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CHARACTERIZATION OF PROTEINS AND OTHER MACROMOLECULES BY AGAROSE GEL CHROMATOGRAPHY

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SUMMARY

A number of proteins and proteoglycans were chromatographed on a Sepharose 4B column at various ionic strengths and after application of various amounts of samples. K_{av} was negligibly affected by the chromatographic conditions. When K_{av} was plotted against Stokes' radius, it was found that molecules with different frictional ratios followed different relationships. These results are discussed in relation to the effects of molecular asymmetry. Physical parameters for Sepharose 4B and 6B gels were calculated from chromatography of two protein mixtures.

INTRODUCTION

Gel chromatography separates molecules with different radii[1]. The relationship between molecular radius and elution volume has been used to determine the size of proteoglycan fragments after calibration of a Sepharose gel with proteins* of known size[2]. This method was used to characterize corneal proteoglycans[3] and fragments of skeletal proteoglycans[4]. It was found, however, that the calibration proteins did not follow the predicted relationships; the deviation was largest for asymmetric proteins. Therefore, it was decided to investigate how the elution volume of macromolecules depends on Stokes' radius, frictional ratio, sample mass, ionic strength and differences between various batches of agarose gels. The results, which are partly inconsistent with some published reports discussed below but confirm recent data from Tanford's group[5], are discussed in the present paper; some data were previously discussed at an ARVO meeting[6].

THEORETICAL

The distribution coefficient, K_d, shows the fraction of the solvent volume inside the gel grains that is accessible to the sample[7], and K_{av} is equal to the fraction of the total gel grain volume that is accessible to the sample[1]. The partial specific volume of

* It should be pointed out that in the plot (Fig. 1) in the paper of Serafini-Fracassini *et al.*[2], the points have been wrongly numbered; they should be numbered from right to the left in the graph.

agarose[8] is 0.6 ml/g. Thus, for Sepharose 4B (nominal agarose concentration 4% w/v), K_{av} equals 0.98 K_d, whereas for Sepharose 6B (nominal agarose concentration 6% w/v), K_{av} equals 0.96 K_d.

The Stokes' radius (r_S) of a molecule is defined as the radius of a hypothetical sphere that in diffusion encounters the same frictional coefficient (f) as the real molecule. According to Stokes' law[9], the relationship between the frictional coefficient and the radius of a sphere is

$$f = 6\pi\eta r_S \tag{1}$$

where η is the viscosity of the solvent. Stokes' radius has also been used as the designation for the radius of the equivalent sphere with the same properties as the molecule in gel chromatography or viscometry. The differences between the various types of Stokes' radii have been extensively discussed by Tanford and co-workers[5,10–12]. In this paper, r_S always refers to Stokes' radius as defined by eqn. 1. The Stokes'–Einstein equation, which is derived from Stokes' law (eqn. 1), gives the basis for calculation of r_S from the diffusion coefficient (D^0):

$$r_S = RT/6\pi N\eta D^0 \tag{2}$$

where R is the gas constant, T the absolute temperature, and N Avogadro's number. If r_S is expressed in nm and $D^0_{20,w}$ in μm^2/sec, and if the viscosity of water at 20 °C and the temperature 293 °K are inserted into eqn. 2, it will be reduced to

$$r_S = 214/D^0_{20\,w} \tag{3}$$

Laurent and Killander[1] have shown that K_{av} for a spherical molecule that is partly included in a chromatographic gel is related to r_S by

$$K_{av} = \exp\left[-\pi L (r_S + r_r)^2\right] \tag{4}$$

which can be rearranged to[13]

$$(-\ln K_{av})^{\frac{1}{2}} = (\pi L)^{\frac{1}{2}} (r_S + r_r) \tag{5}$$

where L is the concentration of fibres in the gel grains (in this investigation the concentration of agarose fibres expressed in m fibre per m^3 gel) and r_r the radius of the fibres.

In some cases, the frictional ratio (f/f_0) for a protein was calculated from other literature data using the equation[14]

$$f/f_0 = 2.89 \times 10^{-3}/D\,(M\,\bar{v})^{\frac{1}{3}} \tag{6}$$

where M is the molecular weight and $\bar{v}$ the partial specific volume.

