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GEL CHROMATOGRAPHY IN ELUENTS CONTAINING POLYMERS

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SUMMARY

In gel chromatographic experiments using Sephadex G-200, human serum albumin has been chromatographed in eluents containing neutral polymers. Each of the four types of polymers studied; dextran, polyethyleneglycol, polyvinylalcohol, and Ficoll, was tested at several concentrations. Albumin is eluted later as the polymer concentration in the eluent is increased. This is ascribed to an activity increase of the protein in the mobile polymer phase that is due mainly to steric exclusion. When the exclusion properties of the gel phase are known one can calculate the exclusion of the test substance from the liquid polymer phase. Measurements made by this method agree well with data derived from solubility studies of the exclusion of albumin from . dextran solutions. They agree exactly with the exclusion measurements on dextran gels. The method should be useful in studies of the exclusion properties and the molecular parameters of polymer solutions.

INTRODUCTION

Gel chromatography (gel filtration)—a kind of partition chromatography—was developed by PORATH AND FLODIN[1]. Several reviews on this subject have appeared, *e.g.* refs. 2 and 3. Many authors have attributed the unequal partition of solutes between the mobile and stationary phases in gel chromatography to steric exclusion of the solute molecules from the gel. One model for the exclusion process was presented by LAURENT AND KILLANDER[4], who calculated the available spaces in dextran gels on the basis of the assumption that the gels consisted of three-dimensional networks of straight polymer fibers. The experimental partition values for several solutes of known molecular sizes were close to the values predicted by the model. The relationship seems valid for several types of gels[5–8]. Although effects other than purely steric ones can influence the activity coefficient of a solute in a polymer phase[9,10], steric factors appear to predominate in neutral polymer gels.

The solubility of a protein is reduced by the inclusion of dextran of high molecular weight in the solution[11,12]. The decrease in solubility depends on the molecular size of the protein and on the concentration of dextran. This phenomenon can also be explained in terms of steric exclusion of the protein from a volume fraction of the solution that is regarded as the domain of the polysaccharide. Furthermore, the degree

to which various proteins were excluded from dextran gels—as determined by chromatography—correlated very well with the diminution in solubility observed for the same proteins in dextran solutions. By this measure, solutions of high molecular weight dextran have almost the same exclusion properties as dextran gels of the same dextran concentration. That is, the exclusion domain of the polysaccharide is the same in the gel as it is in solution[13].

The addition of the appropriate polymer to the eluent used in gel chromatography should thus give the liquid phase in the column properties similar to those of the stationary gel phase. The activity of a solute in the liquid phase will be increased by the presence of the polymer with the result that the distribution of the solute between the gel phase and the liquid phase will change. The purpose of the present investigation has been to study this effect quantitatively. A preliminary communication has been made[14]. The great similarity of this system to the polymer two-phase systems of ALBERTSSON[15] should be pointed out.

Materials

The different dextran fractions and the Ficoll preparation were supplied by Pharmacia AB, Uppsala, Sweden. The following polysaccharides were used (the molecular weights were determined by the manufacturer by end-group analysis and light-scattering): Dextran 10 (Lot No. To 644) number-average molecular weight, $\bar{M}_n$ = 6,200, weight-average molecular weight, $\bar{M}_w$ = 9,400; Dextran 35 (To 740) $\bar{M}_n$ = 22,000; $\bar{M}_w$ = 35,000; Dextran 150 (To 4934) $\bar{M}_n$ = 95,000; $\bar{M}_w$ = 153,000; Dextran 500 (T 5406), $\bar{M}_n$ = 153,000, $\bar{M}_w$ = 420,000; Ficoll (To 5987), $\bar{M}_w$ approx. 400,000; Blue Dextran 2000 $\bar{M}_w$ approx. $2 \cdot 10^6$.

Polyethyleneglycol (PEG) was obtained from L. Light & Co Ltd., Colnbrook, England. It had a molecular weight of 6,000. Polyvinylalcohol (PVA) was obtained from Wacker-Chemie, Munich, Germany. The batch used (trade name Polyviol M 05/20) had an approximate molecular weight of 22,000.

