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OPERATIONAL VARIABLES IN HIGH-PERFORMANCE GEL FILTRATION OF DNA FRAGMENTS AND RNAs

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SUMMARY

Double-stranded DNA fragments and ribosomal and transfer RNAs were measured by high-performance gel filtration on TSK-GEL G2000SW, G3000SW, G4000SW and G5000PW columns to investigate the separation range and resolution of these columns and the effects of eluent ionic strength and flow-rate on retention and resolution. These columns could separate double-stranded DNA fragments up to *ca* $1 \cdot 10^6$ and rRNAs up to *ca* $5 \cdot 10^6$ daltons in molecular weight. However, it was found that the selection of the column is very important to achieve optimum separation, depending on the molecular weight of the sample. Elution is delayed as the eluent ionic strength is increased. An eluent ionic strength of 0.3–0.5 seemed appropriate in most cases. Resolution is greatly increased as the flow-rate is decreased.

INTRODUCTION

High-performance gel filtration was introduced several years ago and has been widely adopted in protein separation. In addition, it has been very useful for the separation of RNAs[1,2] and DNA fragments,[3,4] and is also becoming one of the important means of separation and purification in the field of nucleic acids. In order to take full advantage of this technique, however, fundamental investigations such as a study of operational variables to optimize separation conditions are required. We therefore investigated the separation range and the resolution in gel filtration of double-stranded DNA fragments and ribosomal and transfer RNAs on TSK-GEL SW type and PW type columns. The effects of eluent ionic strength and flow-rate were also studied.

EXPERIMENTAL

Double-stranded DNA fragments were prepared by cleaving plasmid DNA pBR322 with restriction endonuclease HaeIII (a gift of A. Fujiyama) or BstNI (New England Biolabs, U.S.A.). The preparation method has been described elsewhere[4]. HaeIII-cleaved pBR322 DNA contains 22 fragments of 7, 11, 18, 21, 51, 57, 64, 80, 89, 104, 123, 124, 184, 192, 213, 234, 267, 434, 458, 504, 540 and 587 base pairs[5].

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BstNI-cleaved pBR322 DNA contains six fragments of 13, 121, 383, 928, 1060 and 1857 base pairs[5]. The molecular weights of the fragments were determined by multiplying their base pair numbers by 650. Total *Escherichia coli* RNA was purchased from Miles Labs. (Elkhart, IN, U.S.A.). This sample contains four components ; 4S tRNA (mol.wt. 25,000), 5S rRNA (39,000), 16S rRNA (560,000) and 23S rRNA (1,100,000). The average molecular weight of the components is taken from 4S tRNA. Commercial proteins thyroglobulin (660,000), bovine serum albumin (67,000), ovalbumin (43,000), myoglobin (16,900) and cytochrome *C*(12,400), were also measured for comparison.

Gel chromatography was carried out at 25°C with a high-speed liquid chromatograph Model HLC-803D, equipped with a variable-wavelength UV detector (Toyo Soda, Tokyo, Japan) operated at 260 nm. Systems consisting of two G2000SW columns, two G3000SW columns, two G4000SW columns or two G5000PW columns (Toyo Soda) were employed. Each column was 60 × 0.75 cm I.D. The eluent was usually 0.1 *M* phosphate buffer (pH 7.0) containing 0.1 *M* sodium chloride and 1 m*M* EDTA. In the study of eluent ionic strength, however, 0.01 *M* Tris–HCl buffer (pH 7.5) containing 0.025–1.6 *M* sodium chloride and 1 m*M* EDTA was used. The flow-rate was 1 or 1/3 ml/min except in the study of its effect when it was varied over the range of 1/15–1 ml/min. The injection volume was 0.1 ml and the sample concentration was 0.01–0.1 % (w/v).

RESULTS AND DISCUSSION

Separation range

Separation profiles of HaeIII- or BstNI-cleaved pBR322 DNA and RNAs on G4000SW are shown in Fig. 1. The separations of the same samples were also carried out on columns G2000SW, G3000SW and G5000PW (data not shown). Fig. 2 shows the molecular-weight calibration curves for each system of columns. In the case of G5000PW, plots for the components of HaeIII-cleaved pBR322 DNA were omitted because they could not be separated well (see Fig. 3c). For peaks of DNA fragments containing more than one component, the average molecular weights were adopted. Peaks of DNA fragments were assigned by polyacrylamide gel electrophoresis. The exclusion limits of columns estimated from these calibration curves are summarized in Table I, which shows that double-stranded DNA fragments of molecular weight up to $1 \cdot 10^6$ and rRNAs of molecular weight up to *ca.* $5 \cdot 10^6$ can be separated by using TSK-GEL SW type and PW type columns. The exclusion limits for double-stranded DNA fragments are lower than those for rRNAs, indicating that double-stranded DNA fragments have a larger molecular size than rRNAs (or tRNAs) of the same molecular weights in solution. The exclusion limits for protein are even higher than those of rRNA due to the compact conformation of proteins.

