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**Size fractionation of DNA fragments by liquid-liquid chromatography**

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**ABSTRACT**

A method for the fractionation of double-stranded DNA fragments from 150 to 22000 b.p. in size by liquid-liquid chromatography is described. The procedure makes use of the fact that the partitioning of DNA in a polyethylene glycol-dextran system is size dependent and can be altered by alkali metal cations. Cellulose or celite are used as supports for the stationary, dextran-rich phase. Examples show the fractionation of digests of T7 DNA produced by Dpn II and Hind II restriction endonucleases as well as  $\lambda$  DNA digests produced by Hind III and Eco RI restriction endonucleases.

**INTRODUCTION**

The separation of DNA fragments by size is usually performed by preparative gel electrophoresis or chromatography on RPC-5 resins. Both procedures have their merits and are widely used despite some well known limitations. These consist for the first method in the limited capacity, especially for larger fragments, and the difficulty of recovering the DNA in a pure form from the gel. The second method suffers from lack of resolution of fragments above 400-500 base pairs in size, as well as from the limited availability of RPC-5 resin of the right properties.

The chromatographic procedure described in the present paper makes use of the well known fact that polyethylene glycol (PEG) and dextran form two phases in aqueous solution if their concentrations exceed certain values. DNA distributes between these two phases with partition coefficients (K) which depend strongly on the kind and concentrations of salts added. Albertsson, to whom we owe most of the basic knowledge on the partition of biological molecules and particles in such systems, has compiled the relevant data on the behavior of DNA in this system in his book (1).

About 12 years ago it was reported that the partition of DNA in a PEG-dextran system depends markedly on its size (2), a fact which apparently has never been used to fractionate DNA fragments by countercurrent distribution. When we detected that cellulose binds the dextran-rich phase of such systems strongly enough to provide stationary phases for liquid-liquid chromatography, we attempted to adapt this procedure for DNA fractionation. This paper describes the preparation and the use of such columns for size fractionation of DNA fragments up to 40 kilo base pairs (k.b.). It is clear that the technique reported here can also be used for solving other separation problems if the factors affecting the partition of the particles in the phase pair are appropriately adjusted.

### MATERIALS AND METHODS

Cacodylate buffer: 5 mM sodium cacodylate, 5 mM cacodylic acid (from Sigma or Fluka), 2 mM EDTA adjusted to pH 6.0-6.1 by acetic acid if necessary.

Chemicals were purchased from the following sources: Sodium acetate: BDH or Merck AG, Darmstadt; Lithium sulfate: Merck AG, Darmstadt; Lithium acetate: BDH or C.Roth, Basel; Dextranase: Sigma Chemical Co.

Polyethyleneglycol 6000: Unfortunately there are several types of polyethyleneglycol (PEG) "6000" on the market, ranging from 6000 to nearly 9000 in their mean molecular weight. In addition, older products stored for several years often contain substantial amounts of acid, probably due to endgroup oxidation. We always used the product sold by Sigma or Union Carbide. Both products are about 8000-9000 in mol. wt. and contain no more than  $2 \times 10^{-6}$  equiv. of acid per gram (checked by titration). They both contain UV-absorbing materials ( $\lambda$  max. = 287 nm) probably caused by antioxidants added. PEGs of 6000-7500 mol. wt. are not suitable since solutions of higher concentrations are needed in order to obtain a phase separation. This increases unnecessarily the viscosity of the phases and might lead to precipitation or aggregation of DNA.

Dextran T500 was obtained from Pharmacia.

Cellulose, microgranular, type CC31 was purchased from Whatman Ltd. England. A generous gift of a larger quantity is gratefully acknowledged.

Celite 545 (Johns Manville Corp.) was purchased from Serva, Heidelberg. The product was fractionated by size using sieves of 100, 36 and 25  $\mu$ mesh

size. The material larger than 100  $\mu$  was discarded, the fractions between 100 and 36  $\mu$  (designated as "coarse") and the fraction between 36 and 25  $\mu$  (designated as "fine") were used. Material finer than 25  $\mu$  was discarded as well. The sieved material was boiled in a sixfold quantity of 0.05 M EDTA, pH 8, and subsequently in 0.2 M sodium acetate solution pH 8.3 for 5-10 min. After thorough washing with distilled water, it was dried to constant weight at 120°C.

Hind II and Dpn II restriction fragments of T7 DNA were prepared as described elsewhere (R. Haas and P. Cole, manuscript in preparation).

Hae III restriction fragments of Col EI DNA (above 400 b.p.) were kindly supplied by B. Eshaghpour.

$\lambda$ -DNA containing several lac operons of E. coli was a kind gift of M. Fried. This DNA was digested using Eco RI restriction nuclease producing six fragments A to F, ranging respectively from 20.7 to 3.2 k.b. The A and F fragments correspond to the two terminal regions of  $\lambda$  DNA which contain 20 base complementary, single-strand ends.

#### Gel electrophoresis

a) Agarose Gels were run on a Studier-type horizontal electrophoresis apparatus using Seakem agarose in Tris (40 mM)-sodium acetate (20 mM)-EDTA (2 mM) buffer, pH 7.8.

b) Polyacrylamide Gels (3.5 and 5%) were run on a 20 x 40 x 0.3 cm vertical slab apparatus under the conditions given by Maniatis et al. (3).