The polymers were dissolved in 0.05 M phosphate buffer, pH 7.4, containing 0.1 M sodium chloride and 10 % (v/v) of a saturated solution of 5,7-dichloro-8-quinolinol (bacteriostatic and chelating agent). When Dextran 500 was used, the concentration in the eluent was checked by the anthrone reaction[16].

The densities of the solutions were calculated from the concentrations and the partial specific volumes of the dissolved materials (dextran = 0.61[17]; Ficoll = 0.66[18]; sucrose = 0.61[19]) assuming a value of 1.0107 for the density of the buffer at 4° [20]. The densities of the PVA and PEG solutions were estimated from data published by ALBERTSSON[15].

Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) was the only type of dextran gel used in the experiments. However, different batches were employed after storage for varying periods of time in saturated solutions of the above-mentioned chelating agent.

Human serum albumin was kindly supplied by AB Kabi, Stockholm, Sweden. Although it contained some components with the electrophoretic properties of α- and β-globulins, it was used without further purification. α-crystallin with a molecular weight of 820,000 was a gift from Dr. I. BJÖRK[21].

Tritiated water was obtained from the Radiochemical Centre, Amersham, England. Solutions containing 1 μCi/ml were prepared in the above-mentioned buffer.

METHODS

Preparation of columns

Plexiglas tubes with an inner diameter of 2 cm were used for the chromato-graphic experiments. The tubes were fitted at both ends with adjustable plungers containing porous membranes, essentially as described by PORATH AND BENNICH[22]. The columns were packed in the phosphate buffer according to the manufacturer[23]. When the gel had settled, the column was run at a hydrostatic pressure of about 100 cm for 24 h, after which the upper plunger was adjusted to touch the surface of the gel bed. The bed heights used ranged from 52 to 108 cm. The flow of the eluent was then reversed with a peristaltic pump. The flow rate was not allowed to exceed that obtained with a hydrostatic head of 100 cm, usually 3–4 ml/cm$^2 \cdot$h. After an eluent volume corresponding to at least the total volume had passed through the bed the column was ready for use. When the eluent was changed, the column was again allowed to adjust to the new medium at a hydrostatic head of 100 cm. In some experiments, the flow rate could decrease to less than 2 ml/cm$^2 \cdot$h.

Characterization of columns

The void volume (V_0) was determined with Blue Dextran 2000 (3–5 mg) or α-crystallin (15 mg).

The total volume of the column (V_t) was determined with a low molecular weight U.V.-absorbing component of uncertain origin in the albumin preparation and with tritiated water[24]. The differences between these two types of measurements did not exceed ± 1 % of the total volume. V_t was calculated from the U.V.-peak by addition of the volume occupied by the dextran fibres in the gel matrix. When a polymer was included in the eluent, correction was also made for that fraction of the polymer that enters the gel phase. The determinations of V_t were also checked by direct measurements of the volume of the gel bed in the tube. The void volume and the total volume were determined for each eluent used.

Experimental

The transmission of the eluate at 254 nm was continously recorded with a Uvicord absorptiometer (LKB; Stockholm, Sweden) and the exact position of each peak was determined on the recorder strip. The eluate was collected in 2.5–3 ml portions. The fractions were weighed and their exact volumes were then calculated from the density of the solutions. The absorbance of each fraction at 280 nm was also measured.

Human serum albumin was dissolved at a concentration of about 6 % in phos-phate buffer containing the polymer to be investigated. 0.5 or 1.0 ml samples were introduced into the columns followed by 1–2 ml of a 5 % solution of sucrose in the same medium. In this way, convection currents during the introduction of the samples were avoided. All the experiments were performed at +4°.

