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# RAPID SEPARATION OF DNA RESTRICTION FRAGMENTS USING CAPILLARY ELECTROPHORESIS

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## SUMMARY

Open-tube capillary electrophoresis has been applied to the separation of restriction fragments of DNA with a Tris–borate buffer containing 7 *M* urea and 0.1% sodium dodecyl sulfate. The importance of sample pretreatment and of the injection of heated samples has been demonstrated. In one separation, a DNA restriction fragment mixture from 72 to 23 130 base pairs (DRIgest™ III) (molecular weight range from $4.6 \cdot 10^4$ to $1.5 \cdot 10^7$) has been electrophoresed in 10 min on a column of 15 cm effective length. Over 600 000 plates have been obtained for individual peaks. Several of the peaks have been identified, by spiking slab gel electrophoretically purified components. Other examples of restriction fragment separations are illustrated in this paper. The results of this study when further validated with full characterization of individual species, open up the possibility of rapid restriction enzyme mapping.

## INTRODUCTION

The ability to analyze and manipulate DNA conveniently and rapidly is the cornerstone of the recent revolution in the biological sciences. With the discovery of restriction endonucleases, the molecular scissors of biology[1,2], a route was opened for characterization and use of fragments of large DNA molecules. Since individual restriction enzymes cleave at specific DNA base sequences, separation and analysis of individual fragments provides important information on the structure of DNA. Interestingly, the characterization of DNA is not only of importance in molecular biology but has also recently been promoted as a tool in forensic science for human identification[3].

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Restriction mapping, a method of separation and analysis of DNA fragments, is currently a major application of slab gel electrophoresis[4]. The basis of separation is believed to be size related since the charge density of large individual fragments is similar, except for conformational changes[5]. While successful, the slab gel method can be characterized as long and tedious, prone to error (particularly in not detecting all fragments), non-quantitative and cumbersome to use for isolation of individual fragments.

Capillary electrophoresis has recently emerged as an instrumental approach to electrophoresis (*e.g.*, refs. 6–13). Both open-tube and gel-filled capillaries can be used for separation. Remarkably high-performance separations are possible due to the high applied electric fields and operation in a manner that axial diffusion is the main cause of band broadening[14]. The method can be automated and used both for analysis and isolation of purified fragments.

This laboratory has previously separated small oligonucleotides by open-tube capillary electrophoresis using a metal–sodium dodecyl sulfate (SDS) micelle system[15]. In addition, we have used polyacrylamide gel-filled columns for separation of oligonucleotides differing by single bases and have isolated purified fractions[16]. The time required for isolation was greatly reduced over conventional methods for slab gel electrophoresis, elution and desalting. Others have separated small oligonucleotides[17] and eluted $\lambda$ DNA [48 kilobase (kb) pairs][18] in open-tube electrophoresis. Since samples of restriction fragments will normally consist of polynucleotides with a wide molecular weight range, we decided to examine the possibility of DNA restriction fragment separations with open-tube capillaries. This approach should in principle permit the migration of all molecular weight species. In this paper we report initial studies on the rapid separation of restriction fragments using open-tube capillary electrophoresis. These promising results offer the potential of a new approach to restriction mapping. Relative to slab gel electrophoresis the possibility exists of higher speed of separation, higher resolution, greater ease of isolation of individual fragments and a greatly increased dynamic molecular weight range for separation.

## EXPERIMENTAL

### *Apparatus*

Capillary gel electrophoresis was performed in fused-silica tubing (Scientific Glass Engineering, Austin, TX, U.S.A.), 75 $\mu$m I.D. with column lengths of 300–650 mm, depending on the experiment. The polyimide coating was carefully burned off at approximately the midpoint of the capillary for on-column detection. A 30-kV high-voltage d.c. power supply (Model LG-30R-5, Glassman, Whitehouse Station, NJ, U.S.A.) was used to produce the potential across the capillary. A plexiglass lock system with cut-off the circuit when opened was placed on the high-voltage side for safety. A UV detector (Soma S-3702, IR & D, Kingston, MA, U.S.A.), modified as previously described[15], was employed at a wavelength of 260 nm. The tubing and the detector were cooled using a thermostated air bath. The power supply outlets were connected to platinum electrodes, immersed in buffer reservoirs (for analytical runs) or in a microfuge vial (for collection). An analog–digital interface (Nelson Analytical, Cupertino, CA, U.S.A.) attached to a recorder and IBM PC/XT computer system were used to record the results and process the data.

