Journal of Chromatography, 210 (1981) 443–452
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,714

HIGH-PERFORMANCE SIZE EXCLUSION CHROMATOGRAPHY OF SEA WORM CHLOROCRUORIN AND OTHER LARGE PROTEINS, VIRUSES AND POLYSACCHARIDES ON A TSK G5000 PW PREPARATIVE COLUMN

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(Received January 22nd, 1981)

SUMMARY

The elution parameters of large enzymes, viruses, ribosomes and other "supramolecular" structures are studied using the preparative TSK G5000 PW type column. The pigmented protein, chlorocruorin, isolated from the sea worm *Potamilla leptochaeta*, was found to serve as an excellent high-molecular-weight marker for size exclusion liquid chromatography. This is due to its high degree of molecular stability and a molecular weight, found to be $2.9 \cdot 10^6$ by sedimentation velocity analysis, which is located in a zone formed between viruses and enzymes that is largely devoid of macromolecular markers. Calibration constants for this chromatography column are found for both molecular weight and molecular radii. The data found for hydrodynamic molecular radii are further extended to non-globular, swollen macromolecules, such as polysaccharides, using dextran fractions sized by alcohol precipitation.

INTRODUCTION

The rapid and gentle purification of large macromolecules, such as viruses, ribosomes and polysaccharides, is of great concern to many workers in the biophysical sciences. The traditional use of Sepharose-type support materials has long been the method of choice for the fractionation of high-molecular-weight macromolecules; however, the low flow-rates used to prevent compression of the chromatography beads lead to very high column residence times which are undesirable for purification of hydrolase-sensitive molecules.

In a previous study we described[1] the elution behavior of 26 standard proteins and peptides on TSK G3000 SW columns and developed a new method for treating size exclusion chromatography (SEC) data. This method appears to give a more linear relationship between elution volumes and molecular size than do other methods currently in use. However, the rather low molecular-weight fractionation range of this TSK G3000 SW column limited the study to proteins of molecular weight less than 325,000. The TSK G5000 PW packing is reported by the manufacturer to have a higher molecular-weight fractionation range which has not been precisely defined.

This laboratory is engaged in the purification of bacterial ribosomes, proteins,

0021-9673/81/0000–0000/$02.50

polysaccharides and lipopolysaccharides. Ribosomes in particular are sensitive, during purification, to both proteases and nucleases. These objectives led to the studies reported here.

EXPERIMENTAL

Chromatography elution samples

Southern bean mosaic virus (SBMV) and tomato bushy stunt virus (TBSV) were generous gifts from Dr. Robert J. Shepherd, University of California at Davis, CA, U.S.A. Tobacco mosaic virus (TMV) was obtained from Dr. A. T. Tu from this department and turnip yellow mosaic virus (TYMV) was a gift from Professor R. E. F. Matthews, University of Auckland, New Zealand. The bovine heart myoglobin, human hemoglobin (oxy) and the sea worm chlorocruorin (a hemoglobin-like protein) were generously supplied by students and visiting faculty from the laboratory of Dr. W. S. Caughey. The sea worm chlorocruorin was isolated from *Potamilla leptochaeta* by the method of Orii and Washio[2]. *Escherichia coli* ribosomes were prepared by alternate high- and low-speed centrifugation in 50 m*M* Tris buffer, pH 8.0, containing 10 m*M* $MgCl_2$, as described by Tissieres *et al.*[3]. Chromatography of the ribosomes was carried out in the same buffer. Cytochrome *c* and apoferritin were obtained from Sigma (St. Louis, MO, U.S.A.). Pig thyroglobulin was from Mann Research Labs. (New York, NY, U.S.A.). Bovine serum albumin was from Armour (Kankakee, IL, U.S.A.) and sodium azide was obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). The dextran samples used in this study were obtained from Sigma Biochemicals. These fractions, obtained from *Leuconostoc mesenteroides* by alcohol fractionation, had viscosity-average molecular weights ($\bar{M}_v$) of 500,000, 70,000 and 9700.