The chromatographic properties of the various polymers were studied in separate experiments, the details of which are given in the legend of Fig. 1. Dextran and Ficoll were determined in the eluates by means of the anthrone reaction[10], while PEG and PVA were determined with a titrimetric method[25].
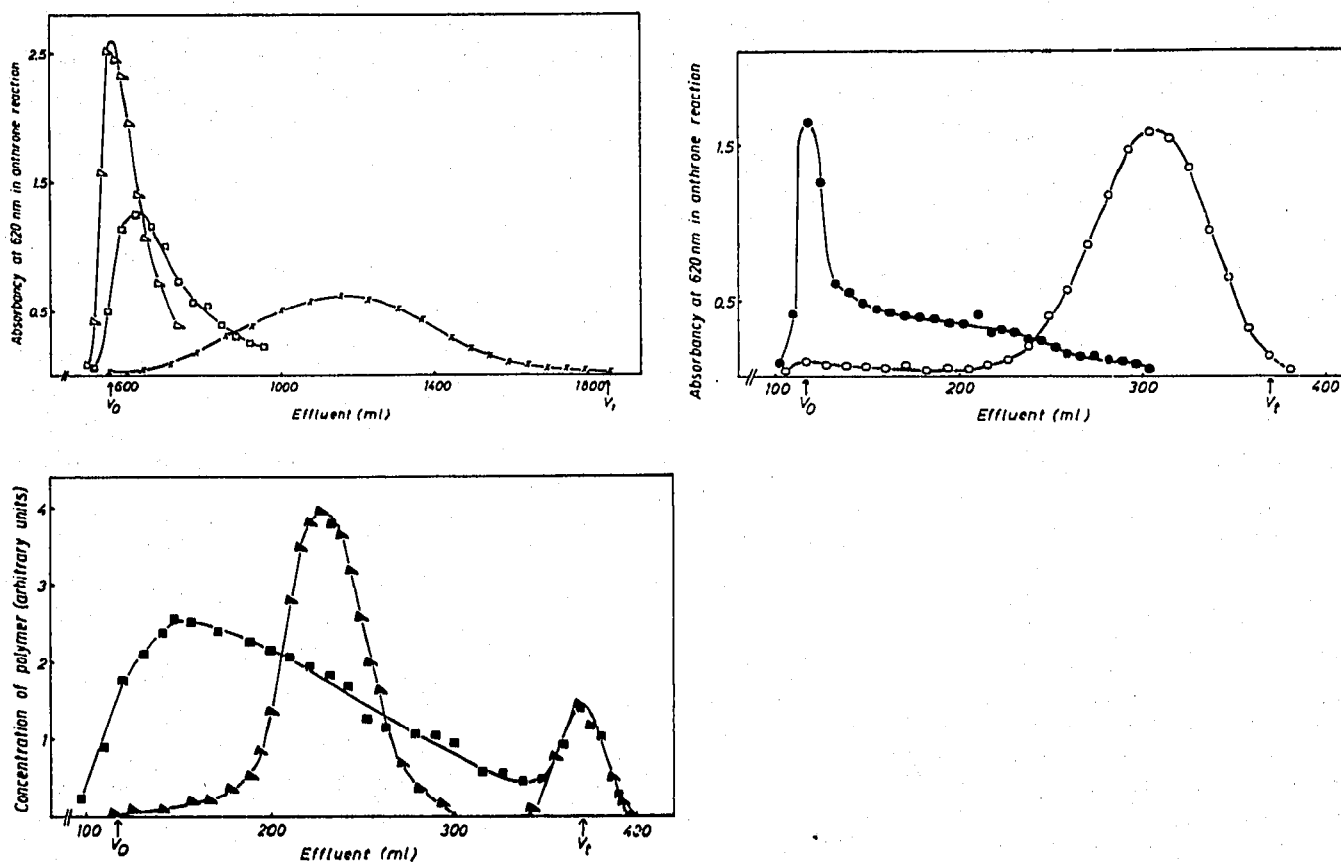
Fig. 1. (a) Chromatography of 20 mg each of Dextran 500 (△); Dextran 150 (□); and Dextran 35 (×) on a 5 × 94-cm column of Sephadex G-200 (Column A in Table I). (b) Chromatography of 10 mg each of Ficoll (●); and Dextran 10 (○) on a 2 × 113 cm column of Sephadex G-200 (Column B in Table I). (c) Chromatography of 10 mg PEG (▲) and 40 mg PVA (■) on the column B. The peak at V_t is due to a non-specific reaction with salt in the titrimetric method used for the polymer determination[23].