*Materials*

DRIgest™ III ($\lambda$ DNA-Hind III/$\Phi$X174 DNA-Hae III), DRIgest™ II ($\lambda$ DNA-Hind III/$\Phi$X174 DNA-Hinc II) and $\Phi$X174 DNA-Hinc II digest were purchased from Pharmacia, (Piscataway, NJ, U.S.A.) and used as received. All other reagents were of protein-research grade (Schwartz/Mann Biolab, Cambridge, MA, U.S.A.). All buffer solutions were filtered through a Nylon GC filter unit of 0.2 $\mu$m pore size (Schleicher and Schuell, Keene, NH, U.S.A.). Samples were kept frozen at $-20$°C and sample solutions were stored at 4°C before use. Samples were heated at 60–65°C for 20 min prior to injection and were injected hot. Buffer solutions were carefully vacuum degassed.

*Procedure*

The fused-silica capillary tubing was filled with the desired buffer (0.1 *M* Tris–borate pH 8.1, 2.5 m*M* EDTA, 7 *M* urea and 0.1% SDS = buffer A). Both ends of the tube were then dipped into separate 5-ml reservoirs filled with buffer. The end in which the sample was introduced was connected with a platinum electrode to the positive high voltage side of the power supply. The reservoir at the detector end was connected with a platinum electrode to ground. Hot samples at a concentration of 0.25 mg/ml were introduced by siphoning with an estimated injection volume of 3–4 nl.

Before each run, the capillary was purged with 100 $\mu$l of 0.1 *M* sodium hydroxide solution followed by 250 $\mu$l of triply distilled water. Care was taken to equilibrate the capillary with buffer prior to operation. The reproducibility of retention was better than $\pm$ 3% relative standard deviation from run to run. It has been found that reproducibility is sensitive to temperature control.

## RESULTS AND DISCUSSION

We selected for study a standard digest —DRIgest III, which consists of a combination of the restriction enzyme, Hind III, digest of $\lambda$ DNA (48 kb) and the enzyme Hae III digest of $\Phi$X174 DNA (5 kb). This mixture, based on the known sequences of the DNAs, is expected to yield 19 fragments from 72 to 23 130 base pairs; however, the molecular weight difference of several pairs is not great (and generally not resolved) and several only appear faintly on gels upon staining[19]. It is to be noted that the molecular weight range of this sample is very wide ($4.6 \cdot 10^4$ to $1.5 \cdot 10^7$), necessitating different polyacrylamide or agarose gel compositions to encompass the whole mixture[20].

It is known that a sample of DNA fragments must first be carefully treated in order to break aggregated base-pair species. Moreover, DNA can break into smaller fragments as a result of shear forces, the lability being a function of the size of the DNA. Care must therefore be exercised in sample handling. In addition, samples were always freshly used and stored at $-20$°C.

Direct injection of an untreated sample of the DRIgest III mixture resulted in poor separation, with very broad bands under a variety of buffer and column temperature conditions. This is possibly due to the sticky ends of $\lambda$DNA aggregating together[19]. We then turned to the standard pretreatment procedure for dissociating aggregates, namely heating the sample at 60°C for 15–20 min. This approach most likely does not break base-paired fragments, since 7 *M* urea is not present[21].

Fig. 1 shows an electropherogram after cooling the heat treated sample to 25°C and then injecting into a column at room temperature containing buffer A. Improved separation resulted over an untreated sample; however, a broad early eluting band was observed. This band was suspected to be aggregated fragments of deoxyoligonucleotides, perhaps arising from reaggregation of the small fragments upon cooling.

Successful separation required injection of the hot sample solution on the column and rapid application of the electric field. In this work, 3–4 nl of the heat-treated sample was siphoned into the column, and an electric field of 500 V/cm was immediately applied. In this manner high-performance separations were achieved, as shown in Fig. 2A. Evidently, the applied electric field is able to act as a counterbalancing force to aggregation by providing rapid separation of individual fragments. Not only were the sample pretreatment and injection steps important, but the composition of the buffer was also significant. For example, removing 7 *M* urea from buffer A caused formation of a broad band as in Fig. 1. Similarly, 0.1% SDS was found to be a necessary ingredient for achieving successful separation. Finally, substitution of Tris–phosphate for Tris–borate at pH 8 caused a significant loss in