Column injection methods

Chromatography elution samples used in the calibration of the column were injected with a Rheodyne syringe injection valve in volumes of 250 μl. An Altex septum injector, mounted in-line from the Rheodyne valve, was used for the injection of the 1.0-ml samples of polysaccharide. It was felt that the great length of small-bore tubing required to attain the higher sample volume of polysaccharide for injection in the sample loop of the Rheodyne valve might lead to some degree of shearing with an extended macromolecule, hence the use of a direct injecting septum port. To minimize column bed shock, the pump flow-rate was decreased to 0.1 ml min^{-1} and the 1.0-ml capacity syringe needle was carefully pushed through the septum as far as possible. Using the syringe, the sample was then introduced at nearly the same flow-rate as normally used during the run (*i.e.* 1.0 ml min^{-1}) by monitoring the degree of pen deflection on the refractive index (RI) monitor during the injection. When the injection of polysaccharide was complete, the flow-rate was brought up to the running value of 1.0 ml min^{-1}.

Equipment and pre-column

A 600 × 21.5 mm I.D. TSK G5000 PW preparative high-performance chromatography column (Toyo Soda, Tokyo, Japan), was used for these studies. The basic solvent delivery system consisted of the Altex/Beckman 330 HPLC pump and

UV detector. The chromatography equipment used in this study also included an Altex Model 250-24 septum injector, a Rheodyne Model 7020 syringe injector fitted with a 250-μl sample loop and an LDC refractive index monitor equipped with external temperature control. All components were connected with 1/16 in. × 0.020 in. stainless-steel tubing. The LDC monitor was modified in this laboratory to interface with the other equipment in the system by conversion of the sample cell to Swagelok type 1/16 in. stainless-steel fittings. This modification ended an air contamination problem previously encountered with this monitor. The pre-column is packed using standard methods of slurry packing, as described by Kirkland[4]. A pre-column system assembled as described above is currently in use and has withstood over 50 injections, 200 h of running time, and gives the excellent resolution seen with cytochrome *c* in Fig. 1 (sample number 13).