EXPERIMENTAL

Materials

Proteins for calibration came from Sigma Chemical Company, St. Louis, Mo., U.S.A.; Calbiochem AG, Lucerne, Switzerland; and Serva Feinbiochemica GmbH & Co., Heidelberg, G.F.R. (see Table I for details; this table also contains physical data for the proteins). Procedures described elsewhere[28,29] were used to prepare proteoglycans from bovine corneal stroma (designated cornea-50P and cornea-70P[28]) and proteoglycan aggregates from bovine nasal cartilage[29]. Tritiated water was obtained from Packard Instrument Company, Downers Grove, Ill., U.S.A. Sepharose gels were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

TABLE I

DATA FOR PROTEINS USED FOR CALIBRATION OF AGAROSE

Protein	*Commercial designation*	*Stokes' radius (nm)*	*Frictional ratio* (f/f_0)	*Mol. wt.*	*Ref.*
1. Peroxidase (horse-radish)	Sigma P-8250	3.03	1.36	39,800	15
2. Serum albumin (bovine)	Sigma A-4503	3.48	1.30	65,400	16
3. Transferrin (human)	Sigma T-2252	3.55*	1.26**	76,600	17
4. Catalase (bovine liver)	Sigma C-40	5.12	1.24	243,000	18
5. Urease (jack bean)	Sigma U-0376	6.52	1.22**	520,000***	19
6. Apoferritin (horse spleen)	Calbiochem 17836	6.73*	1.32	460,000	20
7. L-Glutamate dehydrogenase (bovine liver)	Sigma G-2501	7.22*	1.58**	316,000	21§
8. α-Casein (cow milk)	Sigma C-3883	7.37	2.25	121,800	22
9. Thyroglobulin (bovine)	Sigma T-1001	8.58	1.49	669,000	23
10. α_2-Macroglobulin (human)	Serva 22390	8.87	1.43	820,000	24
11. Fibrinogen (human)	Calbiochem 341576	10.7	2.34	340,000	25

* Calculated from Svedberg's equation[9] and eqn. 2.
** Calculated from eqn. 6.
*** Calculated from Svedberg's equation[9] using a $\bar{v}$ value from ref. 26.
§ Data on $s^0_{20,w}$ and $\bar{v}$ from ref. 27.

Samples for gel chromatography

Solutions of proteins (0.5 and 1.0 mg/ml), corneal proteoglycans (0.75 and 1.5 mg/ml) and cartilage proteoglycans (1.0 mg/ml) were made. Fibrinogen was dissolved in 1 *M* NaCl–0.05 *M* Tris–HCl (pH 7.0). All other proteins were dissolved in

0.5 *M* Tris–HCl (pH 7.0). Cornea-50P was dissolved in 4 *M* guanidinium chloride–0.05 *M* sodium acetate (pH 5.8) and dialysed extensively against the elution buffer, as it is difficult to dissolve it in the absence of denaturating agents. Cornea-70P was dissolved directly in the elution buffer. L-Glutamate dehydrogenase was obtained as a suspension (20 mg protein/ml). Two portions were diluted 20-fold and 40-fold, respectively, with 0.5 *M* Tris–HCl buffer (pH 7.0) and dialysed against the same buffer in order to get solutions with protein concentrations of *ca.* 1 mg/ml and 0.5 mg/ml, respectively. Crude α-globulin solutions with protein concentrations of 3 and 6 mg/ml were made; this made the α_2-macroglobulin concentration very approximately 0.5 and 1.0 mg/ml, respectively. Solutions of glucuronolactone (0.1 mg/ml) and tritiated water (12% v/v) were made with distilled water.

To simplify the calibration procedure, two mixed solutions with selected proteins in Tris buffer were made. Protein solution A contained cartilage proteoglycan aggregates (1 mg/ml), peroxidase (0.5 mg/ml) and urease (0.5 mg/ml); protein solution B contained thyroglobulin (0.5 mg/ml) and serum albumin (0.5 mg/ml).

The sample volume applied to the columns was 400 μl. Thus, the mass of sample applied to the column was: for proteins, 0.2 or 0.4 mg; for corneal proteoglycans, 0.3 or 0.6 mg; and for cartilage proteoglycan aggregates, 0.4 mg.