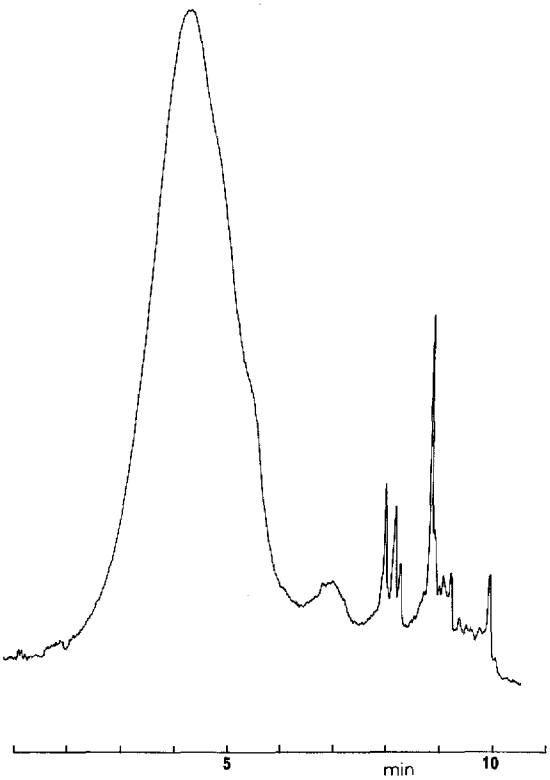


Fig. 1. Influence of sample pretreatment on electropherogram of DRIgest III. Sample heated for 20 min at 60°C, cooled to room temperature, and injected by siphoning. Conditions: Buffer: 0.1 *M* Tris–borate, pH 8.1, 2.5 m*M* EDTA, 0.1% SDS, 7 *M* urea; column 300 × 0.075 mm I.D., effective length 150 mm; applied voltage, 15 kV, 30 μA; detection, UV, 260 nm; temperature, 27 ± 0.5°C.

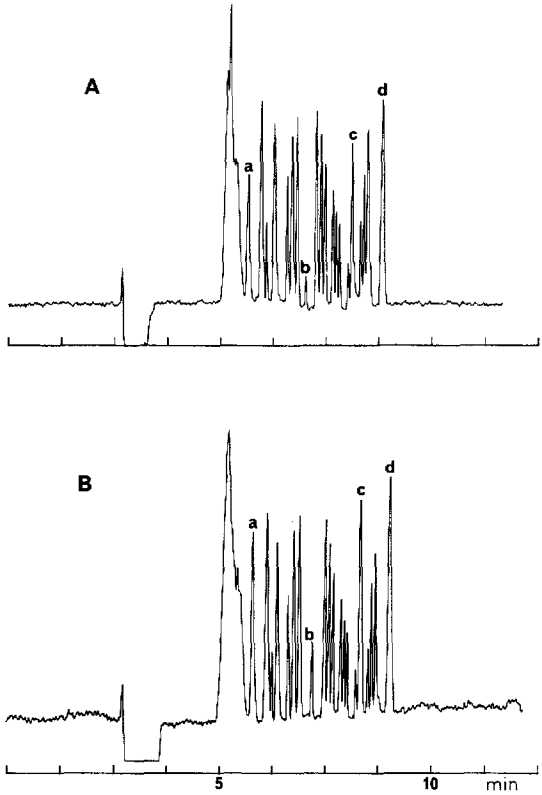


Fig. 2. (A) Separation of DRIgest ($\lambda$ DNA-Hind III/$\Phi$X174 DNA-Hae III). Sample heated for 20 min at 60°C, injected hot by siphoning (3–4 nl). All other conditions in Fig. 1. (B) Separation of DRIgest III sample spiked with 4 slab gel electrophoretically purified fragments: 72 (a), 564 (b), 4362 (c) and 23 130 (d) base pairs. Note the increased peak areas for the spiked peaks relative to those (A). All conditions identical to (A).

resolution. Other systems, perhaps involving complexation[15], may also prove useful for resolution purposes.

Returning to Fig. 2A, high-performance separation is observed, with the appearance of 20 peaks, after a small initial broad band. Electroosmotic flow occurs towards the negatively charged electrode (cathode), due to the negative zeta potential on the walls. Since the restriction fragments are also negatively charged, elution order is in increasing electrophoretic mobility towards the positive electrode. In order to demonstrate that restriction fragments are indeed being separated we next examined the elution order of several purified fragments.

Using standard Hind III digest conditions of $\lambda$ DNA, we separated individual fragments in a conventional manner on a slab gel consisting of 0.8% agarose. Several bands were observed upon staining with ethidium bromide and each band was electroeluted into a dialysis bag. The DNA was recovered by precipitation with ethanol to yield purified fragments.