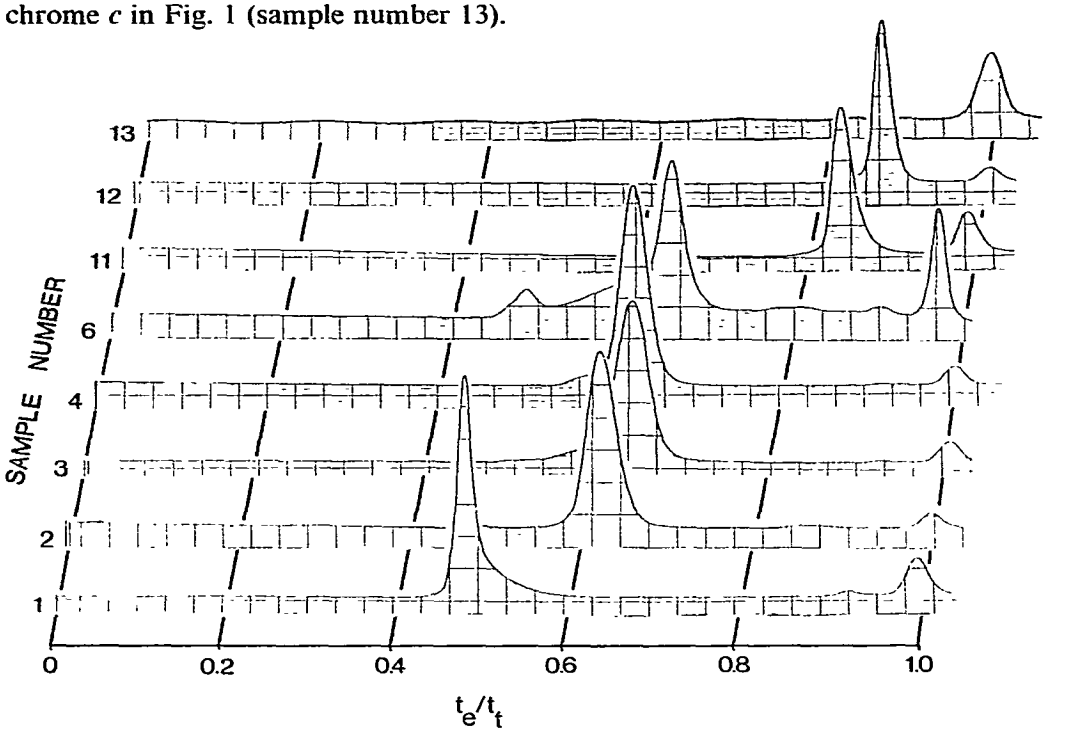

Fig. 1. High-performance gel permeation chromatography elution profile of standard proteins and virus samples used in column calibration. Chromatography was carried out at a flow-rate of 0.96 ml min^{-1} with 10 m*M* phosphate pH 7 buffer in 100 m*M* KCl on a TSK G5000 PW column measuring 600 × 21.5 mm I.D. Chromatography was monitored by absorbance at 280 nm and recorded at a chart speed of 10 cm h^{-1}. Average sample load was 250 μl of a 3 mg ml^{-1} protein solution. Sample numbers from Table I.

Methods and theory

Column parameters are defined as described by Pharmacia[5]. Here, V_0, the void volume, refers to the interstitial volume between the beads, V_i and V_s to the volume within the beads that is accessible and inaccessible to solvent, respectively, and V_e to the elution volume of a solute under study. The analogous parameters t_0, t_t and t_e indicate elution in time found from the chart recorder. The method of calibrating the pump flow-rate during each chromatography run with an internal marker, providing

a normalization factor from run to run, was carried out as described earlier[1]. The column parameter t_t was found with injections of NaN_3 in concentrations of 10–50 μg/ml. We found that NaN_3 eluted slightly after KCl and water (detected refractometrically). We assume that the simple salt, KCl, and water, which has a lower refractive index than the buffer, are probably better markers of the t_t than NaN_3 on the grounds that only late elution can be explained by interaction of the sample with the support when chromatography is performed in a buffer of sufficient ionic strength. It is not practical to use the refractive index monitor in this way for all fractionation experiments, so we included NaN_3 in all samples, and corrected for this small difference in elution time. This correction factor was found to be 0.96. For the column used here, the parameters V_0 and V_t were found by direct volume measurement from the co-chromatography of TYMV and water. These values were V_0 = 109 ml and V_t = 167 ml. The geometrical column volume, V_g, was found for the total chromatography system (*i.e.* column and pre-column) to be 228 ml. Thus V_i and V_s were 58 ml and 61 ml, respectively. As described earlier[1], elution parameters of SEC may be defined by the following relationship

$$F_{(v)} = \frac{V_e^{1/3} - V_0^{1/3}}{V_t^{1/3} - V_0^{1/3}} = \frac{(t_e/t_t)^{1/3} - (t_0/t_t)^{1/3}}{1 - (t_0/t_t)^{1/3}} = b - aM^{1/3} \quad (1)$$

where t_e/t_t is simply the ratio obtained by dividing the time interval from injection to the appearance of the maximum of the sample peak by the interval before appearance of the peak due to NaN_3. Values of $F_{(v)}$ found for the elution of samples in this study are shown in Table I. Eqn. 1 predicts a straight line for a plot of $F_{(v)}$ *vs.* $M^{1/3}$. Furthermore, the constants C and A, defined as the limiting molecular weight values found to elute at V_0 and V_t, respectively, can be obtained from the intersection of the curve with these limits. Thus, when $F_{(v)} = 1$, $M^{1/3} = A^{1/3}$ and when $F_{(v)} = 0$, $M^{1/3} = C^{1/3}$.

Size exclusion of globular proteins is determined by their molecular radii, $r = (3M\bar{v}/4\pi N)^{1/3}$. Since the partial specific volume, $\bar{v}$, varies little from 0.73 for most simple proteins[6], little error is introduced by plotting $F_{(v)}$ *vs.* $M^{1/3}$. For supramolecular aggregates which are also compact, and nearly spherical, but contain variable amounts of nucleic acid, the values of $\bar{v}$ can vary substantially and plotting $F_{(v)}$ *vs.* $(M\bar{v})^{1/3}$ should yield improved linearity. This prediction was confirmed by plotting the data obtained in this study by both methods.

Sedimentation velocity of sea worm chlorocruorin

The sea worm chlorocruorin (SWC) was studied by sedimentation velocity in the Beckman analytical Model E ultracentrifuge at 29,500 rpm and 20°C. The buffer used for these experiments was the same as the chromatography buffer. The light filter was replaced by a red filter (Kodak No. 25) and Kodak 103-F plates were used for photography. The sedimentation coefficient at standard conditions, $s_{20,w}$, was found following the methods of Schachman[21].