## RESULTS

Partitioning of DNA-fragments in PEG-dextran systems. The work of Albertsson (1) showed that the partition coefficient ( $K = \frac{[DNA]_{\text{PEG phase}}}{[DNA]_{\text{dextran phase}}}$ ) of DNA in PEG-dextran systems can be altered drastically by the addition of different salts. The most interesting effect among the cations is exerted by lithium ions which raise the K value, while the other alkali cations show the opposite effect. Among the anions, the multivalent species such as sulfate, citrate, and secondary or tertiary phosphate increase K, while the monovalent species either lower K or have no effect. Albertsson (1) has given an explanation for these phenomena in his book.

The size-dependent partitioning of DNA in PEG 6000-dextran T500 as reported by Favre and Pettijohn (2) could easily be confirmed for various

PEG-dextran systems using DNA restriction fragments of various lengths and measuring their distribution by gel electrophoresis of equal aliquots of the two phases. The overall partition coefficients were adjusted to unity as closely as possible by varying the ratios of  $\text{Li}^+$  to  $\text{Na}^+$  or secondary to primary phosphate ions, respectively. Inspection of the patterns (Fig. 1) reveals a definite preference of the larger fragments for the dextran-rich lower phase while the smaller fragments prefer the PEG-rich upper phase of the systems. More quantitative data are obtained by scanning the negatives.

#### Liquid-liquid Chromatography of DNA-Fragments

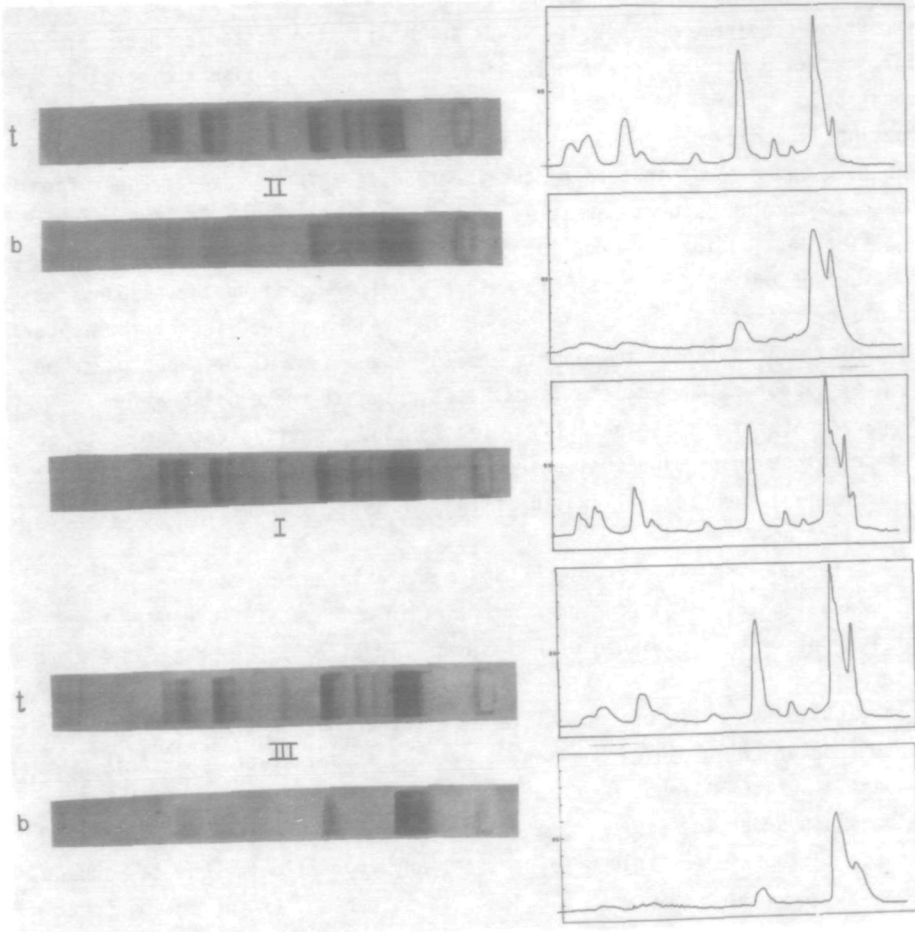
a. General technical observations. Soaking cellulose powder in the lower, dextran-rich phase of a PEG-dextran system at  $80^\circ\text{C}$  for 1 hour causes swelling of the cellulose, which is accompanied by physical binding of 1.2-1.5 g of phase per gram of dry cellulose. The term "physical binding" means that the bound dextran phase is not washed off by extensive rinsing with the PEG-rich upper phase at temperatures between  $25$  and  $37^\circ\text{C}$  either on a Buchner funnel or in a column. In addition to cellulose, celite may also be used as a support although it binds less of the lower phase.

Our first attempts to separate DNA fragments on columns prepared from cellulose or celite coated to saturation by lower phase revealed very broad bands, especially for fragments above 5 k.b. in size, with extensive tailing causing a substantial overlap of the zones. These difficulties were finally overcome by the following means:

- (i) application of salt gradients in the mobile PEG-phase, which gradually increase the partition coefficient of the DNA,
- (ii) careful selection of suitable flow rates which take into account the decreasing equilibration rate with increasing fragment size,
- (iii) coating the support particles by a very thin and uniform film of lower phase.

