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GEL PERMEATION CHROMATOGRAPHY OF PROTEINS IN PARTLY AQUEOUS ELUENTS

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Summary

Gel permeation chromatography of a number of proteins has been performed with Sephadex G-100 and G-200, using mixtures of water and organic solvents as eluents. It appears that the chromatographic distribution coefficients of the proteins are enhanced by the addition of organic co-solvents to the eluent. Possible causes of this phenomenon are (1) adsorption of the proteins to the gel matrix in the partly aqueous eluents and (2) partition of the proteins between two phases of different composition, *viz.*, the eluent and the liquid in the gel beads.

This phenomenon has been applied to resolve a mixture of ribonuclease and cytochrome *c*, using 5% (w/v) polyethylene glycol 20,000 as the eluent. These proteins cannot be separated with an aqueous eluent, due to the small difference between their molecular weights.

INTRODUCTION

Chromatography of proteins with hydrophilic gels is virtually always performed with aqueous eluents. Under these conditions, the retention is governed by steric exclusion of the proteins from part of the liquid in the gel network.

Some years ago Brewer and Söderberg[1] presented data on the chromatography of proteins with Sephadex G-100, using 5% (w/v) polyethylene glycol 6000 as the eluent. In this case, the retention was clearly not governed by steric exclusion. Brewer and Söderberg pointed to the analogy between their results and data obtained by partition of proteins in aqueous two-phase polymer systems.

We prefer a slightly different explanation. In 5% polyethylene glycol, Sephadex G-100 swells to only 36% of its swollen volume in water. This shows that the affinity of the gel for the mixed solvent is less than for water. Further, the mixed solvent is probably a poorer solvent for proteins than water. Adsorption of the proteins to the gel matrix is therefore likely to occur in the mixed solvent. Moreover, the liquid in the gel beads probably has a slightly smaller polyethylene glycol content than the eluent. As a consequence, the proteins will partition between the liquid in the gel beads and the eluent, and at equilibrium their concentration in the interior liquid is

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probably larger than in the eluent. Both mechanisms give rise to an enhancement of the chromatographic distribution coefficients of proteins in the mixed eluent, as observed by Brewer and Söderberg.

If this explanation is correct, the same phenomenon should occur when low-molecular-weight organic solvents are added to the eluent. This might lead to the possibility of separating proteins that cannot be separated with aqueous eluents, due to the closeness of their molecular weights. An investigation of this question is presented in this paper.

As a matter of fact, low-molecular-weight peptides have been separated by gel chromatography on Sephadex G-25, with partly aqueous eluents[2]. As far as we are aware, however, this principle has not been used for the separation of proteins.

EXPERIMENTAL

Chemicals

Sephadex gels and Blue Dextran 2000 were from Pharmacia (Uppsala, Sweden). Aldolase, bovine serum albumin (BSA, fraction V), ovalbumin, β-lactoglobulin, myoglobin (type III), ribonuclease (type I-A) and cytochrome *c* (type III) were from Sigma (St. Louis, MO, U.S.A.), chymotrypsinogen A from E. Merck (Darmstadt, F.R.G.), and trypsin and trypsin-inhibitor were from Boehringer (Mannheim, F.R.G.). A buffer solution was prepared from $Na_2HPO_4 \cdot H_2O$ (Baker Analyzed, J. T. Baker, Deventer, The Netherlands) and $NaH_2PO_4 \cdot 2H_2O$ (Baker Grade). The organic co-solvents were polyethylene glycol (PEG) 4000 and 20,000 (Fluka, Buchs, Switzerland), ethanol (Baker Analyzed), acetone (99%, Aldrich, Beerse, Belgium) and dioxane (zur Analyse, Merck).