Comparison of separation efficiencies

The separation of a sample should depend on the column employed. For example, in the separation of HaeIII-cleaved pBR322 DNA, the best separation was obtained for base pairs 7–21, 51–104 and 123–267 on G2000SW or G3000SW, on G3000SW and on G4000SW, respectively, as can be seen from Figs. 1 a and 3. The separation of two components, base pairs 1060 and 1857, in BstNI-cleaved pBR322

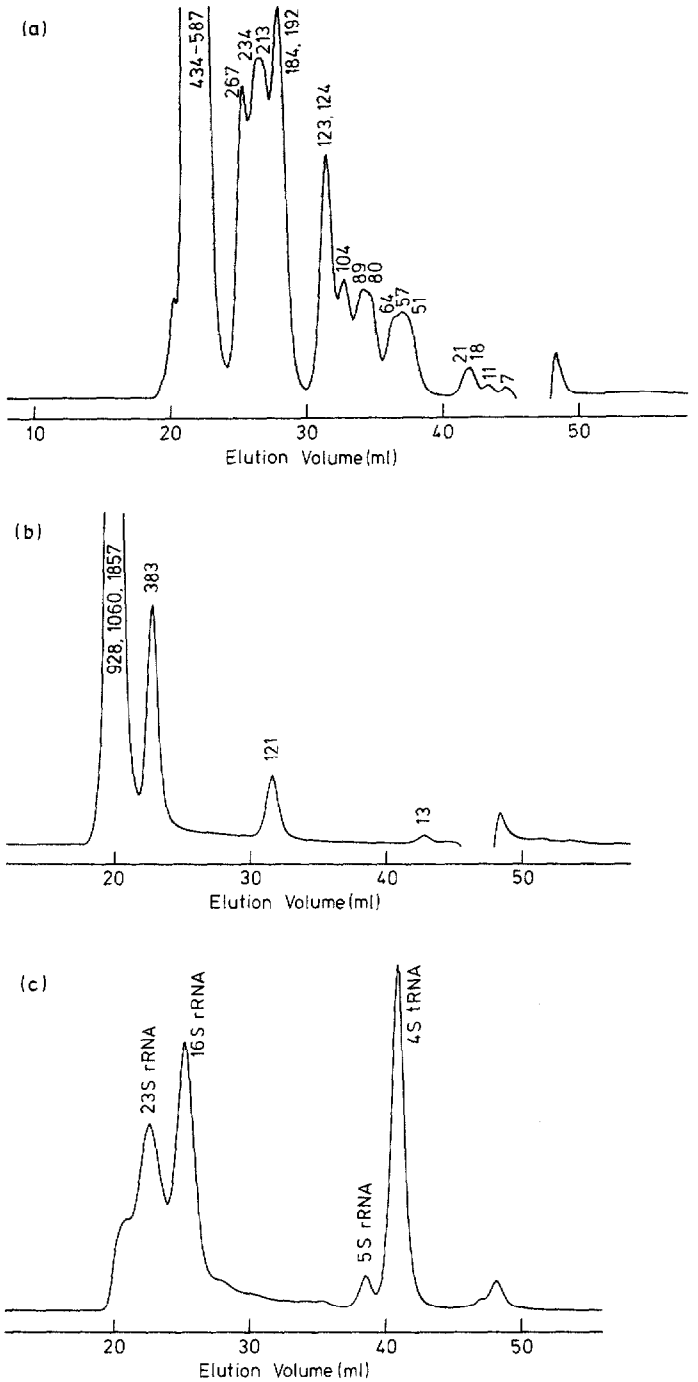

Fig. 1. Chromatograms of HaeIII-cleaved pBR322 DNA(a), BstNI-cleaved pBR322 DNA(b) and Total *E. coli* RNA(c) obtained on a G4000SW two-column system with 0.1 *M* phosphate buffer(pH 7.0) containing 0.1 *M* sodium chloride and 1 m*M* EDTA, at a flow-rate of 1 ml/min.