Concerning the first point, we apply two consecutive salt gradients in the PEG-rich phase, if fragments from 100 base pairs to about 50 k.b. are to be resolved. The first gradient runs from 0.14 M potassium acetate to 0.14 M sodium acetate; the second one runs from 0.14 M sodium acetate to 0.128 M lithium acetate-0.004 M lithium sulfate. Both gradients are linear and have a slope of 0.47 mM salt change per ml, corresponding to  $2 \times 150$  ml of fluid per gradient, which is suitable for columns of  $0.64 \text{ cm}^2$  cross section (0.9 cm diameter) and about 1 m in length. Thicker columns are run with multiples of these volumes proportional to the increase of cross section.

A mean flow rate of about 4 ml/hour was found to be acceptable for



**Figure 1.** 0.7% agarose gels and corresponding densitometer tracings of upper and lower PEG-dextran phases between which Dpn II fragments of T7 DNA have been partitioned. The gels were stained with ethidium bromide (1  $\mu\text{g}/\text{ml}$ ) for 45 min. (I): Total digest. (II): Top (t) and bottom (b) phase of a PEG 6000-dextran T2000 system in cacodylate buffer. The partition coefficient of the added DNA was adjusted to near unity by the addition of lithium and sodium acetate (total cation concentration 30 mM). (III): Top (t) and bottom (b) phase of a PEG 6000-dextran T500 system at a total cation concentration of 150 mM (other conditions as for II).

fragments ranging from 150 base pairs to 15 k.b. when they were run on a column of  $0.64 \text{ cm}^2$  cross section. This rate may be scaled linearly with the cross section of the column.

The thin and uniform coating of the support seems to be the most important requirement since it avoids long diffusion distances necessary for the DNA particles to reach the interphase and establish the partition equilibrium determined by the ion composition of the gradient. This coating is achieved by suspending the dry cellulose or the celite in the PEG-rich upper phase instead of the lower phase. The uptake of water from the phase by the support particles results in a disturbance of the phase equilibrium, leading to formation of lower phase in very fine droplets on the surface of the particles. To ensure a uniform coating the suspensions should be stirred at speeds high enough to avoid any settling of the support particles. Both types of supports are suspended in 7 ml of upper phase per gram of support. When celite is used, stirring at the equilibration temperature of the phase pair ( $T_{eq}$ ) for 15 min. is sufficient for coating. The cellulose suspension must be heated to 80°C for one hour, cooled to 2°C below  $T_{eq}$  and then reequilibrated at  $T_{eq}$ . All steps should be performed with stirring.

After pouring the suspension into a suitable column equipped with a thermostating jacket (if one does not work in a thermostated room), the settled column bed is washed with two bed volumes of upper phase at a flow rate which may be up to 2-3 times higher than the speed of the run. It is important to use cellulose or celite of a rather narrow size distribution. Microgranular cellulose of Whatman, type CC31 is satisfactory in this respect. Celite 545 must be fractionated by sieving (see Materials and Methods) in order to ensure a homogenous packing in the columns. With respect to the kind of column to be used, any type allowing one to work at somewhat elevated pressure (2-5 kg/cm<sup>2</sup>) is suitable. If the column contains sintered teflon disks on the end piece or plunger, these should be exchanged for sintered polyethylene discs since the teflon discs may adsorb DNA from the media used. The pressure required to establish sufficient flow rates may easily be obtained by good peristaltic pumps.

Any UV monitor stable over several days can be used as a detector. The shape of the flow cell was found to be important, however, since the small flow rate of the relatively viscous phase may produce a laminar flow in a cell if the latter is much wider than the tubing used. The best results were obtained with quartz capillaries of 1-1.2 mm I.D. (2.5-3 mm outer diameter) mounted behind a 0.8 mm slit aperture in the light beam of the monitor. It finally should be noted that packed columns may be reused many times when reequilibrated with 2-3 void volumes of fresh PEG-phase

between each run. No decrease of resolving properties could be detected with increased use of a column. It is important, however, to keep the columns at constant temperature (about 1°C below equilibration temperature) when not used.

b. Detailed description of the procedure: (The procedure described was performed at  $T_{eq} = 25^\circ \pm 1^\circ\text{C}$ ;  $T_{eq}$  may be any temperature between 21° and 30°C). 114 g of PEG 6000 (Sigma or Union Carbide) and 8 g of dextran T500 are dissolved in 1878 g of cacodylate buffer at 25°C ( $\pm 1^\circ$ ). (The resultant fluid should be turbid; if a clear solution results, the PEG used is too small in molecular weight). If DNA fragments smaller than 1.5 k.b. are to be separated, about 800 g. of the fluid are made up to 0.14 M in potassium acetate by dissolving the appropriate amount of dry salt in the turbid PEG-dextran solution ("phase I"), a further 800 g is made up to 0.14 M sodium acetate in the same way ("phase II"), and 400 g is made up to 0.128 M lithium acetate and 0.004 M lithium sulfate ("phase III") (4). The phases are cleared either by centrifugation or by storage for 2-3 days at 25°C. The amount of lower phase settled is less than one percent. The upper phases are carefully removed with wide tipped pipettes. The phases should be covered when stored since evaporation of solvent leads to the reappearance of two phases. 30 g of cellulose or celite (coarse or fine) is suspended in 210 g of phase I, degassed and treated as indicated in section a. This amount is sufficient for a column of 100 x 0.9 cm in size. The slurry is poured using a reservoir on top of the column large enough to contain the total amount. The slurry is stirred from time to time in the reservoir. After the support has settled, an adjustable plunger is fitted to the top of the bed and 130 ml of phase I is pumped at 18-24 ml/hr through the column bed (5). The absorbance of the eluate should reach a constant level during this rinsing. To prepare the sample, the DNA (up to 3 mg) in a volume of about 1-4 ml is dialysed against cacodylate buffer. Potassium acetate (4 M) in cacodylate buffer, is added to a final concentration of 0.14 M. Solid PEG is added to give a final concentration of 5.7% (w/w). No dextran is added to the sample. It should be noted that DNA fragments larger than 8 k.b. may be bound irreversibly to the column if samples had been stored in frozen solutions.