Apparatus

Gel permeation chromatography was performed in a Pharmacia K 16/70 column, equipped with a Pharmacia A 16 flow adapter and a Pharmacia LV 4 sample valve. The column was packed by means of a Pharmacia R 15/16 packing reservoir. The sample concentration in the eluate was monitored by a UV detector (Uvicord S 2138, LKB, Bromma, Sweden) or a refractivity detector (R403, Waters Assoc., MA, U.S.A.), connected to a 2210 LKB dual-pen recorder. The flow-rate of the eluent was regulated by a peristaltic pump (Minipuls II, Gilson, Villiers-Le-Bel, France). It was determined gravimetrically three times a day.

Procedure

Columns were packed according to the instructions of the manufacturer[3], using accurately weighed portions of Sephadex that had been swollen for 24 h in the appropriate eluent. All eluents contained a 0.025 *M* NaH_2PO_4 + 0.025 *M* Na_2HPO_4 buffer, of ionic strength 0.1 and pH in water 7.2.

Samples of 0.5 ml, containing 0.5 mg of protein in the appropriate eluent, were used. The chromatographic distribution coefficients K_{av} were calculated from the equation:

$$K_{av} = \frac{V_e - V_0}{V_t - V_0} \quad (1)$$

TABLE I

DATA ON THE SWELLING OF SEPHADEX GELS IN PARTLY AQUEOUS ELUENTS (ml/g)

Eluent	*% (w/v)*	*G-50*		*G-75*		*G-100*		*G-200*	
		V_t	V_0	V_t	V_0	V_t	V_0	V_t	V_0
Water		12.5	5.2	17.5	7.6	21.2	9.1	36.8	16.2
Dioxane	5					19.7	7.8		
	10					20.3	8.3	33.6	14.5
Ethanol	5					20.5	8.5		
	20					16.6	6.9	25.3	10.6
Acetone	20					12.1	5.2	17.8	6.4
PEG 4000	5					11.7	4.5	19.2	7.0
PEG 20,000	5					10.2	4.0	10.2	3.3

where V_e is the retention volume of the protein, V_0 the void volume (equal to the retention volume of Blue Dextran) and V_t the total bed volume, which was calculated from the height and cross-section of the gel bed.

RESULTS AND DISCUSSION

Plate height values were determined for Sephadex G-100 columns in all eluents, using data on BSA, chymotrypsinogen and cytochrome *c*. The plate height did not depend on the eluent or the sample. Its mean value was 0.21 cm.

Data on the swelling of Sephadex G-50 and G-75 in water and of G-100 and G-200 in all the eluents investigated are given in Table I.

From these data it can be calculated that the void volume of the four gels in most eluents is about 43% of the bed volume. Only in the eluents where the swollen

TABLE II

DATA ON THE RETENTION OF PROTEINS ON SEPHADEX G-50 AND G-75 IN WATER AS THE ELUENT (ml/g)

The molecular weight M and the equivalent hydrodynamic radius r_s of the protein molecules[3] are also shown.

Protein	*log M*	$r_s \times 10^8$	*G-50*		*G-75*	
			V_e	K_{av}	V_e	K_{av}
Aldolase	5.20		5.2	0.00	7.6	0.00
BSA	4.83	36.5[4]	5.3	0.01	8.3	0.07
Ovalbumin	4.65	27.3	5.6	0.05	9.0	0.15
β-Lactoglobulin	4.54		5.9	0.09	9.9	0.23
Chymotrypsinogen	4.40	22.4	6.4	0.17	10.7	0.32
Trypsin	4.40	19.4	6.8	0.21	12.0	0.44
Trypsin-inhibitor	4.33	22.6	6.4	0.15	10.5	0.30
Myoglobin	4.25	19.8	7.0	0.25	11.6	0.41
Ribonuclease	4.14	19.2	7.3	0.29	12.1	0.46
Cytochrome *c*	4.10	16.4	7.5	0.31	12.3	0.48

TABLE III

DATA ON THE RETENTION OF PROTEINS ON SEPHADEX G-100 IN VARIOUS ELUENTS (ml/g)

