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Properties of supports for the partition chromatography of proteins[a]

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ABSTRACT

LiParGel 650 and 750 were investigated as supports for the partition chromatography of proteins using aqueous two-phase systems. It was found that the elution pattern of the column is not determined solely by partitioning. Size exclusion properties and interactions with negatively charged residues on the support and hydrophobic effects, typical of polymer matrices, may strongly influence the migration rate of single components. In order to calculate elution volumes from partition coefficients and *vice versa*, the volumes of the stationary and mobile phases, V_s and V_m, respectively, were determined using a set of standards for calibration and a computer fit to correlate the data. The observed deviations from ideality are due to the broad pore-size distribution and unspecific side affinities of the support material, but do not affect the general applicability of the support materials for the chromatographic separation of proteins.

INTRODUCTION

In recent years, several attempts have been made to immobilize the dextran (Dx)-rich bottom phase of an aqueous polyethylene glycol (PEG)–dextran two-phase system and in this way to combine the favourable properties of aqueous phase systems with the advantages of column chromatography [1–3]. The main difficulty in finding a suitable support for this purpose arises from the similarity of the physico-chemical properties of the two phases and their low interfacial tension. This problem was finally solved by Müller [4], combining the incompatibility of polyacrylamide towards PEG with the mechanical strength of silica gel or a hydrophilic methacrylated polymer. Using a method described by Mino and Kaizerman [5], Müller grafted linear polyacrylamide chains of 15–25 monomer units to the hydroxylated support, which was then wetted preferentially by the dextran-rich phase. As long as the corresponding PEG-rich phase is used as the mobile phase the dextran-rich bottom phase is retained as a stationary phase and liquid–liquid partition chromatography can be performed. We examined the general properties of the newly developed material for partition chromatography.

[a] The results presented in this paper are part of the doctoral thesis of A. Walsdorf submitted to the Heinrich Heine Universität Düsseldorf.

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EXPERIMENTAL

Materials

The support materials LiParGel 650 and 750 were a gift from E. Merck (Darmstadt, Germany). Merck also provided experimental samples of polyacrylated LiChrospher Diol 1000/10, a porous spherical silica with 1000-Å pores and 10-μm particle size. The triazine dye Procion Red HE3b was a gift from ICI Germany. PEG 20 000 was obtained from Merck Schuchard (München/Hohenbrunn, Germany and monomethoxy-PEG 5000 from Fluka (Neu Ulm, Germany). Dextran Pl 500 (MW $5 \cdot 10^5$ dalton) and Vc 40 (MW $4 \cdot 10^4$ dalton) were bought from Pfeifer und Langen (Dormagen, Germany). The proteins were purchased from the following sources: albumin and peroxidase (E. Merck), ferritin, thyroglobulin (Pharmacia, Uppsala, Sweden), transferrin, myoglobin (Serva, Heidelberg, Germany), α-chymotrypsinogen A Type II (Sigma, St. Louis, MO, U.S.A.), lysozyme from egg white (Fluka) and formate dehydrogenase (Boehringer, Mannheim, Germany). Dextran blue was obtained from Pharmacia and glycyl-L-tyrosine from Serva. The monoclonal antibody directed against peroxidase was a gift from Dr. Eran Hadas (Department of Biotechnology, University of Tel Aviv, Israel). All other chemicals were of analytical-reagent grade.

Preparation of PEG–dye derivatives

The triazine dye Procion Red HE3b was coupled to monomethoxy-PEG 5000 and PEG 20 000 according to Cordes and Kula [6].

Phase preparation

The concentration of PEG and dextran in various phase systems used for partition chromatography were taken from phase diagrams established by Albertsson [7]. The PEG- and dextran-rich phases were prepared, mixing the polymers with water and salts in the appropriate amounts for 1 h. The pH was adjusted by adding concentrated phosphoric acid or potassium hydroxide solutions. The phases were allowed to settle under gravity in a separating funnel, and after 1–2 h the phases were separated. The PEG-rich phase was left standing to obtain a totally clear top phase, removing a small amount of bottom phase that settled over several days. In order to prevent oxidative degradation of the PEG, the mobile phase was stored under nitrogen.

Preparation of columns

The polyacrylated support particles LiParGel 650 or 750 and LiChrospher Diol 1000/10 were equilibrated with the phase system and packed into the chromatographic columns (Superformance system; E. Merck) according to Müller [4].