The DNA sample is pumped at a speed of 4 ml/hr into the column. Approximately 10 ml of phase I is used to rinse the residual sample onto the column. The gradient is then attached to the column and the column run at the selected speed.

If fragments to be resolved are larger than 450 base pairs in size, the potassium acetate-sodium acetate gradient may be omitted. Coating and rinsing of the support may then be performed in phase II, the run being done with a gradient from phase II to phase III only. The recovery of DNA is greater than 90% even for DNAs of 45 k.b. in size.

For analysis of the fractions by gel electrophoresis, samples are adjusted to a final concentration of 5% glycerol, 0.002% bromophenol blue in 0.1 to 1 x electrophoresis buffer, and loaded on polyacrylamide or agarose gels and run under standard electrophoresis conditions. The dextran binds some of the bromophenol blue without affecting the mobility of large DNA. However, those fragments migrating faster than bromophenol blue may coelectrophorese with the dye, depending upon the amount of dextran present.

Recovery of the DNA from the eluate may be done in one of the following ways:

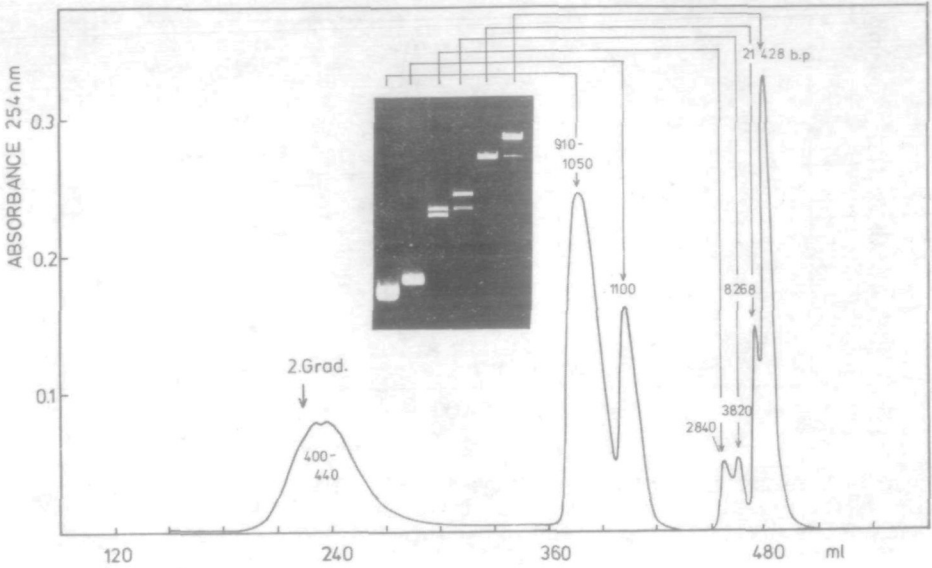
- (1) The combined fractions are extracted twice with chloroform containing 5% isopropanol in order to remove the PEG. Low speed centrifugations may help to clear the phases. The aqueous phase is concentrated in a rotary evaporator at 30°-35°C bath temperature and dialysed against cacodylate or phosphate buffer (pH 6). The DNA may then be precipitated by cetyltrimethyl ammonium bromide and isolated according to Seibert and Zahn (6).

- (2) Smaller fragments (< 1000 b.p.) may be recovered after chloroform extraction by digesting the dextran with dextranase in one of the dialysis buffers indicated above, followed by ethanol precipitation.

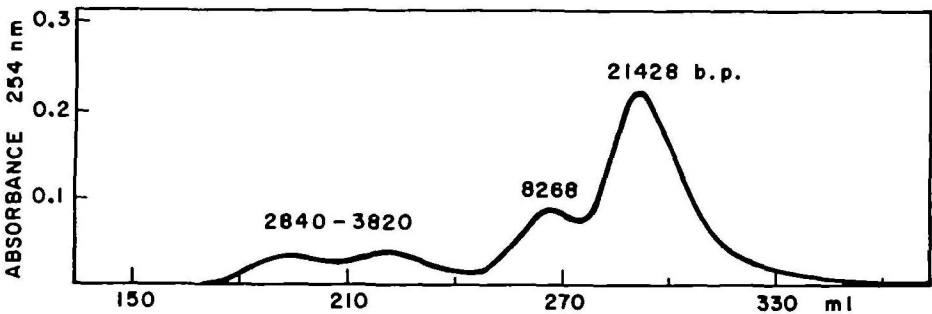
- (3) The DNA plus polymers may be coprecipitated with ethanol and the DNA separated from the mixture by ion exchange chromatography (i.e., diethylaminoethyl-cellulose).

c. Experimental results. Figure 2 shows the elution profile of an artificial mixture of Hae III fragments of Col EI DNA (above 400 b.p.) and Dpn II fragments of T7 DNA from a column containing cellulose as the support. The resolution is not equal over the whole size range, becoming less for the larger fragments. We tried to obtain a better resolution by using a shallower salt gradient. The profile in Fig. 3 for the larger Dpn II-fragments (2,840 to 21,428 b.p.) shows that this does not yield a better resolution but results in a peak broadening only. Figure 4 shows an elution profile obtained with Hind III fragments from  $\lambda$  DNA. The result is very similar; the separation of the two fragments in the 6 k.b. range from the 23 k.b. piece is somewhat clearer, however.