Protein	*Water*		*5% Dioxane*		*10% Dioxane*		*5% Ethanol*		*20% Ethanol*		*20% Acetone*		*5% PEG 4000*		*5% PEG 20,000*	
	V_e	K_{av}	V_e	K_{av}	V_e	K_{av}	V_e	K_{av}	V_e	K_{av}	V_e	K_{av}	V_e	K_{av}	V_e	K_{av}
Aldolase	9.9	0.06	8.7	0.07	9.2	0.07	9.7	0.09			5.3	0.02	5.0	0.07		
BSA	11.4	0.19	10.2	0.20	10.9	0.22	11.2	0.22	9.8	0.29	6.3	0.16	5.9	0.19	6.2	0.35
Ovalbumin	12.6	0.29	11.4	0.30	12.1	0.32	12.1	0.30	10.7	0.40	7.0	0.27	7.0	0.34	8.5	0.72
β-Lactoglobulin	13.4	0.36	12.6	0.40	13.9	0.47	13.5	0.41	11.9	0.52	7.9	0.40	7.1	0.36	8.4	0.70
Chymotrypsinogen	14.8	0.47	13.5	0.48	14.3	0.50	14.7	0.51	13.5	0.68	8.8	0.53	8.1	0.50	8.3	0.69
Trypsin	15.7	0.54	14.3	0.55	15.4	0.59	14.0	0.46	14.5	0.79			9.7	0.72	9.9	0.95
Trypsin inhibitor	14.8	0.47	13.2	0.45	14.0	0.48	14.1	0.47	12.1	0.54	7.9	0.39	7.5	0.42	7.8	0.62
Myoglobin	15.9	0.56	14.4	0.56	15.8	0.63	15.8	0.61	14.3	0.77			10.5	0.83	12.0	1.30
Ribonuclease	16.2	0.59	14.6	0.57	15.9	0.64	15.5	0.58	14.8	0.81			9.9	0.74	10.8	1.09
Cytochrome *c*	16.8	0.63	15.5	0.64	17.4	0.76	16.3	0.65	17.4	1.08	15.1	1.43	12.1	1.05	14.0	1.61

TABLE IV

DATA ON THE RETENTION OF PROTEINS ON SEPHADEX G-200 IN VARIOUS ELUENTS (ml/g)

Proteins	*Water*		*10% Dioxane*		*20% Ethanol*		*20% Acetone*		*5% PEG 4000*		*5% PEG 20,000*	
	V_e	K_{av}	V_e	K_{av}	V_e	K_{av}	V_e	K_{av}	V_e	K_{av}	V_e	K_{av}
Aldolase	21.1	0.24	19.2	0.25	15.5	0.33	8.5	0.19	13.2	0.51	6.0	0.40
BSA	23.8	0.37	22.3	0.41	17.6	0.48	10.3	0.34	13.1	0.50	6.9	0.52
Ovalbumin	26.0	0.48	24.2	0.51	19.6	0.61	11.5	0.45	15.2	0.68	9.5	0.90
β-Lactoglobulin	28.1	0.58	26.5	0.63	21.3	0.72	12.4	0.53	15.6	0.70	9.2	0.85
Chymotrypsinogen	28.3	0.59	27.7	0.69	23.2	0.86			16.5	0.78	8.9	0.81

volume is about half that in water or less, does the void volume occupy a smaller fraction of the bed volume. The smallest fraction is found for Sephadex G-200 in 5% polyethylene glycol 20,000, *i.e.*, 32%. Probably, when only little swelling occurs the mechanical strength of the swollen gels is decreased, and some deformation of the gel beads occurs on packing the columns. The gels swell more in water than in the mixed solvents. This implies that the affinity of Sephadex gel for water is larger than for the organic co-solvents at the concentrations examined. As argued in the Introduction, this means that concentration of the proteins in the gel beads by adsorption to the gel matrix and partition between the liquid in the beads and the eluent can be expected. Data on the retention of the proteins are given in Tables II–IV.