TABLE I

DATA AND ELUTION PARAMETERS FOR G5000 PW SAMPLES

No.	*Sample*	M_r	$(M\bar{v})^{1/3}$*	t_e/t_t**	*Ref.*
1	TMV	$39.4 \cdot 10^6$	306.4	0.495	7
2	TBSV	$8.9 \cdot 10^6$	187.4	0.642	8
3	SBMV	$6.6 \cdot 10^6$	166.2	0.662	8
4	TYMV	$5.4 \cdot 10^6$	153.2	0.651	9
5	SWC	$2.9 \cdot 10^6$	130.2	0.668	This study
6	Apo-ferritin (dimer)	960,000	88.82	0.690	10
7	Thyroglobulin	670,000	78.43	0.736	11
8	Apo-ferritin (monomer)	480,000	70.50	0.743	10
9	Bovine serum albumin (dimer)	132,600	46.00	0.816	12
10	Bovine serum albumin (monomer)	66,300	36.51	0.848	12
11	Hemoglobin	64,300	36.50	0.886	12
12	Myoglobin	16,600	23.10	0.910	12
13	Cytochrome *c*	12,400	20.79	0.935	12
14	Water, KCl	18 and 79	2.0	1.000	
15	NaN_3	64	2.0	1.041	

* The following values were used for $\bar{v}$ (the partial specific volume): No. 1, 0.73[13]; No. 2, 0.739[14]; No. 3, 0.696[15]; No. 4, 0.661[9]; No. 5, 0.746 (estimated from heme proteins in this study); No. 6, 0.73[16]; No. 7, 0.72[11]; No. 9, 0.734[17]; No. 11, 0.750[18]; No. 12, 0.743[19]; No. 13, 0.715[20].

** Corrected to the absolute V_t found for the elution of water (see text).

RESULTS

Gel chromatography of proteins, viruses and ribosomes

As shown in Fig. 1, the protein and virus samples eluted as single, symmetrical peaks of good resolution. Band spreading was low enough to allow resolution of bovine serum albumin into monomer and dimer (not shown). It was found that a sample injected in a 0.25-ml volume eluted in *ca.* 10 ml. The plot of $(M\bar{v})^{1/3}$ *vs.* $F_{(v)}$ for all samples chromatographed is shown in Fig. 2. The parameters A and C obtained from the intersection of $F_{(v)}$ with the horizontals at $F_{(v)} = 0$ and 1.00 are essentially 0 and $1.4 \cdot 10^6$, respectively. This indicates that the effective fractionation range of the G5000 PW column is from rather small molecules to $1.4 \cdot 10^6$ daltons. The regression line calculated for points in the linear region was

$$F_{(v)} = 1.007 - 0.009864\,(M\bar{v})^{1/3} \tag{2}$$

with a standard deviation of $0.3165M$. The column calibration was converted to molecular radius by the relationship

$$r = \left(\frac{3\,M\bar{v}}{4\,N\pi}\right)^{1/3}\left(1 + \frac{w}{\bar{v}\varrho}\right)^{1/3} \tag{3}$$

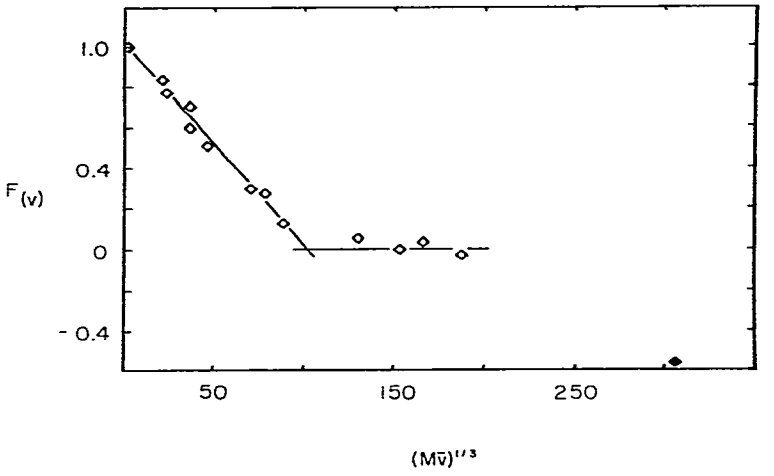

Fig. 2. Plot of $(M\bar{v})^{1/3}$ *vs.* $F_{(v)}$ for all fourteen chromatography samples studied on the G5000 PW column. The filled-in data point is the TMV sample and the next four going from right to left are TBSV, SBMV, TYMV and SWC, respectively.