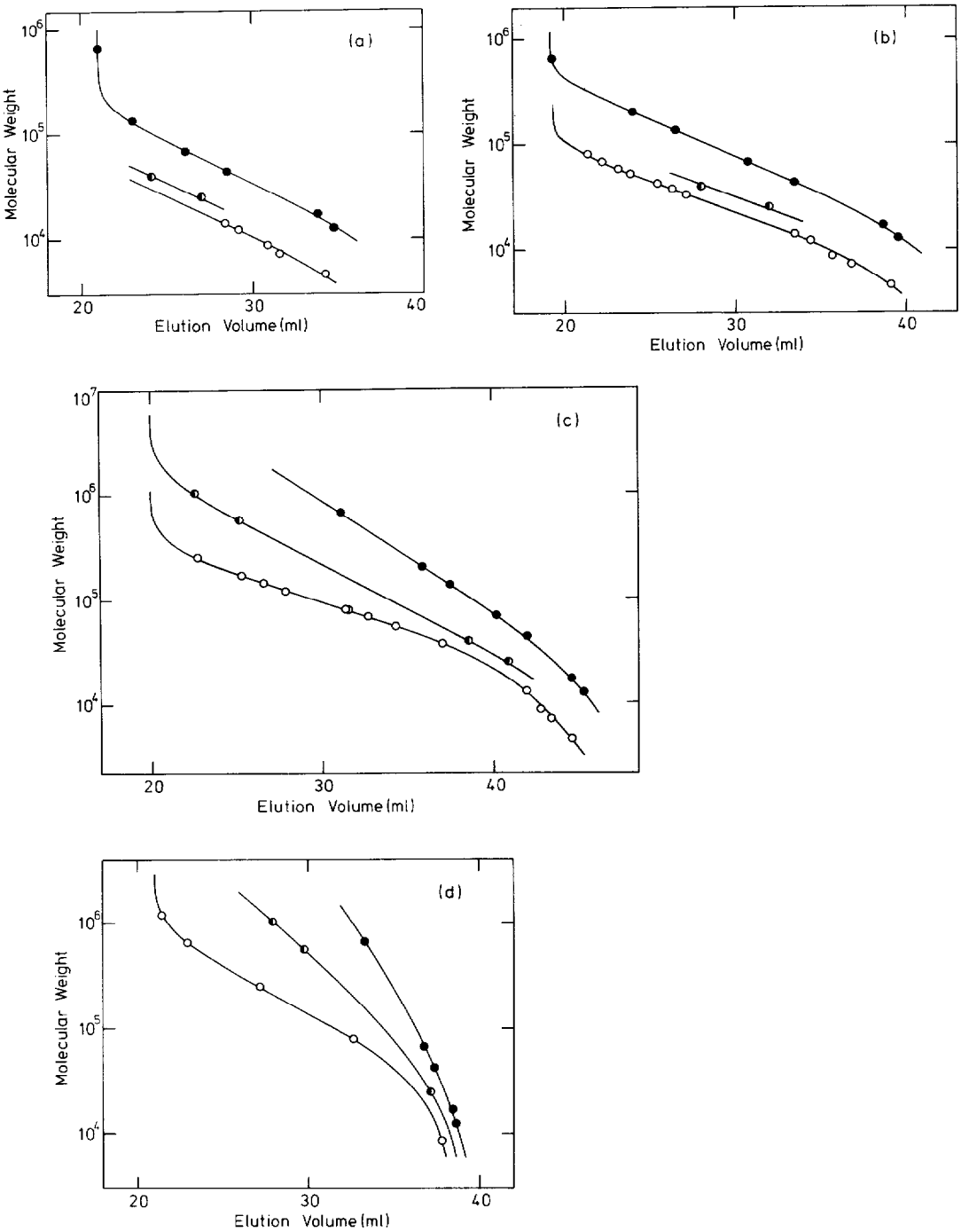

Fig. 2. Molecualr-weight calibration curves of G2000SW(a), G3000SW(b), G4000SW(c) and G5000PW(d) two-column systems with 0.1 *M* phosphate buffer(pH 7.0) containing 0.1 *M* sodium chloride and 1 m*M* EDTA for double-stranded DNA fragments (○), rRNA and tRNA (◐) and protein (●).