Table III contains several K_{av} values that are larger than 1. This points to concentration of the protein concerned in the gel, due to adsorption and/or partition. In Tables III and IV most K_{av} values with the mixed eluents are larger than the corresponding values with water as the eluent. This points also to the occurrence of adsorption and/or partition of the proteins in these eluents. Only in 5% dioxane and ethanol and in 20% acetone are the K_{av} values not enhanced. However, it is possible that in the latter eluents, too, adsorption and/or partition of the proteins occurs: as the gels swell less in mixed eluents than in water, one would expect smaller K_{av} values in mixed eluents than in water, on the basis of steric exclusion alone.

This argument can be expressed in quantitative terms as follows. Ogston[5] has shown that steric exclusion of spherical solute molecules from a gel is governed by the equation:

$$K_{av} = \exp[-\pi L(r_r + r_s)^2] \quad (2)$$

where L is the concentration of the gel fibers in cm/cm^3, r_r is the radius of the gel fibers and r_s is the radius of the solute molecules. One can substitute for L:

$$L = L'/(V_t - V_0) \quad (3)$$

where L' is the length of 1 g of gel fibers. According to Laurent *et al.*[6] the K_{av} values obtained for proteins on Sephadex gels are in excellent agreement with eqns. 2 and 3, with $r_r = 7 \cdot 10^{-8}$ cm and $L' = 3.8 \cdot 10^{13}$ cm/g.

We calculated the values of K_{av} which would correspond to a steric exclusion mechanism, using the above-mentioned values of the parameters r_r and L', the data

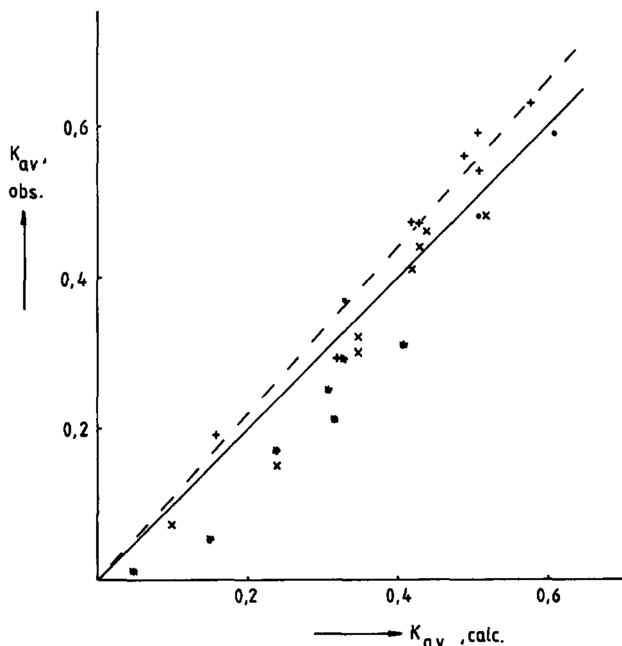


Fig. 1. Comparison of observed and calculated values of K_{av} in water as the eluent, on Sephadex G-50 (✱), G-75 (×), G-100 (+) and G-200 (●). (———)K_{av}, obs. = K_{av}, calc. (---------)K_{av}, obs. = 1.10 K_{av}, calc.

on V_t and V_0 in Table I and the data on r_s in the third column of Table II. The calculated values are compared with the observed values of K_{av} in Figs. 1–6.

The first impression of Fig. 1 is that our data with water as the eluent are well represented by eqns. 2 and 3 with the above-mentioned values of the parameters r_r and L'. However, closer inspection of Fig. 1 shows that the observed values of K_{av} for Sephadex G-50 and G-75 are slightly smaller than the calculated values, and that the observed values for Sephadex G-100 and G-200 are about 10% larger than the calculated values.