The coefficient K for the partition of a substance between the gel phase and the liquid phase was calculated according to LAURENT AND KILLANDER[4]. :

$$K = \frac{V_e - V_0}{V_t - V_0} \tag{1}$$

where V_e is the elution volume and V_0 and V_t are the void volume and the total volume of the column, respectively. $V_t—V_0$ is equal to the volume occupied by the gel phase.

RESULTS

Partition coefficients of the polymers

The polymers to be used in the eluents were chromatographed on two Sephadex G-200 columns (Fig. 1 a-c), as was the serum albumin as well. Despite the apparent polydispersity of several of the polymers, average partition coefficients were calculated for all of them, except Ficoll, by the use of eqn. 1 (Table I). The volume of eluent required to elute half of the amount of polymer applied to the column was taken as the elution volume, V_e.

TABLE I

PARTITION COEFFICIENTS OF VARIOUS POLYMERS ON SEPHADEX G-200 COLUMNS, AS CALCULATED FROM THE EXPERIMENTS IN FIG. I

	Column A	Column B
Serum albumin	0.44	0.47
Dextran 500	0.00	
Dextran 150	0.05	
Dextran 35	0.47	
Dextran 10		0.76
PVA		0.29
PEG		0.44

The gels in columns A and B give different partition coefficients for albumin than do Columns I–IV (Table II). The partition coefficients for the various polymers on the latter columns can be calculated from the ratio between the logarithm of the partition coefficient for albumin and the logarithm of that for the polymer. According to eqn. 2, this ratio should be the same for any dextran gel.

TABLE II

CHROMATOGRAPHY OF HUMAN SERUM ALBUMIN ON SEPHADEX G-200 GELS USING POLYMER-CONTAINING ELUENTS

Column No.	Polymer in eluent	Concn. $g/ml \times 10^2$	$V_t - V_0$ ml	Shrinkage of the gel %	K for albumin
I	None		128	—	0.39
	Dextran 500	1	121	5	0.47
	Dextran 500	2	115	10	0.54
	Dextran 500	3	106	17	0.62
	Dextran 500	4	100	22	0.73
II	None		242	—	0.41
	Dextran 35	1	242	—	0.46
	Dextran 35	2	238	2	0.50
	Dextran 150	1	237	2	0.51
	Dextran 150	2	223	8	0.57
III	None		129	—	0.35
	Dextran 10	4	127	2	0.40
	Ficoll	2	124	4	0.46
	Ficoll	4	116	11	0.57
	(Sucrose)	4	130	1	0.35
IV	None		122	—	0.37
	PVA	2	108	11	0.60
	PEG	2	99	21	0.54
	PEG	4	50	57	0.91

Shrinkage of the gel phase due to polymers in the eluent

When the eluent on the Sephadex column was changed from buffer to a polymer solution, the gel phase shrank somewhat, as evidenced by a decrease in $V_t - V_0$ (Table II). The shrinkage is also expressed in the table as per cent of the original gel volume.