DNA could be achieved only on G5000PW (see Fig. 4). Therefore, it is very important to select the best column depending on the molecular weights of the samples to be separated. Table II summarizes the best columns, established by the results obtained in the above experiments.

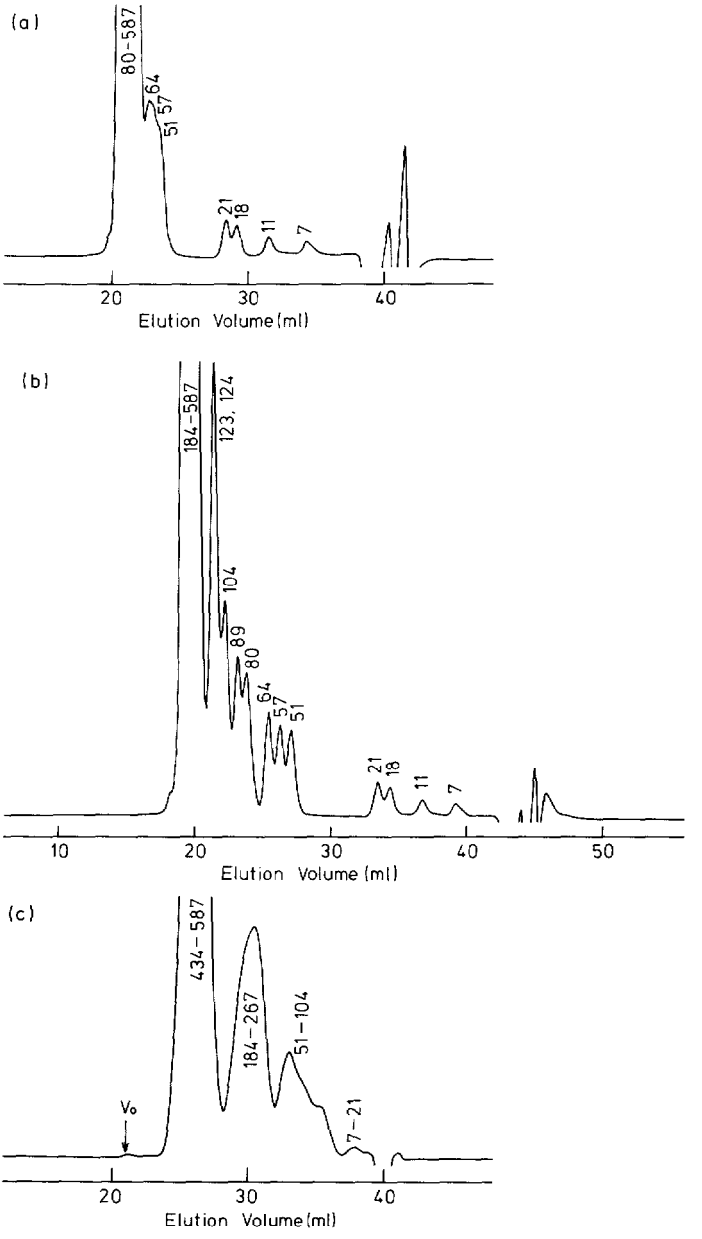

Fig. 3. Chromatograms of HaeIII-cleaved pBR322 DNA obtained on the G2000SW(a), G3000SW(b) and G5000PW(c) two-column systems. Other conditions as in Fig. 1. The arrow in (c) indicates the void volume.

TABLE I

EXCLUSION LIMITS OF G2000SW, G3000SW, G4000SW AND G5000PW FOR DOUBLE-STRANDED DNA FRAGMENTS AND rRNA

Column	*Exclusion limit in molecular weight*	
	Double-stranded DNA fragment	*rRNA*
G2000SW	50,000	70,000
G3000SW	100,000	150,000
G4000SW	300,000	1,500,000
G5000PW	1,000,000	>5,000,000

Effect of eluent ionic strength

Fig. 5 shows the effect of eluent ionic strength on the elution volumes obtained on G3000SW, G4000SW and G5000PW. Elution of both DNA fragments and RNAs was delayed by increasing the eluent ionic strenght. Elution volumes greatly varied in