Figs. 2 and 3 show that the K_{av} values observed on Sephadex in 5% dioxane or ethanol are not larger than expected for a steric exclusion mechanism. However, in 10% dioxane and 20% ethanol the K_{av} values are clearly enhanced. The same conclusion holds for 20% acetone (Fig. 4), and for 5% polyethylene glycol (Fig. 5).

Dioxane can be considered as a low-molecular-weight analogue of polyethylene glycol. Figs. 2 and 5 show that the enhancement of K_{av} increases strongly with the molecular weight of the organic co-solvent.

The same conclusions hold for the data obtained on Sephadex G-200 (Fig. 6).

The ratio α of the K_{av} values of two proteins can depend markedly on the co-solvent, as is illustrated in Table V. This implies that separations that are impossible with water as the eluent, due to the closeness of the molecular weights of the proteins concerned, may be easily accomplished with a suitable co-solvent. This is illustrated in Fig. 7.

We suggested, above, that the enhanced K_{av} values are due to adsorption of

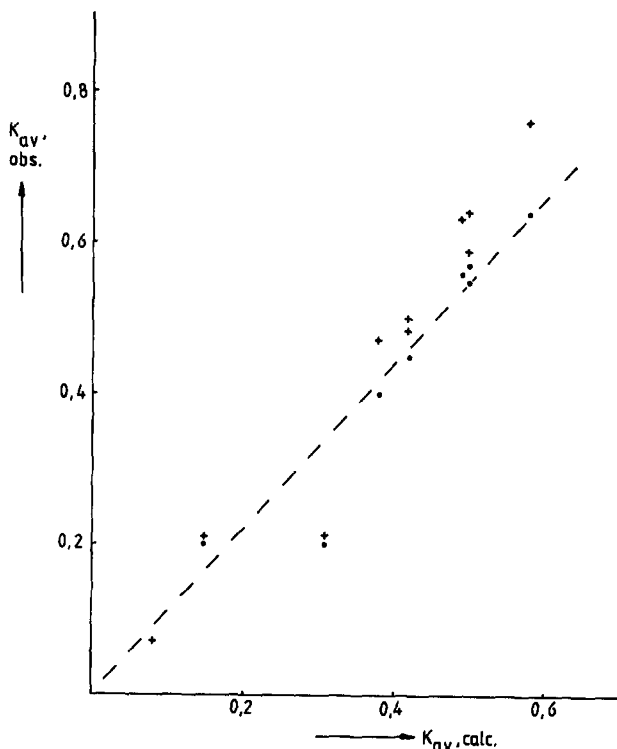


Fig. 2. Comparison of observed and calculated values of K_{av} on Sephadex G-100, in 5% dioxane (●) and 10% dioxane (+). (---------)K_{av}, obs. = 1.10 K_{av}, calc.

the proteins to the gel matrix and/or to partition between the liquid in the gel beads and the eluent. The latter mechanism can only occur when the composition of the liquid in the gel beads is slightly different from that of the surrounding liquid. Further, other causes for the enhanced K_{av} values can be envisaged, *e.g.*, a change in the solvation of the proteins in the mixed solvents, compared with the situation in water, which results in a change (*i.e.*, a decrease) of the solvated volume. Nor can denaturation of some proteins in the mixed solvents be ruled out, *a priori*. These points are discussed below.

Several investigators have observed that dextran gel has a greater affinity for water than for polar organic solvents. The K_{av} values of the lower primary alcohols on Sephadex G-100 are about 0.65[7]. In dioxane–water mixtures Sephadex G-25 takes up an excess amount of water of 0.2–0.4 g/g of dry gel, even at a very low dioxane concentration[8]. Polyethylene glycol 6000 shows a K_{av} value of 0.34 on Sephadex G-200 in a mixed eluent containing 4% PEG 6000[9]. There is, therefore, ample evidence that the composition of the liquid in the gel beads is different from that of the surrounding liquid, in partly aqueous eluents.