The regression of $F_{(v)}$ and r (cm) is given by

$$F_{(v)} = 1.0063 - 763{,}000r \tag{4}$$

Gel chromatography of ribosomes and ribosome subunits (30S and 50S) isolated from *E. coli* showed co-elution of these components at the void volume, indicating that the size of these macromolecules exceeds the available chromatography bead pore openings. Chromatography of ribosomes on TSK columns may be useful, however, as a preparatory tool during isolation and "cleanup".

Properties of sea worm chlorocruorin

SWC, which has previously received little study, provided a valuable molecular-weight marker since it lies in a molecular-weight range for which we had no suitable standards. Sedimentation velocity analysis was used to determine the molecular weight. The sedimentation velocity study showed a major component which was highly symmetrical as well as a minor component of lower s. Application of the relationship[6], $M = 922\,(S/(1 - \bar{v}\varrho))^{3/2}$, where $S_{20,w} = 56.3$ (in Svedberg units, $S = 10^{13}\,s$) and $\bar{v} = 0.746$ (estimated from the other hemeproteins in this study), yielded a molecular weight of $2.9 \cdot 10^6$ for the main component.

Gel elution analysis of the SWC is shown in Fig. 3. The principle component was shown to elute at the void volume of the column, with *ca.* 20% of the material eluting in a smaller peak at $t_e/t_t = 0.776$. From eqn. 2, this corresponds to a molecular weight of 326,000. These data suggest that the principal SWC species may be a decamer or octamer of the smaller unit.

Gel chromatography of dextran fractions

The refractive index detector was required for the SEC studies of the dextran fractions. With sample loads of 20 mg per run, the RI deflection provided peak shapes

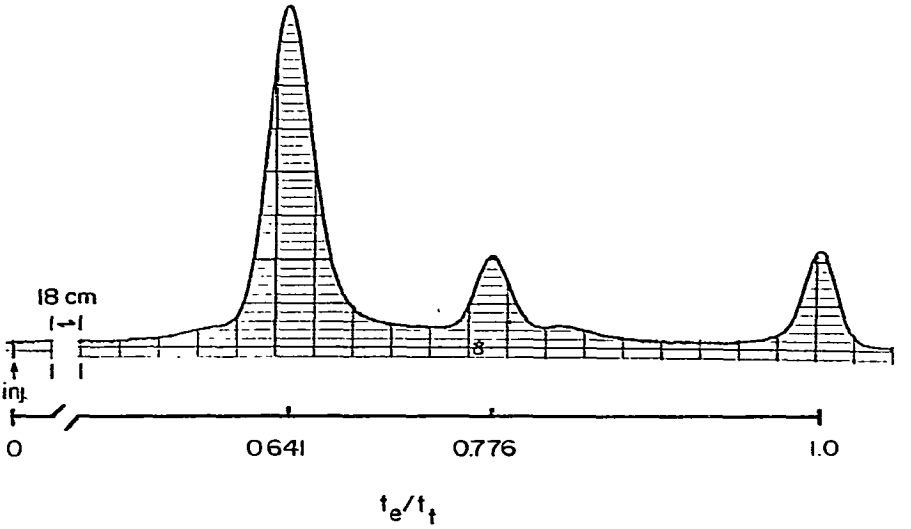

Fig. 3. Elution profile of sea worm chlorocruorin on G5000 PW. The principal component of SWC eluted at the void volume (t_0), with a secondary peak at elution ratio 0.776. For molecular-weight calibration studies, 0.02% sodium azide was included in the sample and can be seen eluting at the total column volume. Similar runs with no azide gave identical elution profiles.