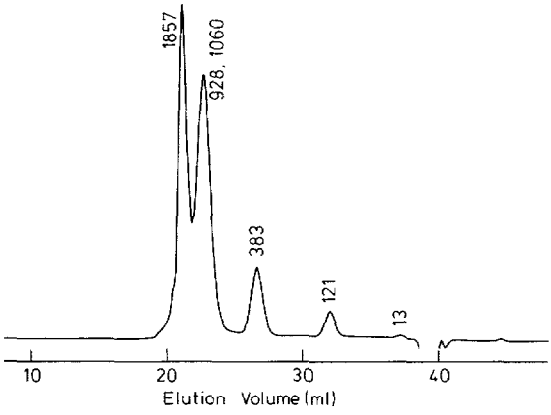

Fig. 4. Chromatogram of BstNI-cleaved pBR322 DNA obtained on a G5000PW two-column system. Conditions as in Fig. 1 except the flow-rate was 1/3 ml/min.

TABLE II

BEST COLUMNS FOR THE SEPARATIONS

Molecular weight range	*Best column*
Double-stranded DNA fragments	
<40,000	G2000SW or G3000SW
40,000 – 80,000	G3000SW
80,000 – 250,000	G4000SW
250,000 – 1,000,000	G5000PW
rRnas	
<60,000	G2000SW or G3000SW
60,000 – 120,000	G3000SW
120,000 – 1,200,000	G4000SW
1,200,000 – 10,000,000	G5000PW

the low ionic strength region, but at high ionic strengths the elution volumes seemed to become constant. Furthermore, the elution volumes of small molecules were more markedly affected than those of large molecules. The peak widths broadened very

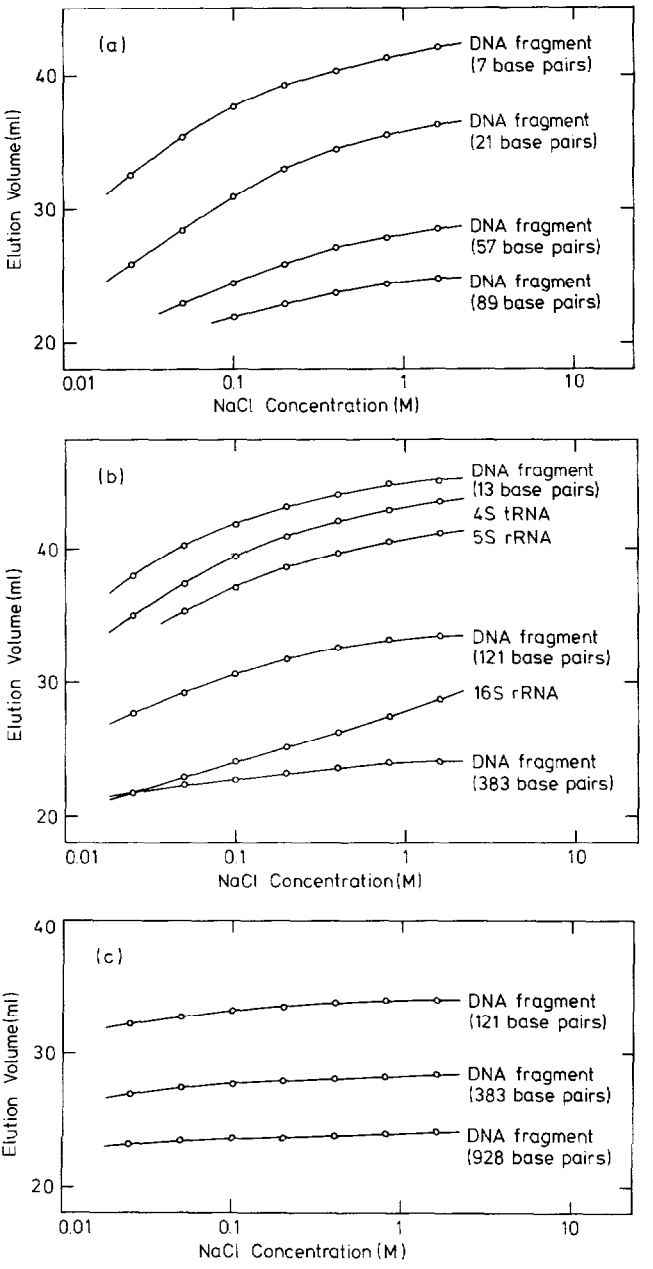

Fig. 5. Dependence of elution volume on eluent ionic strength obtained on G3000SW(a), G4000SW(b) and G5000PW(c) two-column systems with 0.01 *M* Tris–HCl buffer(pH 7.5) containing 0.025–1.6 *M* sodium chloride and 1 m*M* EDTA, at a flow-rate of 1 ml/min.