Partition coefficients of albumin eluted with polymer solutions

The chromatography of serum albumin on a Sephadex G-200 column (Column No. I in Table II) is shown in Fig. 2. The partition coefficient, K, rather than the elution volume, has been plotted on the abscissa so that experiments involving different concentrations of Dextran 500 and thus different bed volumes can be compared. The partition coefficient for albumin increases very rapidly with increasing dextran concentration. The partition coefficients obtained under various conditions are tabulated in Table II.
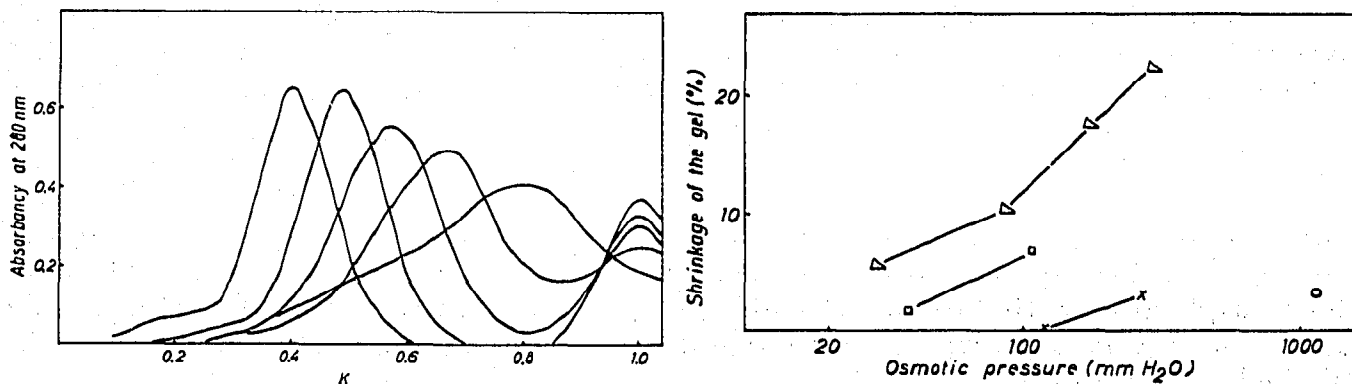
Fig. 2. Chromatography of albumin on a Sephadex G-200 column eluted with buffer containing 0, 1, 2, 3, and 4 % of Dextran 500 (curves in the order from left to right). The albumin polymer fraction that emerges at V_0 ($K = 0$) has been excluded from the chromatogram. The peak at V_f ($K = 1$) is due to an unknown U.V.-absorbing contaminant in the albumin preparation.

Fig. 3. Shrinkage of Sephadex G-200 upon exposure to polymer-containing eluents plotted *versus* the calculated osmotic pressures of the eluents. Dextran 500 (△); Dextran 150 (□); Dextran 35 (×); and Dextran 10 (O).

The effects of polymers on the partition coefficient of serum albumin and the gel bed volume were completely reversible upon switching back and forth between polymer solution and pure buffer as eluent with any given column.

Sucrose in 4 % concentration did not cause any shrinkage of the gel and had no effect on the partition coefficient of albumin.

DISCUSSION

Shrinkage of the gel

The correlation between the shrinkage of the gel and the osmotic pressure of the dextran solutions, as calculated according to HINT[20], is demonstrated in Fig. 3. The low molecular weight dextrans which penetrate the gel te some extent do not exert the same effect as does Dextran 500, which is completely excluded. The shrinkage is probably due to the osmotic force exerted by the excess soluble dextran in the liquid as compared to the gel phase.

Calculation of the exclusion properties of the gel after shrinking

The theoretical expression for the volume available to a spherical particle in a three-dimensional network of randomly oriented long, rigid fibres was derived by

OGSTON[27] and is written here with the symbols used by LAURENT AND KILLANDER[4]:

$$K_{av} = e^{-\pi \cdot L(r_s + r_r)^2} \tag{2}$$

K_{av} is the fraction of the system available to a sphere with the radius r_s; L is the total concentration of fiber expressed in length per unit volume; and r_r is the radius of the fibres. LAURENT AND KILLANDER found that this equation describes correctly the partition of various substances between dextran gels and buffer, where K_{av} is the partition coefficient, and they concluded therefrom that the exclusion is due mainly to steric effects. The fact that other effects might contribute to some extent[9],[10] does not invalidate the following discussion.