Gel chromatography

One column of Sepharose 4B and one of Sepharose 6B were used. Both were packed in glass tubes of I.D. 0.6 cm. A peristaltic pump maintained the flow-rate at 2.4 ml/h through both columns, and 0.58-ml fractions were collected. If the columns were packed with a slightly higher buffer flow than used for elution, the system was very stable. The Sepharose 4B column was used for *ca.* 100 chromatographic runs during more than 2 years without any detectable changes in V_0 (the void volume) or V_i (the solvent volume inside and outside the grains in the gel). This column had a length of 133 cm, with V_i = 38.5 ml and V_0 = 13 ml. The Sepharose 6B column had a bed length of 145 cm, with V_i = 42 ml and V_0 = 16 ml.

Two elution buffers were used: 0.15 *M* NaCl–5 m*M* diemal sodium buffer–0.02% NaN_3 (pH 7.0), and 1 *M* NaCl–5 m*M* diemal sodium buffer–0.02% NaN_3 (pH 7.0). Diemal buffers do not interfere with the Folin procedure, in contrast to Tris or phosphate buffers. The bacteriostatic agent sodium azide must be deleted when the carbazole method is used for analysis of column effluents.

Analytical procedures

Automatic methods[30] were used for the analysis of proteins[31] and glucuronolactone[32] in column effluents. Tritiated water was measured by liquid scintillation counting.

The elution volume for a sample was taken as the elution volume of the fraction with the highest concentration. However, if the peak was asymmetric and the second highest fraction had a concentration greater than 90% of that in the fraction with the peak concentration, the midpoint between the two fractions was taken as the elution volume (Fig. 1). Tris buffer often eluted as a peak with several top fractions within the same range of concentration; the midpoint of this plateau was used as a marker for V_i (Fig. 1). Urease gave only one significant peak, in contrast to other preparations[13]. This peak was considered to correspond to the main fraction described by

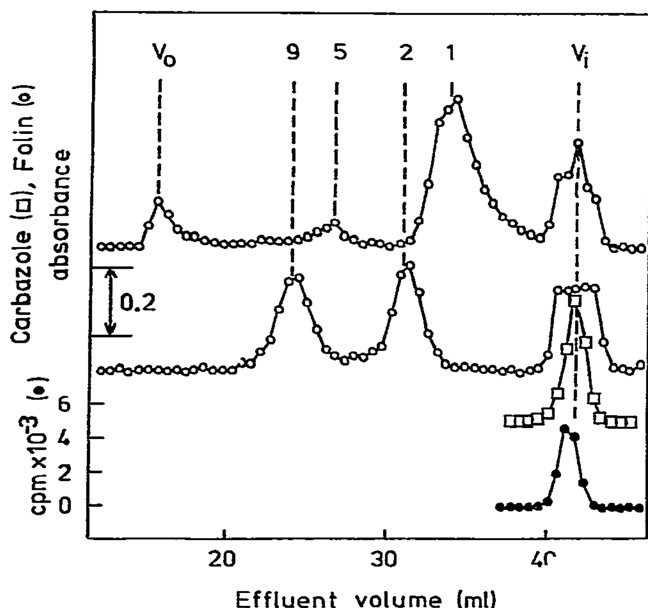

Fig. 1. Calibration of the Sepharose 6B column eluted with the 0.15 M NaCl buffer. The curves are, from the top downwards: protein solution A; protein solution B; glucuronic acid lactone; and tritiated water. The numbers refer to Table I. The urease (5) sample (total 0.2 mg) contained not only urease but also some NaCl; this probably explains the low absorbance given by this protein.