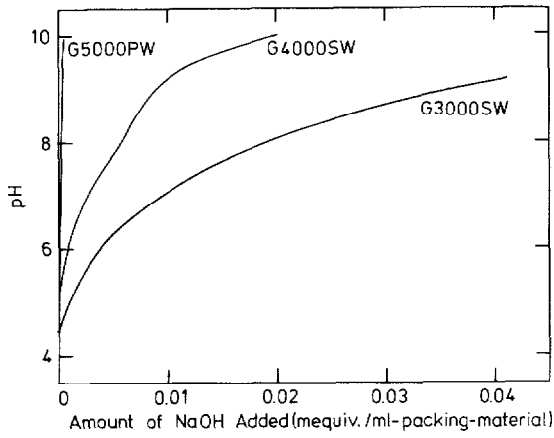

Fig. 6. Titration curves of G3000SW, G4000SW and G5000PW gels. Gels of 10 ml each were washed on a glass filter with 30 ml 0.1 *M* hydrochloric acid and then with distilled water until the pH of the filtrate became nearly neutral. The resultant gels were titrated in 100 ml 0.5 *M* sodium chloride with 0.5 *M* NaOH using an autotitrator.

slightly with increasing eluent ionic strength. Accordingly, an eluent ionic strength of 0.3–0.5 may be appropriate in general.

The phenomenon of increasing elution volume with increasing eluent ionic strength has been observed also on Sephadex[6] and agarose gels[7,8] and was attributed to the repulsive ionic interaction between samples and carboxyl groups on the gels, the change in conformation of samples or the adsorption of samples on the gels. TSK-GEL SW type gels are based on silica and contain some residual silanol groups, whereas TSK-GEL PW type gels are based on hydrophilic synthetic polymer and contain some carboxyl groups. Fig. 6 shows the titration curves of G3000SW, G4000SW and G5000PW gels, which indicate that the number of acidic groups increases in that order. This order coincides with the degree of variation of the elution volume observed in Fig. 5. therefore, it seems reasonable to assume that the ionic repulsion between samples and gels is the main source of variation of elution volume in the separation of nucleic

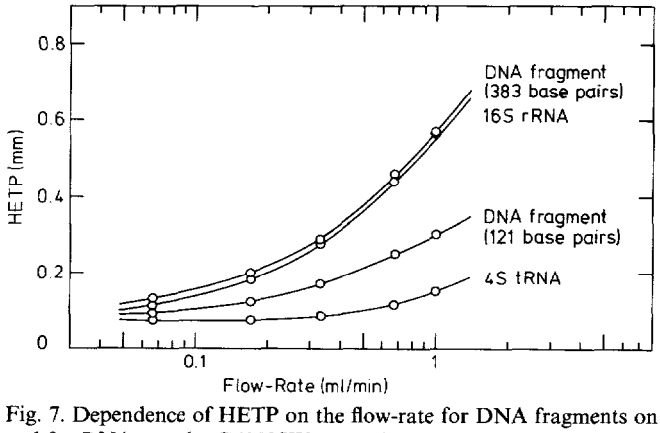

Fig. 7. Dependence of HETP on the flow-rate for DNA fragments on the G5000PW two-column system and for RNAs on the G4000SW two-column system.

acids on TSK-GEL SW type and PW type columns. However, other sources such as adsorption on the gels should also be considered in the case of 16S and 23S rRNAs because their elution volumes increased regularly with eluent ionic strength, even in the high ionic strength region where ionic interactions should diminish (see 16S rRNA in Fig. 5 b).

Effect of flow-rate

Fig. 7 shows the dependence of height equivalent to a theoretical plate (HETP) on flow-rate obtained by measuring BstNI-cleaved pBR322 DNA on G5000PW and for total *E. coli.* RNA on G4000SW. The HETP decreased with decreasing flow-rate throughout the range investigated. Especially in the case of high molecular weight samples, the HETP was significantly dependent on flow-rate and reached a minimum at flow-rates lower than 0.1 ml/min. Flow-rates of 0.3–0.5 ml/min seems to be a good compromise when separation time and resolution are taken into consideration.

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