If the coefficient K_{av} for the partition of a substance, e.g., albumin, between a dextran gel and a buffer is known, eqn. 2 can be used to calculate the parameter L, which is proportional to the dextran concentration in the gel:

$$L = -\frac{\log_e K_{av}}{\pi \cdot (r_s + r_r)^2} \tag{3}$$

r_s for albumin is 35.5×10^{-8} cm (ref. 4), r_r is 6×10^{-8} cm (ref. 28). If the gel should shrink, as it does upon exposure to a polymer solution, the new value of L is obtained by multiplication with the factor $(V_t - V_0)_B / (V_t - V_0)_P$, where $V_t - V_0$ is the volume of the gel phase and the subscripts B and P denote buffer and polymer solution, respectively, as the liquid medium.

Changes in available volume, K_{av}, for a substance in the gel phase after shrinking can also be calculated from eqn. 2 in the following form:

$$\log_e (K_{av})_P = \frac{\log_e (K_{av})_B \cdot (V_t - V_0)_B}{V(t - V_0)_P} \tag{4}$$

Determination of the exclusion properties of the polymer eluents

The coefficient K for the partition of a substance between the gel phase and the liquid phase is related to the available volume for the protein in the gel phase, $(K_{av})_G$ and that in the eluent $(K_{av})_E$:

$$K = \frac{(K_{av})_G}{(K_{av})_E} \tag{5}$$

Analysis of experimental data

The discussion will be divided into three parts; experiments with Dextran 500, which is completely excluded from the gel; experiments with the low molecular weight dextrans; and experiments with the other polymers.

Dextran 500. The values for the partition coefficient, K, in Table II and the recalculated values (eqn. 4) for the available volume for albumin in the dextran gel after shrinkage have been used to obtain the available volume in the eluent, $(K_{av})_E$ from eqn. 5. The data are given in Table III and are plotted in Fig. 4 versus the dextran concentration. They are compared with the data reported by LAURENT for various studies, e.g., solubility measurements[13]. A fair correlation is observed between the results obtained by the different techniques. The decrease in $(K_{av})_E$ is an exponential function, as demonstrated in Fig. 5 by a logarithmic plot versus the dextran concentration. This would be expected if the available volume in a dextran solution like that in a dextran gel, is also given by eqn. 2. Further evidence that eqn. 2 is valid for dextran solutions

TABLE III

THE AVAILABLE VOLUME FOR HUMAN SERUM ALBUMIN IN DIFFERENT POLYMER SOLUTIONS $(K_{av})_E$ CALCULATED AS DESCRIBED IN THE TEXT

Polymer	Concn. of polymer $(g/ml \times 10^2)$			
	1	2	3	4
Dextran 500	0.81	0.67	0.53	0.42
Dextran 150	0.81	0.68		
Dextran 35	0.83	0.66		
Dextran 10				0.46
PVA		0.46		
PEG		0.43		0.05
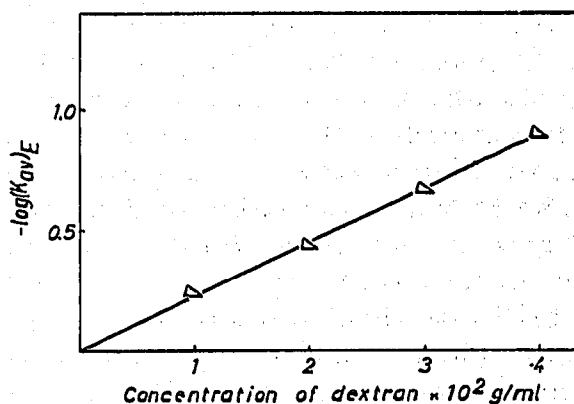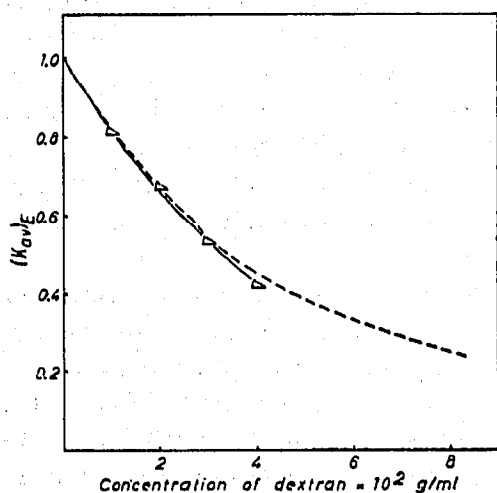
Fig. 4. The available volume for serum albumin in Dextran 500 solutions as calculated from gel chromatography experiments (△) compared with values obtained by other techniques (broken line, from Fig. 2 in ref. 13).