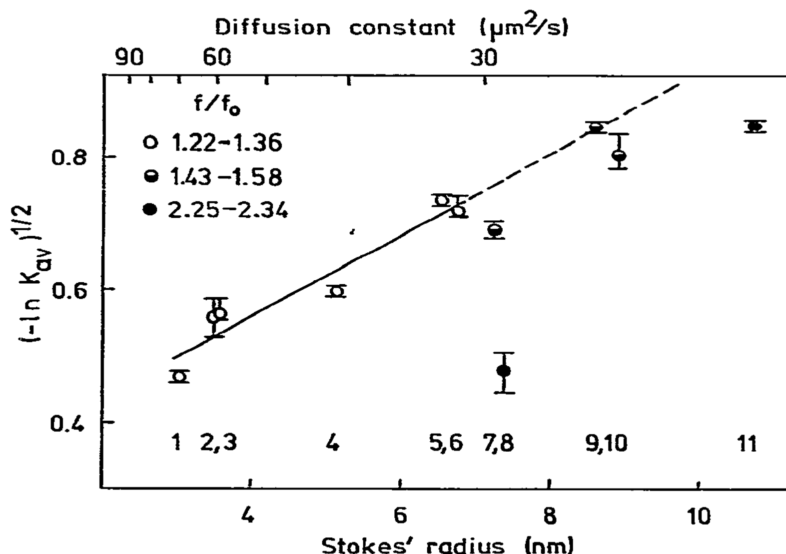

Fig. 2. Chromatography of proteins on the Sepharose 4B column eluted with the 0.15 M NaCl buffer. The horizontal bars indicate the range and the circles the average of $(-\ln K_{av})^{\frac{1}{2}}$ calculated from three runs of each protein (0.2 mg). The straight line is a best least-squares line for proteins with f/f_0 values in the range 1.22–1.36. The numbers on the proteins refer to Table I.

Sumner *et al.*[26]. α_2-Macroglobulin was part of a crude α-globulin preparation; the α_2-macroglobulin peak was distinguished from the other α-globulins as it has a larger molecular size and thus a lower K_{av}.

RESULTS

Calibration of V_0 and V_i

Proteoglycan aggregates from bovine hyaline cartilage are eluted in the void volume of Sepharose 2B columns[33] and can thus be used to indicate V_0 (Fig. 1). Tritiated water and glucuronic acid have been widely used to indicate V_i. Tris has the same elution volume (Fig. 1) and was preferred as V_i indicator as it can be detected by the Folin procedure used for the analysis of proteins in the column effluents.

Chromatography of individual proteins and proteoglycan samples on Sepharose 4B

Each protein was chromatographed separately on the Sepharose 4B column. Three runs were made with 0.2 mg of protein eluted with 0.15 M NaCl buffer (Fig. 2), one run with 0.4 mg of protein eluted with 0.15 M NaCl buffer (Table II) and one

TABLE II

EFFECT OF SAMPLE MASS AND IONIC STRENGTH OF ELUTION BUFFER ON ELUTION VOLUME OF SAMPLES

The first column of K_d values for proteins are those used in Figs. 2 and 4.

Protein	K_d		
	Sample mass 0.2 mg; ionic strength 0.16; average of three runs	*Sample mass 0.4 mg; ionic strength 0.16; one run*	*Sample mass 0.4 mg; ionic strength 1.0; one run*
Peroxidase	0.82	0.82	0.82
Serum albumin	0.75	0.75	0.74
Transferrin	0.74	0.73	0.74
Catalase	0.71	0.70	0.72
Urease	0.59	0.59	0.60
Apoferritin	0.61	0.60	0.59
1-Glutamate dehydrogenase	0.63	0.63	0.63
α-Casein	0.81	0.76	0.78
Thyroglobulin	0.50	0.50	0.48
α_2-Macroglobulin	0.53	0.51	0.55
Fibrinogen	0.50	0.49	0.49
Proteoglycan	K_d		
	Sample mass 0.3 mg; ionic strength 0.16; one run	*Sample mass 0.6 mg; ionic strength 0.16; one run*	*Sample mass 0.3 mg; ionic strength 1.0; one run*
Cornea-50P, peak C (ref. 28, Fig. 4b)	0.29	0.29	0.31
Cornea-70PA*	0.33 (0.31)	(0.32)	(0.29)
Cornea-70PB*	0.56 (0.54)	(0.55)	(0.55)

* Cornea-70PA and cornea-70PB refer to the first and second peaks that cornea-70P shows when chromatographed on Sepharose 4B. Data within brackets were obtained with a batch of Sepharose 4B different from the one used for other experiments presented in this paper.

run with 0.4 mg of protein eluted with 1.0 *M* NaCl buffer (Table II). Similar runs were made with corneal proteoglycans (Table II). α-Casein occurs in different types of aggregate depending on buffer composition[34]. Therefore a control experiment was made with the buffer used by Sullivan *et al.*[22] (0.08 *M* NaCl, 0.02 *M* diemal-Na, pH 7.8). The sample was dissolved in and eluted with this buffer. The K_d value obtained (0.80) is within the range of the other K_d values for α-casein (Table II).