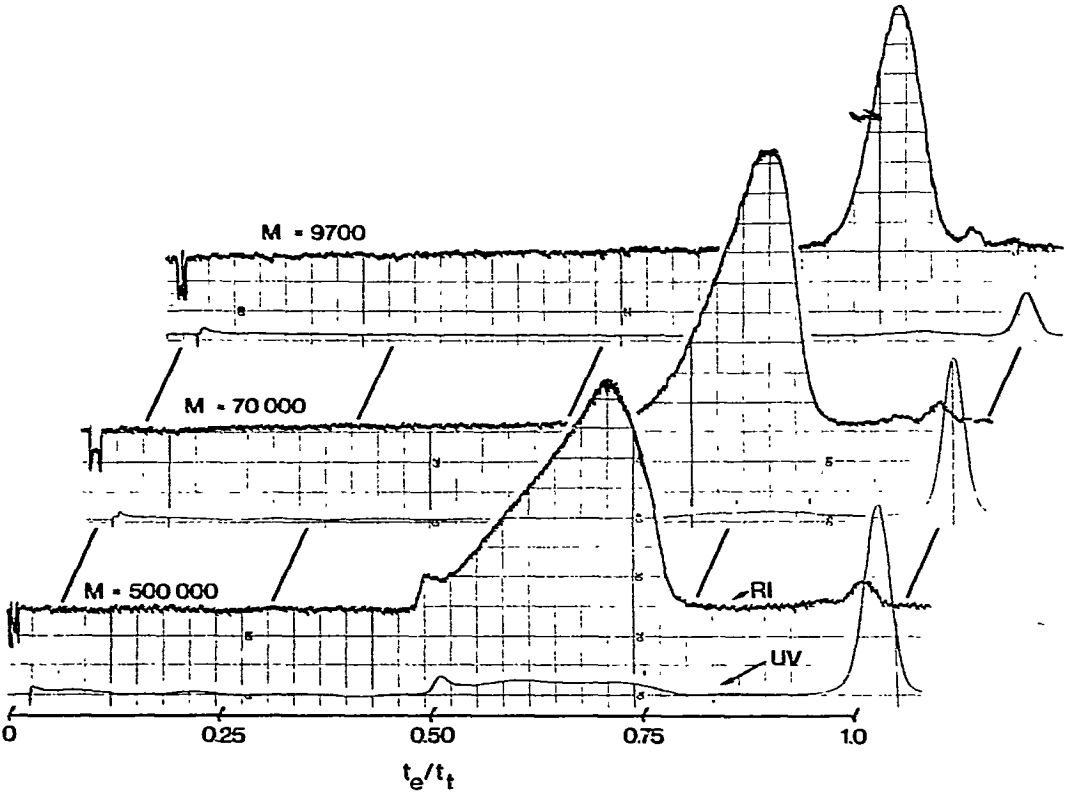

Fig. 4. Elution profiles of dextran fractions on TSK G5000 PW. Chromatography was performed in the standard phosphate buffer described earlier, with detector settings for UV and RI monitors at 0.16 absorbance units and 16, respectively. The injection interval is shown as a zone of pen deflection at the start of each run.

which would be required to calculate molecular weight distributions (Fig. 4). The maximum ordinate was used to mark t_e, thus our elution data correspond to the mode-average, rather than the weight-average or viscosity-average elution times. These data are in Fig. 5. Linear regression yields a correlation coefficient of 0.9991 and

$$F_{(v)} = 0.8979 - 0.00829\,(\bar{M}_v)^{1/3} \tag{5}$$

where $\bar{M}_v$ is the viscosity-average molecular weight.

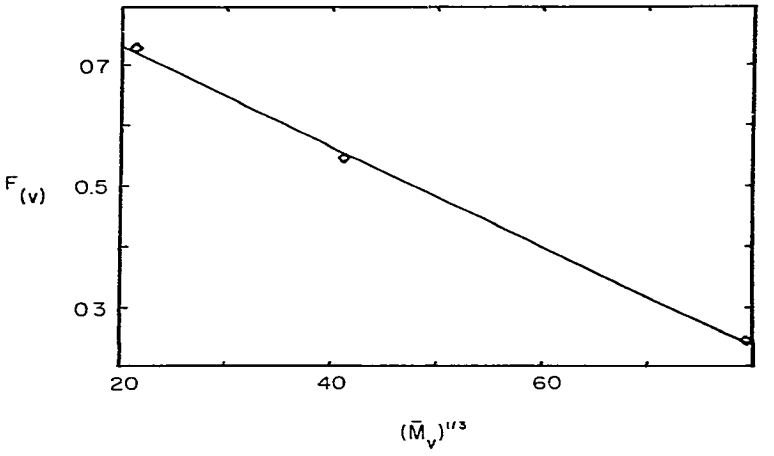

Fig. 5. Size exclusion chromatography of dextran fractions at 25°C, plotted according to eqn. 1. Viscosity-average molecular weights, $\bar{M}_v$, were provided by Sigma Biochemicals.