Two fragments were isolated in sufficient quantity for examination: 564 and

23 130 base pairs. On the agarose gel, these fractions appeared pure on the basis of ethidium bromide staining. Fig. 3B and D show the elution of these two substances from the capillary electrophoresis column, using identical conditions as in Fig. 2A. The electropherograms appear fairly pure in both cases.

Fig. 3A shows the electropherogram of a purified 72-base pair fragment obtained from the plasmid M13 mp18. The fragment was purified by slab gel electrophoresis using 2% agarose. After excision and electroelution into a dialysis bag, the fragment was recovered and further purified by ion exchange. Finally, Fig. 3C shows the electropherogram of a 4362-base pairs fragment obtained from the plasmid pBr 322 which was again digested with Eco RI. Note that for 72 and 4362 base pairs, the base pair number agrees with a fragment of DRIgest III, but the sequence is not the same.

We next added a small amount of each purified fragment to a DRIgest III sample, and the electropherogram of this sample is shown in Fig. 2B. A rudimentary comparison of peaks a–d in Fig. 2A and B reveals that increases in peak height occur for all four bands. This result strongly suggests that elution is in order of increasing molecular weight, *i.e.* the highest molecular weight has the largest electrophoretic mobility. These promising results require further validation, both by examining a larger number of purified fractions and also by collecting fractions from the capillary[16] followed by slab gel electroporesis. Nevertheless, restriction fragment separations have been achieved. Let us next examine several aspects of the separation.

It is generally believed that electrophoretic mobility differences in free solution are independent of the moleclar weight of the restriction fragment.[4] The fact that separation is observed in an open capillary format as in Fig. 2 may in part be related to the high resolving power of the capillary electrophoretic approach. While plate number ($N$) varies from peak to peak, an average value would appear to be 600 000 plates. With such large plate numbers rapidly generated, the power of the high-performance capillary electrophoresis approach can be understood in the following way.

The resolution ($R_s$) of two adjacent peaks can be written as

$$R_s = \left(\frac{N^{1/2}}{2}\right)\left(\frac{\Delta\mu}{\bar{\mu}}\right) \tag{1}$$

where $\Delta\mu$ is the difference in electrophoretic mobilities of adjacent pairs and $\bar{\mu}$ is the average mobility for the set of fragments. If $R_s = 1$ is assumed to be minimal resolution, then for $N = 6 \cdot 10^5$, $\Delta\mu/\bar{\mu} = 5.3 \cdot 10^{-3}$. In other words, an electrophoretic mobility difference of 0.5% will lead to baseline resolution. Thus the high plate count permits subtle differences in mobility to be observed.

Based on retention time and a hold-up time ($t_0$) of 2.8 min, it is possible to calculate the apparent mobility $\mu_{app}$ and the true electrophoretic mobility $\mu_e$

$$\mu_{app} = \mu_e + \mu_0^* \tag{2}$$

where $\mu_0^*$ is the effective mobility of the electroosmotic flow. For the 72-base pairs fragment $\mu_{app} = 9 \cdot 10^{-5}$ cm$^2$/V s and for the 23 130-base pairs fragment $\mu_{app} = 5.4 \cdot 10^{-5}$ cm$^2$/V s. The electrophoretic mobilities $\mu_e$ are then $0.9 \cdot 10^{-4}$cm$^2$/V s and