Chromatography of calibration solutions on Sepharose 4B and 6B

Chromatography of calibration solutions A and B on Sepharose 6B is shown in Fig. 1. Values of $(-\ln K_{av})^{\frac{1}{2}}$ from these runs were plotted against the Stokes' radii (Fig. 3). For comparison, the calibration curves obtained by Laurent[8] with Ficoll samples are also shown in the graph. The elution volumes on Sepharose 4B of the individual proteins in the calibration solutions were in all cases the same as those obtained when the proteins were chromatographed separately. The equation for the regression line in Fig. 2 is $(-\ln K_{av})^{\frac{1}{2}} = 0.061\, r_S + 0.32$. The equation for the regression line which shows chromatography of protein solutions A and B on Sepharose 4B (Fig. 3) is $(-\ln K_{av})^{\frac{1}{2}} = 0.064\, r_S + 0.31$. The differences between the equations are small. It is thus sufficient to use solutions A and B for calibration of 4–6% agarose columns.

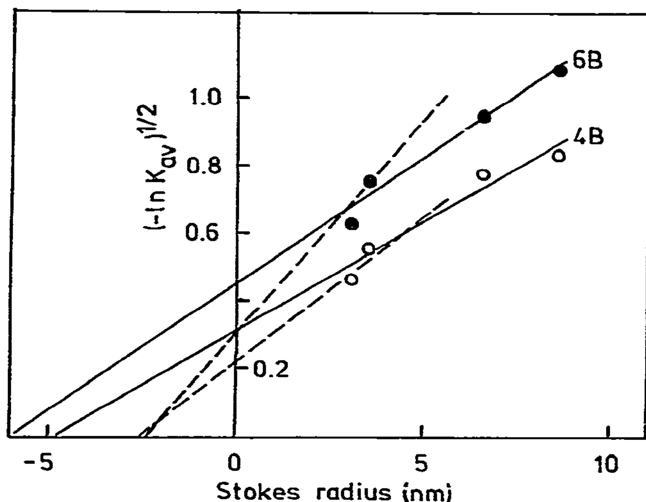

Fig. 3. Calibration of the Sepharose columns with protein solutions A and B. One run was made with each solution on each column. Solid lines indicate the best least-square lines for these runs; dashed lines show the corresponding lines obtained by Laurent[8] for 4% and 6% agarose, respectively.

DISCUSSION

Factors of importance for the elution volume of proteins and proteoglycans

Sample mass. An increased amount of sample may increase the K_{av} value of the sample[35,36]. Small sample weights were used in order to avoid such non-ideal chromatographic behaviour. The effects of sample mass can be neglected for the interpretation of the results in the present investigation as no such effects could be

detected within the mass range used (Table II). This does not, however, rule out the possibility of significant non-ideality when the sample weights are increased beyond those used in this investigation.

Ionic strength. The volume of many polyanions, *e.g.* proteoglycans, glycoproteins and proteins, increases when the ionic strength of the solvent is decreased[37,38]. Moreover, Sephadex and Sepharose gels have carboxyl and sulphate groups that cause ion-exclusion effects[39] when low ionic strength buffers are used for elution. Therefore, Crone[39] suggests that elution buffers for Sepharose columns should have an ionic strength not less than 0.2. Bovine corneal keratan sulphate proteoglycans, for example, are completely excluded from Sepharose 4B at pH 4.0 and ionic strength 0.001 but only partly excluded at higher ionic strength (unpublished results). For proteins and proteoglycans at pH 7, two ionic strengths, 0.16 and 1.0, were chosen. No significant differences in K_{av} were obtained (Table II). It thus seems that physiological ionic strength could be used for the chromatography of the proteins and proteoglycans investigated.

Stokes' radius. It follows from eqn. 5 that $(-\ln K_{av})^{\frac{1}{2}}$ is a linear function of r_S. However, most of the $(-\ln K_{av})^{\frac{1}{2}}$ values in Fig. 2 deviate from the regression line despite the good reproducibility of the K_{av} values. It is probable that inaccurate diffusion constants are partly responsible for the scatter of the points in Fig. 2. However, the role of asymmetry must also be considered, as the two asymmetric proteins investigated (α-casein and fibrinogen) show much lower $(-\ln K_{av})^{\frac{1}{2}}$ values than expected.

Asymmetry. If the proteins are grouped according to their f/f_0 values, it seems that those with higher frictional ratios have lower $(-\ln K_{av})^{\frac{1}{2}}$ values than expected from those with lower frictional ratios (Fig. 2). It may be concluded that molecular asymmetry causes retardation in agarose gel chromatography. This effect of asymmetry may be obscured by the facts that f/f_0 is a function of both shape and hydration, and that many diffusion constants probably are inaccurately determined.

