenum, New York, 1978, pp. 219–231.
22 S. Hjertén and D. Yang, *J. Chromatogr.*, 316 (1984) 301–309.
23 S. Hjertén, in G. Milazzo (Editor), *Topics in Bioelectrochemistry and Bioenergetics*, Wiley, New York, 1978, pp. 89–128.
24 S. Hjertén and R. Mosbach, *Anal. Biochem.*, 3 (1962) 109–118.
25 B. Gelotte, *J. Chromatogr.*, 3 (1960) 330–342.