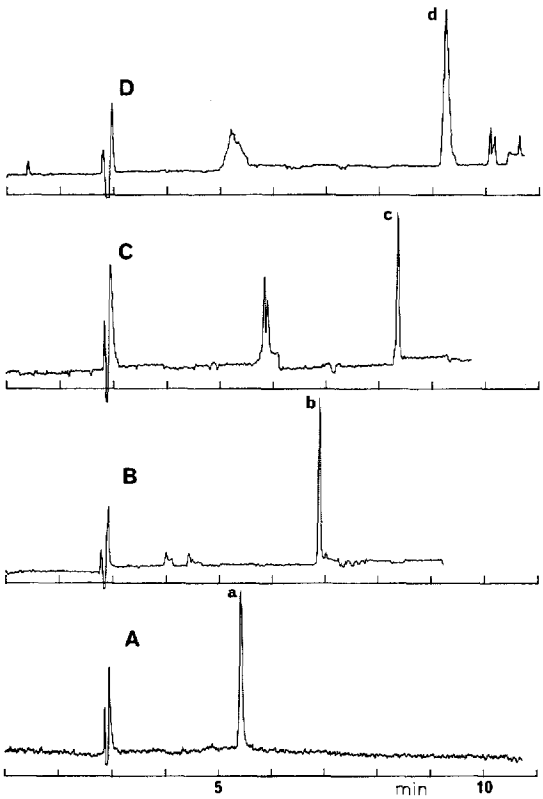


Fig. 3. Electropherograms of individual restriction fragments used in the spiked sample of Fig. 2B. Conditions as in Fig. 2A. (A) 72-base pairs gel-purified fragment from plasmid M13 mp18 DNA. After electroelution the fragment was further purified using an ion-exchange column. (B) 564-base pairs fraction purified from a $\lambda$ DNA-Hind III digest using slab gel electrophoresis, 0.8% agarose. Gel slice of this fraction (stained with ethidium bromide) was electroeluted into a dialysis bag. The fraction was recovered by precipitation with ethanol. The precipitate was then washed with 80% aq. ethanol, suspended in deionized water and lyophilized. (C) 4362-base pairs fraction from plasmid DNA pBr 322 was linearized by Eco RI digestion. (D) 23 130-base pairs fraction purified from a $\lambda$ DNA-Hind III using a slab gel electrophoresis.

$1.3 \cdot 10^{-4}$cm$^2$/V s for the smaller and larger fragments, respectively. These values are in the proper order of magnitude for base pair restriction fragments in solution[22]. The mobility change is seen to be small given a molecular weight variation of 300-fold. In terms of electrophoretic mobility, the column is thus able to achieve baseline resolution of differences of *ca.* $5 \cdot 10^{-7}$cm$^2$/V s. Clearly, column efficiency is very important in restriction fragment separations. Further improvement in resolution and column performance may be anticipated.

A second factor in the high resolution of the restriction fragments is the buffer selected. As already noted, Tris–phosphate did not yield the resolving power of Tris–borate. Undoubtedly, borate complexation with the sugar moieties affects mobility (charge and conformation) in a manner to alter electrophoretic mobility differences[23]. Secondly, SDS and urea were both found to be important in yielding

sharp bands. This may be related to the disaggregating effect of these species[24]. In addition, it is possible that SDS hydrophobically binds to oligonucleotides resulting in mobility changes. Further studies are underway to understand in more depth the retention and separation mechanism, as well as to examine other possible chemistries for selectivity.

Returning to Fig. 2B, four peaks have been identified by spiking of a DRIgest III sample. Interestingly, there are three peaks observed between 4362 and 23 130 base pairs. Theoretically, two peaks are expected (6557 and 9416 base pairs). The extra peak could arise from an impurity in the sample; however, collection and identification would be necessary to clarify this point.

That extra peaks can occur after slab gel purification can be seen in Fig. 3. The electropherogram for the 23 130-base pairs fraction reveals several peaks eluting later than the major peak, and these peaks presumably represent higher-molecular-weight polynucleotides. These could arise from incomplete cleavage (*e.g.*, a 2000-base pairs fragment is adjacent to the 23 130-base pairs fragment) as well as association of the sticky ends of $\lambda$ DNA after fragmentation. It is interesting to note that only a single stained band of the 23 130-base pairs fragment was observed on agarose and this was excised from the gel; however, extra peaks are observed in capillary electrophoresis.

We also note two extra bands of presumably lower-molecular-weight fragments with the 4362-base pairs peak (Fig. 3C). These species arise either from further fragmentation upon sample handling or the possibility of entrapment of small fragments in the duplex helical structure.

Finally, in Fig. 2 the broad peak eluting early in the electropherogram of DRIgest III was investigated. The peak was collected in water (2 $\mu$l) and then analyzed by polyacrylamide slab gel electrophoresis. The peak was assumed to consist of small oligonucleotide fragments (less than 72 base pairs) based on ethidium bromide staining. Thus, the band may be broad as a result of incomplete resolution of small fragments.

Having examined DRIgest III, we next turned to several other restriction fragments to assess separation. Fig. 4 shows the separation under identical mobile phase conditions of Fig. 2 of DRIgest II which is a combination of $\lambda$ DNA-Hind III and $\Phi$X174 DNA-Hinc II. A total of 21 peaks are expected, again with a few components either difficult to resolve or detect. A total of 21 peaks are observed with high resolution in a narrow time window. It is to be noted that for this example (as well as for Fig. 5), a longer column was employed and a slightly lower field (300 *vs.* 500 V/cm). Hence, elution required a longer time than in Fig. 2. Finally, Fig. 5 shows a corresponding separation of $\Phi$X174-Hinc II. Here, a maximum total of 13 peaks are expected (though several are quite difficult to observe by slab gel electrophoresis). A separation with 12–13 peaks is found in this figure. As with DRIgest III, it was necessary to inject hot into the column samples in both Figs. 4 and 5.