The observations in this investigation on the role of molecular asymmetry in gel chromatography are not compatible with some earlier findings. Siegel and Monty[13] found that bovine fibrinogen ($f/f_0 = 2.35$) was perfectly adapted to the regression line for globular proteins in a plot of $(-\ln K_{av})^{\frac{1}{2}}$ against Stokes' radius for Sephadex G-200 eluted with 0.04 *M* phosphate buffer–5 m*M* EDTA (pH 8.0). Demassieux and Lachance[40] also used bovine fibrinogen and globular proteins to calibrate a Sepharose 6B column. They found a linear relationship between log r_S and K_d. However, they used a 0.1 *M* phosphate buffer (pH 7.0) containing the reducing agent dithiothreitol (1 m*M*) and EDTA (1 m*M*) that may influence the conformations of the proteins. These latter authors also state that globular and asymmetric proteins fit to a common regression line if log $[(f/f_0)\,M^{\frac{1}{3}}]$ is plotted against K_d. Therefore, the chromatographic data from Fig. 2 were plotted as log $[(f/f_0)\,M^{\frac{1}{3}}]$ against K_d (Fig. 4). The points are widely scattered and the reliability of the empiric equations formulated by these authors seems limited. Elution buffers with reducing agents were not used in the present investigation as there is little reason to believe that the varying degree of unfolding caused by reduction in the absence of dissociating agents should make K_d a linear function of log $[(f/f_0)\,M^{\frac{1}{3}}]$.

However, a review of gel chromatographic data in the literature revealed several indications that molecular asymmetry may influence results in gel chromato-

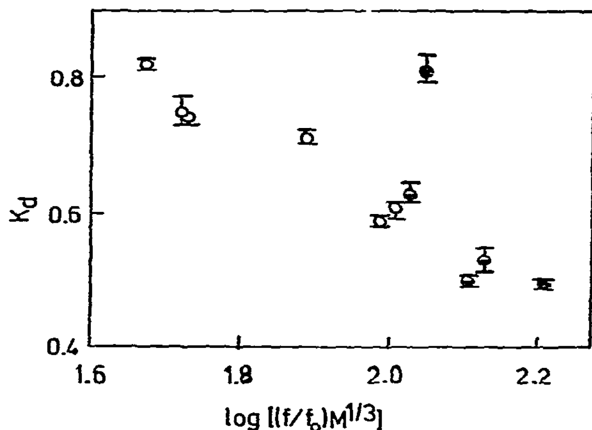

Fig. 4. The data from Fig. 2 plotted as suggested by Demassieux and Lachance[40]. See Fig. 2 for explanation of symbols. The proteins are in order from left (No. 1, Table I) to right (No. 11).

graphy. Laurent and Killander[1] stressed that eqn. 4 is derived for spherical particles only. The data of Warshaw and Ackers[41] suggest that molecular asymmetry may influence the gel chromatographic behaviour of proteins. Laurent *et al.*[42] state that asymmetric molecules penetrate polysaccharide gels more readily than globular molecules with equal Stokes' radius. As K_{av} indicates the fraction of gel accessible for the molecular species, this observation is consistent with higher K_{av} values for asymmetric molecules than for globular molecules with equal r_S. Tanford *et al.*[12] stated in 1974 that "no systematic studies using a mixture of globular and highly asymmetric molecules have been carried out to determine whether partition in the gel is consistently responsive to the R_S [Stokes' radius] value based on either frictional coefficient or intrinsic viscosity". Recently, Nozaki *et al.*[5] described the retardation of fibrinogen and myosin and some denaturated proteins on Sepharose 4B. The present investigation corroborates these data on a pronounced retention of asymmetric proteins and previous data[41] on a slight retention of 'globular' proteins with f/f_0 in the range 1.4–1.6*.

The role of molecular asymmetry has, with few exceptions, been overlooked in the interpretation of data from gel chromatography, indicating that the relationship between molecular shape and exclusion from gels should be systematically investigated.

Differences between gels. Batches of agarose with the same nominal concentrations sometimes do not exhibit similar properties. Laurent[8] and Nozaki *et al.*[5], for example, found different properties for different batches of 4% agarose from Pharmacia. Sepharose 2B is used for determination of the ratio of aggregate to monomer for cartilage proteoglycans because aggregates are completely excluded from the gel whereas monomers are partly included in the gel[33]. However, the same batch of proteoglycans chromatographed on different batches of Sepharose 2B gave different elution profiles, with considerable variation in the amount of completely excluded material and thus different values for the ratio of aggregate to monomer[43].