**Figure 2.** Column fractionation of an artificial mixture containing Hae III fragments (> 400 b.p.) of Col EI DNA and Dpn II fragments of T7 DNA. Column: 100 x 0.9 cm; support: cellulose (Whatman CC31) coated with PEG 6000-dextran T500 described in the text. First gradient: 0.14 M KOAc + 0.14 M NaOAc (2 x 125 ml, linear); flow rate: 6 ml/hr. Second gradient: 0.14 M NaOAc + 0.120 M LiOAc, 0.02 M NaOAc (2 x 125 ml); flow rate: 3 ml/hr.



**Figure 3.** Elution profile of Dpn II fragments of T7 DNA (ranging from 2840 to 21428 b.p.). Column and phase pair as for Fig. 2, eluting gradient: 0.07 M NaOAc, 0.07 M LiOAc + 0.120 M LiOAc, 0.02 M NaOAc (2 x 200 ml); flow rate: 2 ml/hr.

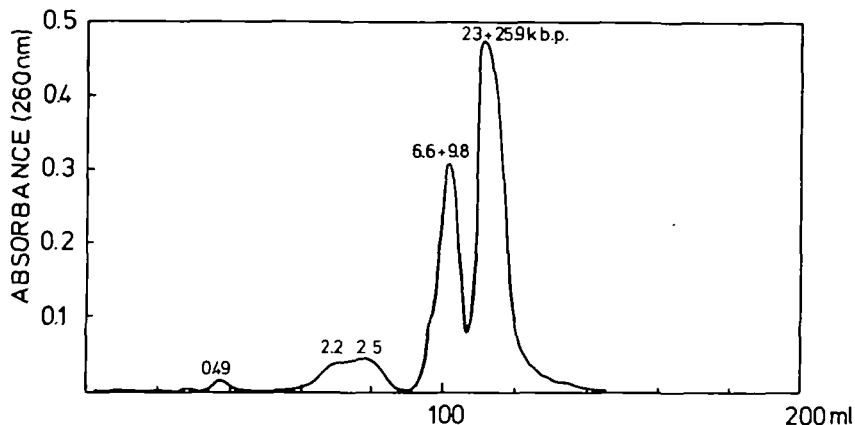
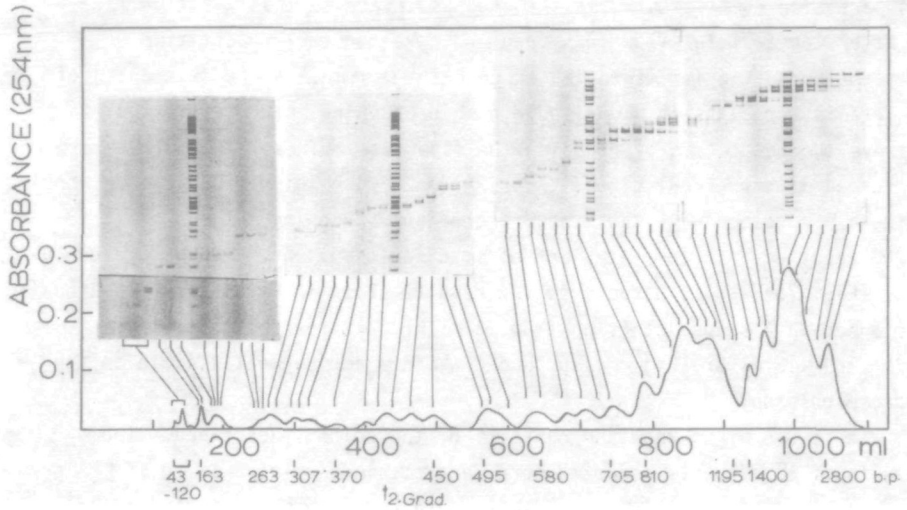