Fig. 5. Logarithmic plot of data from Fig. 4.

is given in Fig. 6. The parameter L for the polymer solution has been calculated from eqn. 3 and the experimentally obtained values of $(K_{av})_E \cdot L$ have been plotted versus the dextran concentration. The linear relationship obtained is identical to that given by LAURENT AND KILLANDER for dextran gels (Fig. 6 in ref. 4).

These results confirm the earlier conclusion[13] that the exclusion properties of a polymer gel are identical to those of a solution of the same polymer at the same concentration, indicating that the fundamental exclusion process in the gel does not involve the cross-linkages. This similarity in exclusion properties is valid only when the soluble polymer is sufficiently large and probably also when the degree of cross-linkage of the gel is rather low.

Low molecular weight dextrans. When lower molecular weight dextrans are used, part of the material enters the gel, thereby increasing the concentration of dextran inside the gel. To obtain the total dextran concentration in the gel, we have to add the concentration of the gel material, corrected for shrinkage, to the concentration of soluble polymer in the gel. The concentration of the soluble polymer in the gel has
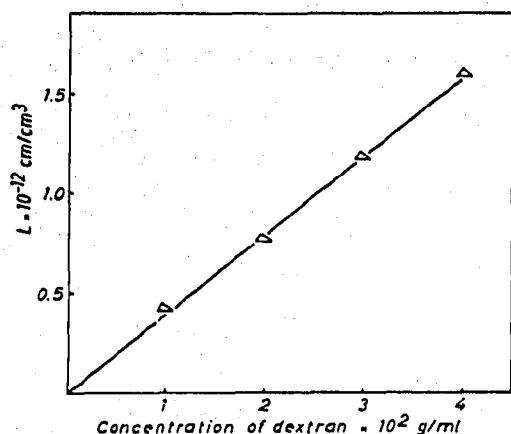
Fig. 6. The relationship between the dextran concentration and the parameter L in eqn. 2. L was calculated from the available volume for albumin in Dextran 500 solutions. The relationship is identical with that given for dextran gels[4].

been calculated from its concentration in the liquid phase and its available volume in the contracted gel.

The concentration of gel material after shrinkage, expressed as L (cm/cm³), is obtained from eqn. 3. The available volume for the soluble dextran in the contracted gel is obtained from the data in Table I (corrected as described in the footnote) and eqn. 4. The absolute concentration of soluble dextran inside the gel can thus be calculated easily when the dextran concentration in the eluent is known. The concentration in g/ml can be expressed in cm/cm³ by the aid of Fig. 6.

The total concentration of dextran (gel + soluble) in the gel grains expressed in cm/cm³ can be introduced into eqn. 2 in order to calculate the available volume for albumin in the gel grains ($(K_{av})_G$ in eqn. 5). The latter equation can thus be used to calculate $(K_{av})_E$. The values obtained for the various dextrans are given in Table III.

The calculation performed above is based on the assumption that the soluble dextran entering the gel grains has the same exclusion properties towards albumin as does the dextran of the gel matrix. The results given in Table III are consistent with this assumption. Dextran 35 and Dextran 150 have essentially the same exclusion properties as Dextran 500 and hence are also equivalent to dextran gels.