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* An arbitrary but widely accepted definition of globular proteins is proteins with f/f_0 less than 1.50 (ref. 24, p. C3).

In Fig. 3 and Table III some data on the Sepharose 4B and 6B gels are compared with the data obtained by Laurent[8] from calibration of 4% and 6% pearl-condensed agarose gels with various fractions of Ficoll, a highly branched polysaccharide. Our values for fibre radius, fibre volume and hydration are larger than those of Laurent and the values for fibre length are consequently smaller. This may reflect both differences in the nature of the calibration molecules (globular protein as against polysaccharide) and differences between various batches of agarose. The latter explanation is more probable for the following reason. Laurent compared human serum albumin with Ficoll and found a K_{av} for albumin equal to the K_{av} for Ficoll with the same r_S; therefore it seems probable that Ficoll behaves like globular proteins. The influence of gel fibre branching must, however, also be considered as a possible explanation for the discrepancy between the two investigations.

TABLE III

DATA FOR THE AGAROSE GELS

Gel type	*Fibre radius, r_r (nm)*	*Total length of fibres, L ([m/m³] × 10^{-15})*	*Fibre volume (m³ fibre/m³ gel grains)*	*Hydration of fibres (H_2O, % w/w)**
4% Agarose				
Data from this paper	4.8	1.3	0.095	64
Data from Laurent[8]	2.6	2.4	0 049**	37**
6% Agarose				
Data from this paper	5 9	1.8	0.20	73
Data from Laurent[8]	2.4	5.1	0.092**	48**

* Calculated as g water per 100 g of hydrated agarose fibre using 1.6 g/ml as the density for pure unhydrated agarose[8].

** Data calculated from other data given by Laurent[8].

Branching of gel fibres. It is assumed in eqn. 4 that the gel consists of randomly distributed, linear, rigid rods of infinite length; deviation from this will lead to apparent thickening and shortening of the rods[1]. The agarose fibre network does contain branching points. One explanation for the discrepancy between the data in this investigation (Fig. 3 and Table III) and the data of Laurent[8] may be that the smaller molecules (Stokes' radii 1.9–5.6 nm, K_{av} on 4% agarose 0.6–0.85) used by Laurent obey eqn. 4 better than the larger molecules (Stokes' radii 3.0–10.7 nm, K_{av} on 4% agarose 0.5–0.8) used in the present investigation. The error caused by the branching of the fibres implies that eqn. 4 is an approximation not only for asymmetric molecules but also for spherical molecules.

The physical models for gel chromatography

The present investigation clearly shows that the physical models for gel chromatography are inadequate for a universal theory for gel chromatography. The present data suggest that the effects of molecular asymmetry and gel fibre branching invalidate simple physical models. A thermodynamic treatment of gel chromatography needs no physical model for the separation mechanism. Hjertén[36] has shown, by using thermodynamic theory, that the distribution of a solute between the gel grains and the

mobile phase is influenced by the activity of the solute, the temperature and pressure in the gel, the interfacial tension at the interface between the solute and the solvent (and thus the area of the solute molecules), the electric charges on the solute and the solvent, and the adsorption of the solute to the gel matrix. A physical model that will account for all the factors involved in gel chromatographic separation will be much more complicated than the present models.

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NOTE ADDED IN PROOF

Conformational changes of macromolecules entering a gel have recently been described in a preliminary communication[44] which may be of considerable importance in the explanation of *e.g.* the gel chromatographic behaviour of denatured proteins.