A decrease of the solvated volume of the proteins investigated in the mixed solvents might, in principle, contribute to the observed enhancement of the K_{av} values. However, we think it improbable that this phenomenon plays an important role.

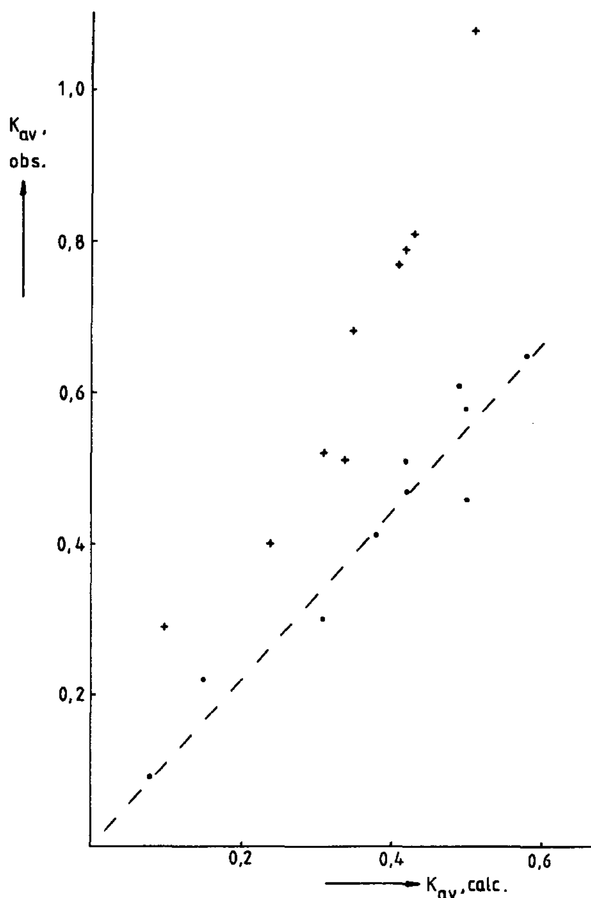
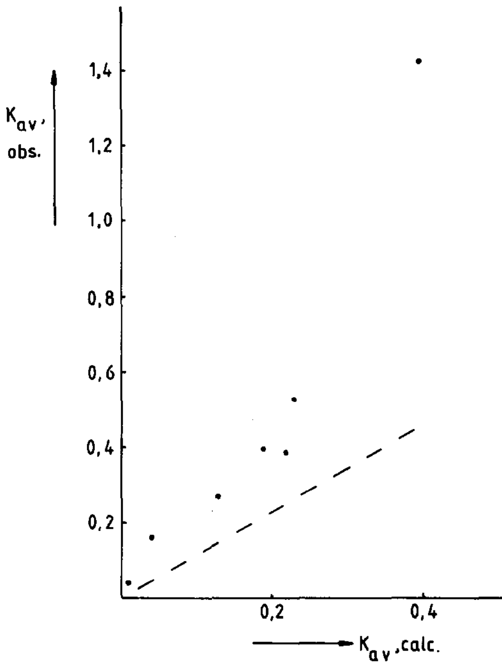


Fig. 3. Comparison of observed and calculated values of K_{av} on Sephadex G-100, in 5% ethanol (●) and 20% ethanol (+).

TABLE V

INFLUENCE OF THE CO-SOLVENT ON THE RATIO α OF THE K_{av} VALUES OF TWO PROTEINS

Proteins	*Gel*	*Water*	*10% Dioxane*	*20% Ethanol*	*20% Acetone*	*5% PEG 4000*	*5% PEG 20,000*
Ovalbumin–BSA	G-200	1.30	1.24	1.27	1.32	1.36	1.73
Myoglobin–trypsin inhibitor	G-100	1.19	1.31	1.43		1.97	2.10
Cytochrome–ribonuclease	G-100	1.07	1.19	1.33		1.42	1.48