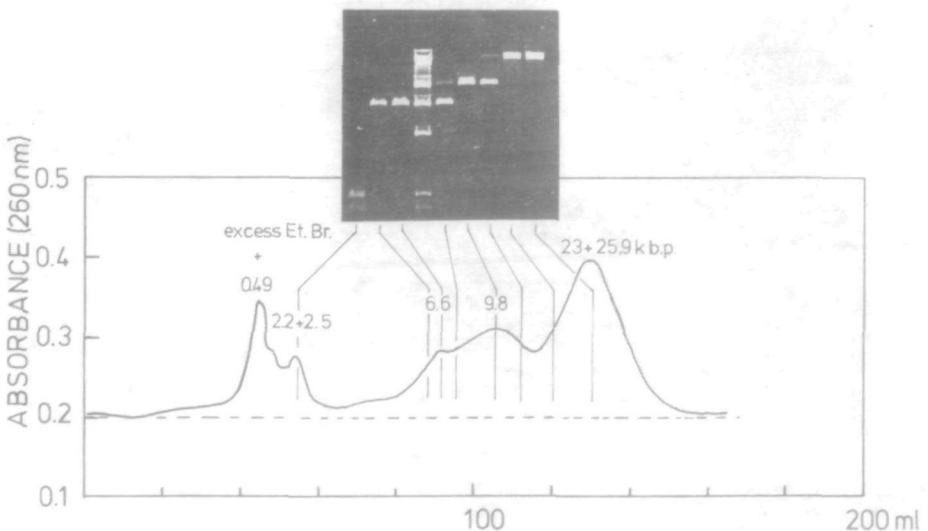
Figure 4. Separation of Hind III fragments of  $\lambda$  DNA: Column and phase pair as for Fig. 2; eluting gradient: 0.1 M Na OAc, 0.04 M LiOAc + 0.128 M LiOAc, 0.004 M  $\text{Li}_2\text{SO}_4$  (2 x 150 ml); flow rate: 4 ml/hr.

In order to find out in what size range the procedure gives the best resolution, a Hind II digest of T7 DNA was run through a 1.6 x 100 cm column. This digest consists of about 60 fragments resolving into approximately 50 bands on polyacrylamide gels ranging from around 40 to 2,800 b.p. (11). The elution profile shown in Fig. 5 indicates that fragments smaller than 150 b.p. are not resolved but are eluted at the front of the first gradient. Inspection of the peaks reveals that the best resolution is obtained between 300 and 1,200 b.p. under the conditions chosen.

Exchanging the cellulose support for the fine celite fraction, we expected a better resolution due to the narrower size range of the support particles. While attempting to verify this using Hind III fragments of  $\lambda$  DNA, we discovered that ethidium bromide may be used at 1  $\mu\text{g}/\text{ml}$  in the phases, allowing one to follow the position of the bands on the column by UV-light of 364 nm. This revealed that when the fragments begin to move in the gradient they form rather sharp bands which broaden somewhat during the run, in agreement with the common theories on liquid-liquid chromatography (7,8). The resolution, however, was not much better than on a column with cellulose support (compare Fig. 4 and Fig. 6); perhaps a somewhat better resolution was obtained for the fragments in the 6 k.b. range. We were surprised, however, to observe that largest fragment (23 k.b.), once started, moved much faster through the column than one might expect on the basis of



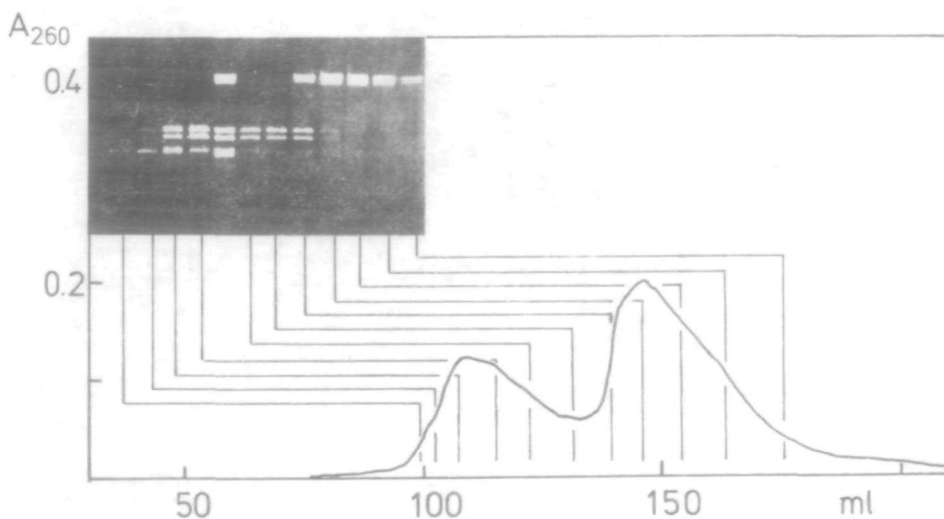
**Figure 5.** Separation of Hind II fragments of T7 DNA (3.2 mgs): Column: 1.6 x 100 cm; support: cellulose Whatman CC31; system: PEG 6000-dextran T500; eluting gradients: (1) 0.14 M KOAc  $\rightarrow$  0.14 M NaOAc (2 x 200 ml). (2) 0.14 M NaOAc  $\rightarrow$  0.12 M LiOAc, 0.02 M NaOAc (2 x 360 ml); flow rate: 10 ml/hr.