REFERENCES

1 T. C. Laurent and J. Killander, *J. Chromatogr.*, 14 (1964) 317.
2 A. Serafini-Fracassini, N. H. Stimson and L. Floreani, *Biochem. J.*, 122 (1971) 101.
3 I. Axelsson and D. Heinegård, *Biochem. J.*, 169 (1987) in press.
4 D. Heinegård , I. Axelsson and S. Inerot, in preparation.
5 Y. Nozaki, N. M. Schechter, J. A. Reynolds and C. Tanford, *Biochemistry*, 15 (1976) 3884.
6 I. Axelsson and D. Heinegård, *The Association for Research in Vision and Ophthalmology, Spring Meeting, Sarasota, Fla., 1976*, Abstract No. 6, p. 24.
7 B. Gelotte, *J. Chromatogr.*, 3 (1960) 330.
8 T. C. Laurent, *Biochim. Biophys. Acta*, 136 (1967) 199.
9 K. E. van Holde, *Physical Biochemistry*, Prentice-Hall, Englewood Cliffs, N.J., 1971.
10 J. A. Reynolds and C. Tanford, *J. Biol. Chem.*, 245 (1970) 5161.
11 W. W. Fish, J. A. Reynolds and C. Tanford, *J. Biol. Chem.*, 245 (1970) 5166.
12 C. Tanford, Y. Nozaki, J. A. Reynolds and S. Makino, *Biochemistry*, 13 (1974) 2369.
13 L. M. Siegel and K. J. Monty, *Biochim. Biophys. Acta*, 112 (1966) 346.
14 C. F. Phelps, in H. Gutfreud (Editor), *MTP International Review of Science, Biochemistry Series One*, Butterworths, London, 1974, pp. 325–373.
15 R. Cecil and A. G. Ogston, *Biochem. J.*, 49 (1951) 105.
16 J. M. Creeth, *Biochem. J.*, 51 (1952) 10.
17 K. G. Mann, W. W. Fish, A. C. Cox and C. Tanford, *Biochemistry*, 9 (1970) 1348.
18 H. Sund, K. Weber and E. Mölbert, *Eur. J. Biochem.*, 1 (1967) 400.
19 J. M. Creeth and L. W. Nichol, *Biochem. J.*, 77 (1960) 230.
20 I. Björk, *Eur. J. Biochem.*, 36 (1973) 178.
21 H. Eisenberg, R. Josephs and E. Reisler, in C. B. Anfinsen, J. T. Edsall and F. M. Richards (Editors), *Advances in Protein Chemistry*, Vol. 30, Academic Press, New York, 1976, pp. 101–181.
22 R. A. Sullivan, M. M. Fitzpatrick, E. K. Stanton, R. Annino, G. Kissel and F. Palermiti, *Arch. Biochem. Biophys.*, 55 (1955) 455.
23 H. Edelhoch, *J. Biol. Chem.*, 235 (1969) 1326.

24 H. A. Sober (Editor), *Handbook of Biochemistry*, The Chemical Rubber Co., Cleveland, Ohio, 1970, p. C-37.
25 R. F. Doolittle, *Advan. Protein Chem.*, 27 (1973) 1.
26 J. B. Sumner, N. Gralén and I.-B. Eriksson-Quensel, *J. Biol. Chem.*, 125 (1938) 37.
27 K. S. Rogers, L. Hellerman and T. E. Thompson, *J. Biol. Chem.*, 240 (1965) 198.
28 I. Axelsson and D. Heinegård, *Biochem. J.*, 145 (1975) 491.
29 D. Heinegård, *Biochim. Biophys. Acta*, 285 (1972) 181.
30 D. Heinegård, *Chem. Scripta*, 4 (1973) 199.
31 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randell, *J. Biol. Chem.*, 193 (1951) 265.
32 T. Bitter and H. M. Muir, *Anal. Biochem.*, 4 (1962) 330.
33 T. E. Hardingham and H. Muir, *Biochim. Biophys. Acta*, 279 (1972) 401.
34 M. P. Thompson, in H. A. McKenzie (Editor), *Milk Proteins, Chemistry and Molecular Biology*, Vol. II, Academic Press, New York, 1971. pp. 117–173.
35 T. C. Laurent and K. A. Granath, *Biochim. Biophys. Acta*, 136 (1967) 191.
36 S. Hjertén, *J. Chromatogr.*, 50 (1970) 189.
37 S. G. Pasternack, A. Veis and M. Breen, *J. Biol. Chem.*, 249 (1974) 2206.
38 C. Tanford, *Physical Chemistry of Macromolecules*, Wiley, New York, 1961, pp. 489–499.
39 H. D. Crone, *J. Chromatogr.*, 92 (1974) 127.
40 S. Demassieux and J.-P. Lachance, *J. Chromatogr.*, 89 (1974) 251.
41 H. S. Warshaw and G. K. Ackers, *Anal. Biochem.*, 42 (1971) 405.
42 T. C. Laurent, B. N. Preston, H. Pertoft, B. Gustafsson and M. McCabe, *Eur. J. Biochem.*, 53 (1975) 129.
43 D. Heinegård and S. Inerot, personal communication.
44 B. N. Preston, *Uppsala J. Med. Sci.*, 82 (1977) 92,