DISCUSSION

These data demonstrate the usefulness of the TSK G5000 PW preparative column for the purification and characterization of biological macromolecules and supramolecular aggregates up to $(\bar{M}v)^{1/3} = 100$ or $M\bar{v} = 10^6$. For compact spheres of $\bar{v} = 0.73$, this corresponds to $M = 1.4 \cdot 10^6$ and by eqn. 3, a radius of 105 Å.

This high-performance chromatography column proved to have roughly the same fractionation range as Sepharose CL-6B and Sephacryl S-300[5]. However, the G5000 PW gel bead has an average diameter of 18 μm, compared with 105 μm for Sepharose CL-6B and 70 μm for Sephacryl S-300, which allows for a substantial enhancement in column efficiency with the PW gel[22]. Although the column used in this study was run at a linear flow-rate of 16 ml cm^{-2} h^{-1}, which is twice the standard operational flow-rates of the linked dextran–Sepharose supports, data from Toyo Soda indicate the PW gels are stable at linear flow-rates as high as 60 ml cm^{-2} h^{-1}.

The gel chromatography of the sized dextran samples produced results (Fig. 4) which indicate that high-performance gel chromatography is a suitable means of fractionation for extended structures. The usefulness of TSK SW columns for this purpose, in a lower molecular-weight range, had also been demonstrated[23]. It must be noted, however, that dextran is a non-charged and only slightly branched polysac-

charide of solution conformation specific to the α-(1,4) bond. It is therefore unlikely that the molecular-weight calibration given in eqn. 4 will strictly apply to highly varied polysaccharides.

It is worthwhile to compare radii calculated from the experimental light-scattering data of Senti *et al.*[24], using the relationship $R = 0.66\ M^{0.43}$, with those found from experimental elution data. Here, experimental radii of the dextran fractions is found using the elution parameter, $F_{(v)}$, from dextran chromatography, and applying it to eqn. 4. These values are shown in Table II. The agreement between these sets of data is quite good for the two lower-molecular-weight dextran fractions. The prediction of a somewhat lower molecular weight from elution data for the 500,000 molecular-weight fraction when compared to Senti's data is due in part to an error introduced in taking the elution time at maximum ordinate with this highly asymmetric elution profile (Fig. 4).

TABLE II

CALCULATED AND EXPERIMENTAL RADII FOR DEXTRAN

	R from TSK PW (Å)	$R = 0.66\ M^{0.43}$ *(Å)**
$\bar{M}_v = 9700$	36	34
$\bar{M}_v = 70{,}000$	61	80
$\bar{M}_v = 500{,}000$	100	186

* From Senti[24].

An interesting anomaly in the study of virus elution was the very early elution of TMV (Fig. 1). The application of the elution function plot $(M\bar{v})^{1/3}$ *vs.* $F_{(v)}$ (Fig. 2) clearly showed that the three smaller viruses and SWC marked the void volume and the TMV eluted early. The void volume molecular-weight cutoff of $1.4 \cdot 10^6$ was further substantiated by the failure of the G5000 PW to resolve 30S from 50S ribosomes, which correspond approximately to molecular weights of $1.2 \cdot 10^6$ and $2.0 \cdot 10^6$, respectively. It seems likely that the cause is external size exclusion of these very large rods whose length (0.3 μm) is substantial compared with the interstitial spaces between the spherical packing beads of *ca.* 9 μm radius. Work directed toward a quantitative test of this hypothesis is in progress[25].

ACKNOWLEDGEMENTS

This paper has been assigned Scientific Series No. 2607 by the Colorado State University Experiment Station and was supported by project No. 15-1870-63, the USDA Animal Health and Disease Research Program, and a Biomedical Research Support Grant. We are indebted to Dr. Shinya Yoshikawa for providing sea worm chlorocruorin and to Mr. Christopher Morris for preparations of *E. coli* ribosomes and subunits.

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