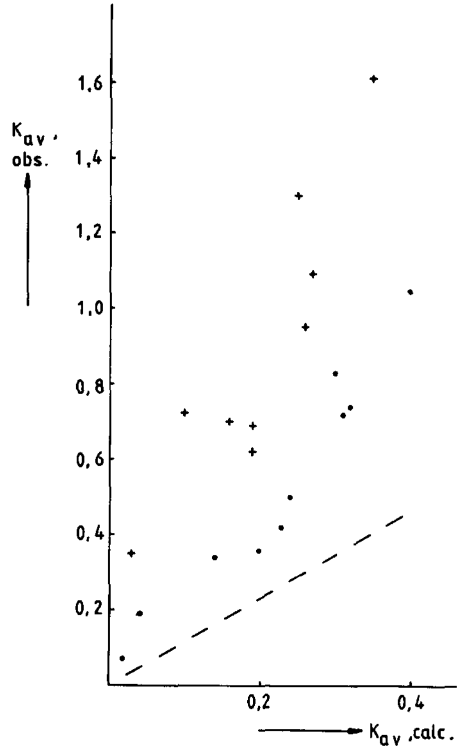


Fig. 4. Comparison of observed and calculated values of K_{av} on Sephadex G-100, in 20% acetone.

Fig. 5. Comparison of observed and calculated values of K_{av} on Sephadex G-100, in 5% polyethylene glycol 4000 (●) and 5% polyethylene glycol 20,000 (+).

TABLE VI

LITERATURE DATA ON THE UNFOLDING OF THE PROTEINS INVESTIGATED

Protein	*Concentration (w/v, %) required to produce 50% denaturation*			
	Ethanol[11,12]		*Propanol-2*[12]	*Diethylene glycol*[12]
BSA	29			
Ovalbumin	22			
β-Lactoglobulin	27			
Chymotrypsinogen	26	17	16	
Myoglobin	27	24	20	84
Cytochrome *c*	26	33	28	80

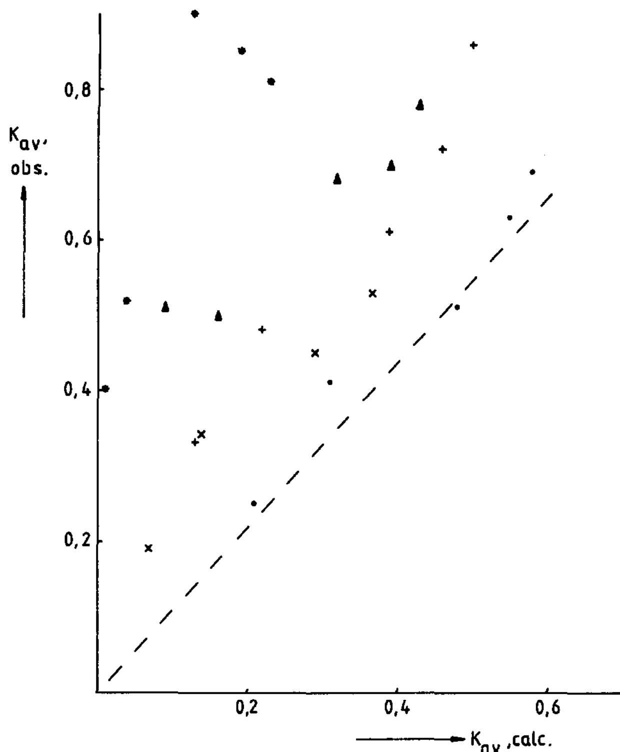


Fig. 6. Comparison of abserved and calculated values of K_{av} on Sephadex G-200, in 10% dioxane (●), 20% ethanol (+), 20% acetone (×), 5% polyethylene glycol 4000 (△) and 5% polyethylene glycol 20,000 (✱).