Polyvinylalcohol and polyethyleneglycol. These two polymers can not be treated quantitatively as above, as it is probably not strictly valid to sum the effects of the gel matrix and the dissimilar soluble polymer in the grains. However, if one assumes that the soluble polymers in the grains do behave like dextran, a value of the available volume for serum albumin in the liquid phase $(K_{av})_E$ can be calculated as above. The results of the calculations are given in Table III. The exclusion properties are obviously more pronounced for these polymers than for dextran, indicating that the assumption of identical behavior is wrong and that the values given for the available volumes of albumin in PVA and PEG solutions in Table III are too high.

Some experiments have been performed with Ficoll, but due to its great polydispersity (Fig. 1b) no attempts have been made to make any calculations with the data. Apparently, however, the available volume for albumin in Ficoll is lower than that in dextran (Table II).

Polymer structural aspects

The above discussion is not based on any *a priori* assumptions regarding the basic mechanisms of the 'exclusion' or change in 'available volume'. The use of eqn. 2

in the calculations is justified because numerous experiments have shown that this equation correctly predicts the observed partition of substances between a dextran gel and a buffer. The terms 'exclusion' and 'available volume' imply, however, that the main selection process is steric.

Strong support for the hypothesis that the partition pattern of albumin depends mainly on steric exclusion is afforded by the fact that the $(K_{av})_E$ values for albumin in soluble dextran follow eqn. 2, which has been deduced theoretically for sterical interactions. Furthermore, the $(K_{av})_E$ values are independent of the molecular weight of the dextran, at least for Dextran 35 and larger dextrans. Finally, the linear PEG and PVA molecules have larger exclusion effects than the branched dextran, whereas the compact Ficoll polymer has a smaller one; these findings are in accord with the concept that the excluded domain of a polymer increases with increasing assymetry. The extended shape of the PVA and PEG chains is also reflected by their gel chromatography behaviour, since they are eluted much earlier in the gel chromatogram (Fig. 1c) than are "globular" substances of similar molecular weights. In fact, PEG with a molecular weight of 6,000 elutes earlier than albumin, which has a molecular weight of 69,000[29].

The volume excluded for a substance by the polymer chains (expressed as ml/g polymer) can be calculated from the available volume. Since the excluded volume decreases with increasing polymer concentration it is convenient to extrapolate to zero concentration. From the present data the volume excluded for human serum albumin by Dextran 500 and PEG can be calculated as 20 and 33 ml/g, respectively.

If one accepts the steric hypothesis and the validity of eqn. 2, partition data can be used to characterize polymers in terms of fiber radii (r_r) and fiber length (L). LAURENT and others have used this approach to characterize a number of polymer gels[5-8]. By the use of the present technique, namely, gel chromatography with polymer solutions as eluents, soluble polymers can be characterized in terms of the same parameters. In such experiments one should use polymers that are completely excluded from the gel. This can be achieved by using high molecular weight polymers and sufficiently tight gels.

The method presented here is similar, in principle, to the equilibrium dialysis method introduced by OGSTON[30] to study exclusion in hyaluronic acid solutions, but has the advantage that it can be used even when the difference in molecular size between the polymer and the excluded compound is relatively small whereas the dialysis method for practical reasons requires a large difference.

Practical aspects

Since the addition of a polymer to the eluent increases the elution volumes of various substances, it seems reasonable to propose that the technique could be used to increase the resolution in gel chromatography, especially in the case of high-molecular weight substances that elute early in the chromatogram. Unfortunately, polymers decrease the solubility of high-molecular weight proteins[31]. In preliminary experiments where human serum was chromatographed on Sephadex G-200 in the presence of Dextran 500, the three major peaks were eluted later than they are in the absence of Dextran 500. However, the size of the peak containing the material of highest molecular weight decreased with increasing dextran concentrations, indicating losses probably due to precipitation on the column. Another drawback is the broad-

ening of the peaks with increasing polymer concentrations. Calculations of the equivalent height of one theoretical plate (EHTP) (ref. 2) for the Dextran 500 experiments show a six-fold increase of EHTP upon increasing the polymer concentration from 0 to 4%. The possible physiologal importance of a two-phase polymer system like this has been pointed out[32].

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