## CONCLUSIONS

This paper has presented initial results on the open-tube capillary separation of restriction fragments. An open tube has been selected in order to encompass the wide molecular weight range in one run. Successful operaton required careful attention to

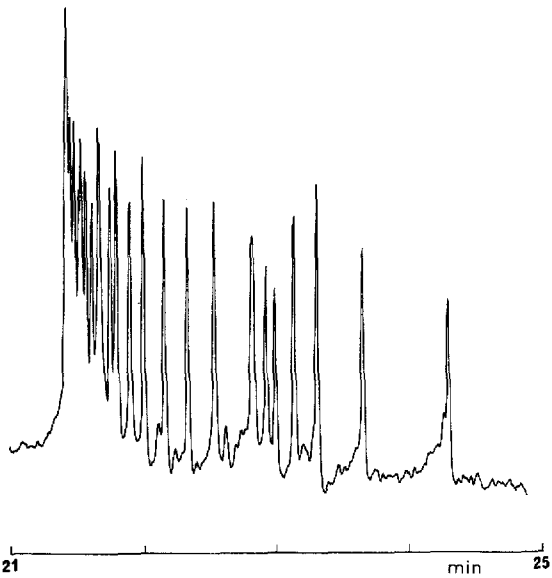


Fig. 4. Separation of DRIgest II (λ DNA-Hind III/ΦX174 DNA-Hinc II). Conditions as in Fig. 2A except column: 500 × 0.075 mm I.D., effective length 250 mm; applied voltage 15 kV, 18 μA.

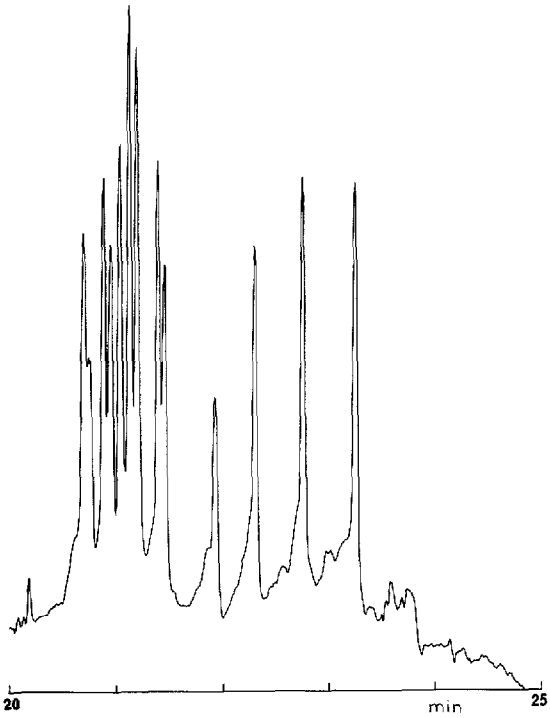


Fig. 5. Separation of ΦX174 DNA-Hinc II. Conditions as in Fig. 4.

sample handling and pretreatment. Hot sample injection produced the best results.

Given the similarity of mobilities of the restriction fragments, even with a molecular weight difference of 300-fold, high efficiency was found to be essential for success. With columns of 600 000 plates, mobilities differing by 0.5% or less should be resolvable. This translates to $5 \cdot 10^{-7}$ cm$^2$/V s difference with a restriction fragment mobility of $1 \cdot 10^{-4}$ cm$^2$/V s.

We have been able to identify several peaks in the electropherogram of DRIgest III by spiking the sample with purified fragments. More work is necessary in identifying a number of other fragments. The strategy to be taken will be to use other purified fragments, as well as a collection of electropherogram peaks (or peak clusters) and identification by agarose or polyacrylamide slab gel electrophoresis.

Ultimately, capillary electrophoresis should be useful in restriction mapping as well as optimization of digestion conditions for DNA. In addition, with appropriate resolution, it should be possible to isolate individual fragments[16] for further study (*e.g.* sequencing) as well as for subcloning. Work is continuing in our laboratory in this area.

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