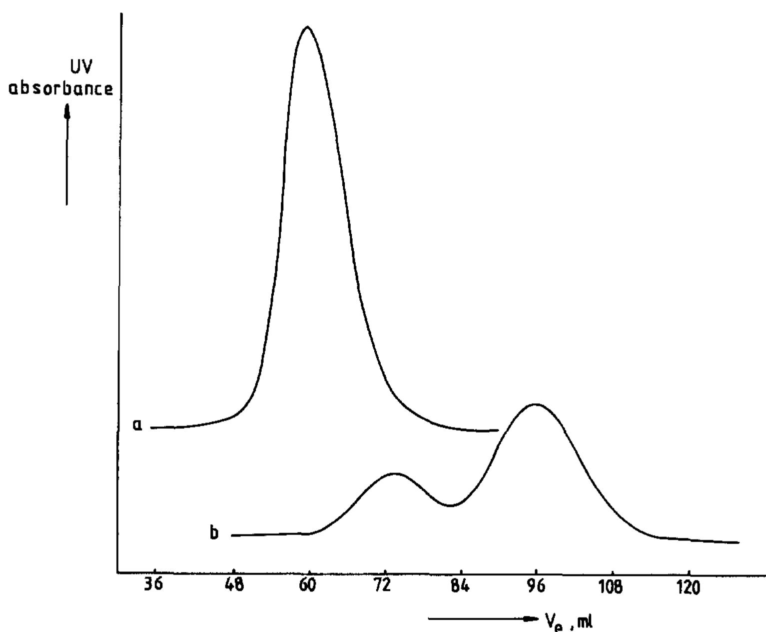


Fig. 7. Chromatograms of a mixture of cytochrome *c* and ribonuclease with water (a) and 5% polyethylene glycol (b) as the eluent. Conditions: a, column length 39.4 cm, amount of Sephadex G-100 gel 3.99 g, eluent flow-rate 18.4 ml/h; b, column length 33.5 cm, amount of Sephadex G-100 gel 7.41 g, eluent flow-rate 17.5 ml/h.

Schellman[10] states that there is no clear way to demonstrate that attractive interactions between protein and solvent molecules are taking place in excess of random encounters. So, it is probably the unsolvated volume of the protein molecule that determines its K_{av} value in gel permeation chromatography.

If partial unfolding of the protein molecules occurs in the mixed solvents, their equivalent hydrodynamic radii would increase. This would cause their K_{av} values to decrease, instead of to increase as is observed. Moreover, the concentration of the organic co-solvents is kept below the value where appreciable unfolding is expected to occur, on the basis of the available literature data (see Table VI). As denaturation appears to depend on the hydrophobic groups in the denaturant molecule[12], it may be expected that the data for propanol-2 in this table will hold approximately for acetone, and the data for diethylene glycol will hold approximately for dioxane. Polyethylene glycols are not strong protein denaturants either. It can be concluded from Table VI that appreciable unfolding may only be expected to occur in 20% ethanol and acetone, for some proteins. For BSA and cytochrome *c* unfolding is not expected in these solvents. Yet, the values for these proteins in Figs. 3 and 4 do not deviate from the expected positions. We conclude, therefore, that, although partial unfolding of some proteins in the solvents 20% ethanol and acetone may have occurred, this phenomenon has not influenced our results appreciably.

CONCLUSIONS

When proteins are chromatographed on Sephadex G-100 or G-200 gels with partly aqueous eluents, enhanced values of K_{av} are obtained. Probably, the enhancement is caused by adsorption of the proteins to the gel matrix and/or to partition between the liquid in the gel beads and the eluent, the composition of which is slightly different.

When the percentage (w/v) of the organic co-solvent is kept constant, the enhancement increases with increasing molecular weight of the co-solvent.

The ratio α of the K_{av} values of two proteins depends on the co-solvent. This phenomenon can be used to perform separations that are impossible with water as the eluent, due to the closeness of the molecular weights of the proteins concerned.

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