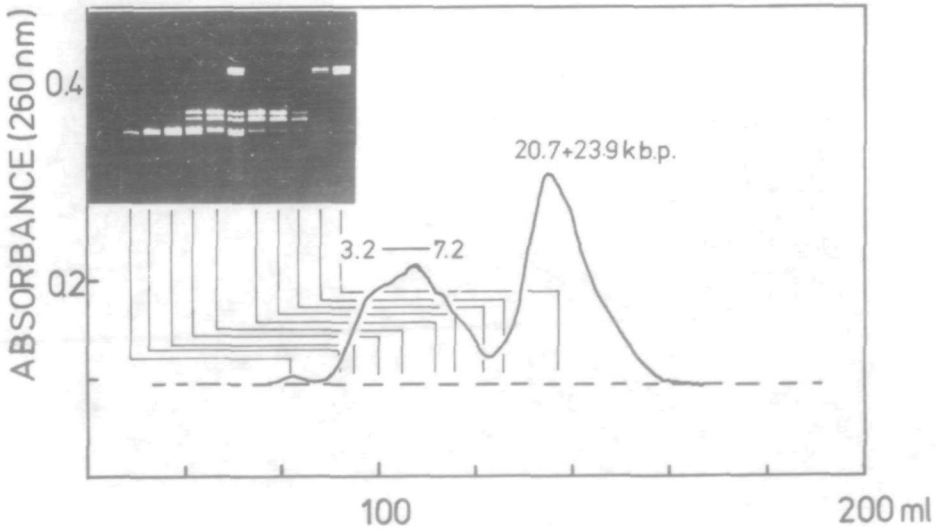
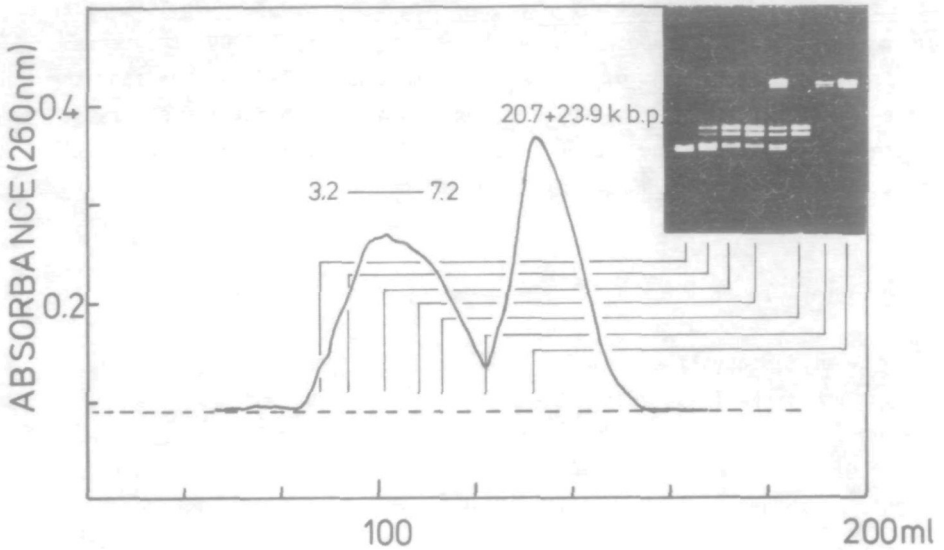
**Figure 6.** Separation of Hind III fragments of  $\lambda$  DNA: Column size and phase pair as for Fig. 4. Support of the stationary phase: celite (fine). Eluting gradient and flow rate: as for Fig. 4. The broken line indicates the background formed by ethidium bromide and EDTA.

its partition coefficient. This caused us to assume that the flow rate might be too fast to allow this fragment size to stay in partition equilibrium during its migration through the column. Since flattening of the gradient results in undesirable band broadening, we tried instead to improve the resolution by reducing the flow rate. These experiments were performed with an EcoRI digest of  $\lambda$  DNA containing lac operons from *E. coli* using a 9mm column with the standard gradient. Running the column at 18 ml/hr resulted in a complete lack of fractionation. At 4 ml/hr the resolution (Fig. 7b) was comparable to the results shown in Figs. 2-7a. Decreasing the speed in 4 steps, starting from 18 ml/hr, progressing to 6 ml/hr, 3 ml/hr and 1.5 ml/hr (Fig. 7c) produced a minor improvement visible on the agarose gels only.

It is useful to know the capacity of the columns described. The results of experiments reported here were obtained using 8-10  $\mu$ g of DNA per ml of column bed. Preliminary results obtained with Hae III digests of calf thymus DNA have shown that resolution of individual restriction fragments in the 100-300 b.p. range is possible with as much of 50  $\mu$ g of DNA per ml of bed volume. The influence of fragment size on the capacity cannot be ascertained without further experiments.



**Figure 7a.** Separation of EcoRI fragments of  $\lambda$  DNA (containing several tandem copies of the lac region of *E. coli*). Column, phase pair and eluting gradient: as for Fig. 6; flow rate: 4 ml/hr; support: cellulose, Whatman CC31.



**Figure 7b.** As for 7a; flow rate: 4 ml/hr; support: celite (fine).

**Figure 7c.** As for 7a; support: celite, fine; the flow rates were step-wise decreased from 18 ml/hr to 6, 3 and 1.5 ml/hr. The first reduction was made when 24 ml of phase has passed the column after loading the sample. The subsequent reductions are indicated by arrows.

For the coated cellulose support, we tried to obtain saturation of a given volume of suspension by adding increasing amounts of DNA fragments and monitoring the amount of DNA in the supernatant. A deviation from the linear increase governed by the partition law should indicate a saturation of the lower phase on the support. As seen from Fig. 8, this point must be greater than 160  $\mu\text{g}$  of DNA per ml of packed cellulose.

Discussion

The elution profiles shown in the previous section demonstrate high resolution of DNA fragments in the range of 150 to 700 b.p., moderate resolution up to 2,800 b.p. and low resolution above this size. It seems rather difficult to us to rationalize this behavior since the chromatographic procedure involves a combination of classical liquid-liquid

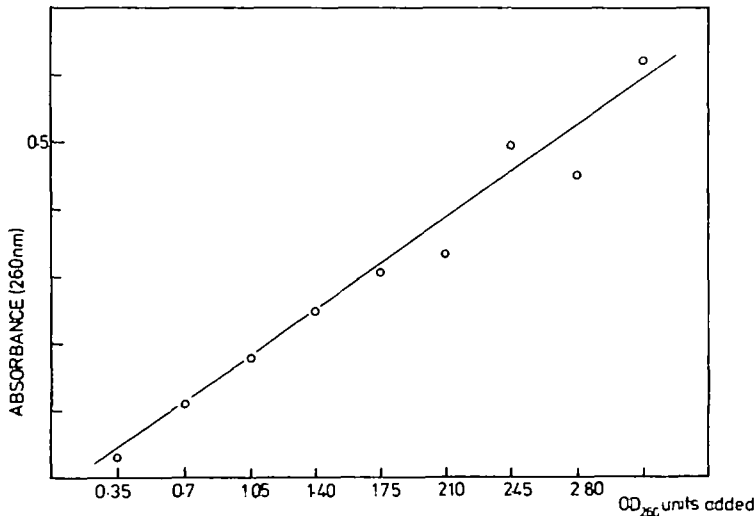


Figure 8. Partitioning of Hae III fragments of Col EI (910-1050 b.p.) between PEG-rich phase and coated cellulose: 1 ml of coated and sedimented cellulose (corresponding to 0.4 g of dry cellulose) was resuspended in PEG-rich phase (0.14 M in NaOAc) to yield a total volume of 2.1 ml. The DNA solution (5.7% w/w in PEG) was added in 20  $\mu\text{l}$  portions. After equilibration by gentle stirring for 2 min and centrifugation in an Eppendorf centrifuge for 2 min, the absorbance at 260 nm was measured in the supernatant against a blank without DNA. The readings were corrected for volume increase.

partition chromatography with a gradient elution technique. In the absence of a salt gradient during elution, one could calculate the retention volume to be expected, since it is known from the work of Lif *et al.* (8) on a similar system that the logarithm of the partition coefficient of DNA decreases linearly with sedimentation coefficient. Eigel (9) has shown that this relationship is also valid for PEG-dextran systems. Such calculations reveal that the retention volume should increase nearly linearly with fragment size up to 1,200 b.p. Above 1200 b.p., the elution volume should increase with greater than the first power of fragment size, and one would expect the resolution to increase for larger fragments. Since the opposite was observed, we must assume that the salt gradient applied counteracts the basic behavior implied by partition theory. The salt gradient, however, seems to be indispensable in order to elute the DNA fragments in reasonable volumes from the column. This means that one has to deal with a compromise which requires adjustment of all parameters for optimal results. Although we have performed over 50 runs with comparable DNA samples, we are far from convinced that the best conditions have been found. The parameters not yet varied include the temperature and the molecular weight of the phase-forming polymers to be used. The temperature variation is somewhat limited, not only for practical reasons but also because of the temperature dependence of the phase composition. Since higher temperatures are expected to increase the rate of equilibration, one might expect a better resolution. Higher polymer concentrations, however, are required for phase separation at higher temperatures. The resultant increased phase viscosity may thus partly counteract the thermally enhanced equilibration rate.

The influence of the molecular weight of the polymer used for the phase preparation is essentially unknown. A test run using dextran T2000 ( $2 \times 10^6$  mean mol. wt.) showed a shift of the retention volumes to smaller values (as expected according to Albertsson (1)) without yielding better resolution. Whether enhanced resolution can be achieved by varying the PEG molecular weight remains to be tested.

The fact that either celite or cellulose can be used as support for the stationary phase without changes in retention volumes and only minor changes in resolution reconfirms that we are dealing with a two-phase partition instead of an adsorption process in our system. Since celite is available in smaller particle sizes, we attempted to test such materials for enhanced resolution of larger fragments since the number of theoretical

plates per unit column length should increase with decreasing support particle size. Unfortunately, celite seems to contain residual metal ions (probably  $\text{Ca}^{2+}$  ions) which produce a steady leak of UV-absorbing material, when EDTA-containing phases are used. Removal of these impurities by more extensive pretreatment is under investigation.

Only small improvements in resolution were obtained by reducing the flow rate to a practical limit. These results indicate that DNA fragments up to 20 k.b. stay in partition equilibrium during a run at 4 ml/hr through a cross-section of  $0.64 \text{ cm}^2$ , and that this equilibrium state is essentially retained even when the flow rate is increased by 30%.

In all fractionation experiments performed with DNA samples above 3 k.b. in size, the restriction fragments used had short, single stranded complementary ends. It is possible that weak interactions between the ends of these fragments may cause broadening of homogeneous fragment bands and counteract the separation of similar sized fragments. It is difficult to estimate the importance of these effects. The fact that two EcoRI  $\lambda$  DNA terminal fragments (A and F), each containing 20 base complementary ends (12), comigrate in our system, indicates that these interactions may be important. These effects are currently under investigation using  $S_1$ -treated fragments missing the complementary ends.

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4. The use of lithium sulfate in phase III is necessary for complete elution of fragments larger than 20 k.b. Otherwise, 0.14 M lithium acetate is adequate.
5. If celite is used as support for EDTA-containing phases, the flow rate during the equilibration should not exceed the flow rate during the run by more than a factor of two, since unsteady base lines may result



during the run.

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