Column chromatography

The chromatography columns were integrated into a Pharmacia FPLC system. The fractions were collected using a Frac 100 fraction collector (Pharmacia).

Sample preparation

Exactly weighed amounts of the substances to be analysed were dissolved in the

mobile phase and centrifuged (3000 *g*, 5 min) to remove undissolved solid material. The samples were applied to the column using an MV 7 valve (Pharmacia).

The isoelectric points of the different proteins were determined using the Pharmacia Phast System with Phast gel IEF 3–9. The isoelectric points of chymotrypsinogen A and lysozyme were taken from the literature [8].

To determine the partition coefficient k, the individual proteins were mixed with equal volumes of top and bottom phases. The phases were separated by low-speed centrifugation (1800 *g*, 10 min) and the protein concentation determined in each phase measuring the absorbance at 280 nm (Shimadzu, UV 160). The activity of formate dehydrogenase was determined according to Schütte *et al.* [9] by an enzymatic assay. The k value was calculated as the ratio of the absorbances or enzyme activities in the top and bottom phases according to the equation [7]

$$\frac{C_T}{C_B} = \frac{f_T}{f_B} = k = \text{constant} \tag{1}$$

where k = partition coefficient, C_T and C_B = concentration of the component in the top and bottom phase, respectively, and f_T and f_B = activity of the component in the top and bottom phase, respectively.

RESULTS

Calibration of the column

Liquid–liquid partition chromatography (LLPC) was developed by Martin and Synge [10] and the following equations can be derived to calculate the elution volume of a given substance from the known partition coefficient in the phase system used and *vice versa*:

$$V_s = \frac{V_{el(2)} - V_{el(1)}}{1/k_{(2)} - 1/k_{(1)}} \tag{2}$$

$$V_m = V_{el(1)} - V_s(1/k_{(1)}) \tag{3}$$

$$V_{el} = V_s/k + V_m \tag{4}$$

$$k_{(n)} = V_s/(V_{el} - V_m) \tag{5}$$

where $V_{el(1,2)}$ elution volume of components 1 and 2, V_s = volume of the stationary phase, V_m = volume of the mobile phase and $k_{(n)}$ = partition coefficient.

To do so, the volumes of the stationary phase, V_s, and mobile phase, v_m, in the column must be known. Fig. 1 illustrates the relationship between the partition coefficient and elution volume in liquid–liquid partition chromatography, which is a hyperbolic function described by eqn. 4. At high k values the term V_s/k tends to zero and $V_{el} = V_m$. To determine V_s and V_m (eqns. 2 and 3) the elution volume of at least two substances in the column and their respective partition coefficients in the given phase system are required. The latter are determined by independent batch experiments.

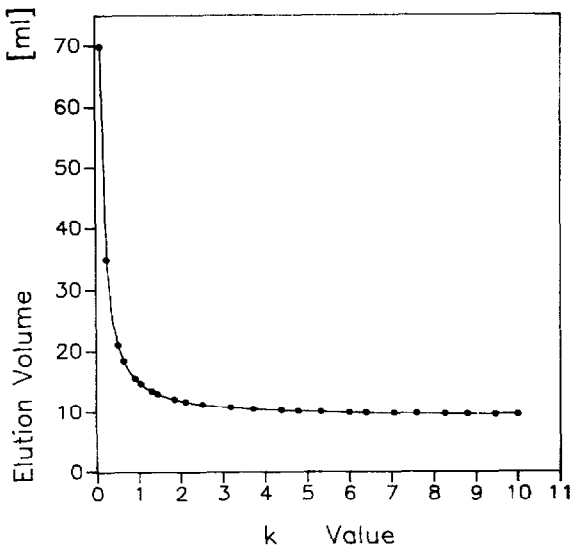

Fig. 1. Relationship between elution volume and partition coefficient in liquid–liquid partition chromatography. The hyperbolic function is derived by the equation $V_{el} = V_s/k + V_m$, where V_{el} is the elution volume, V_s the volume of the stationary phase, V_m the volume of the mobile phase and k the partition coefficient. In this example the column has the dimensions 30 × 1 cm I.D., with V_s = 5 ml and V_m = 10 ml.

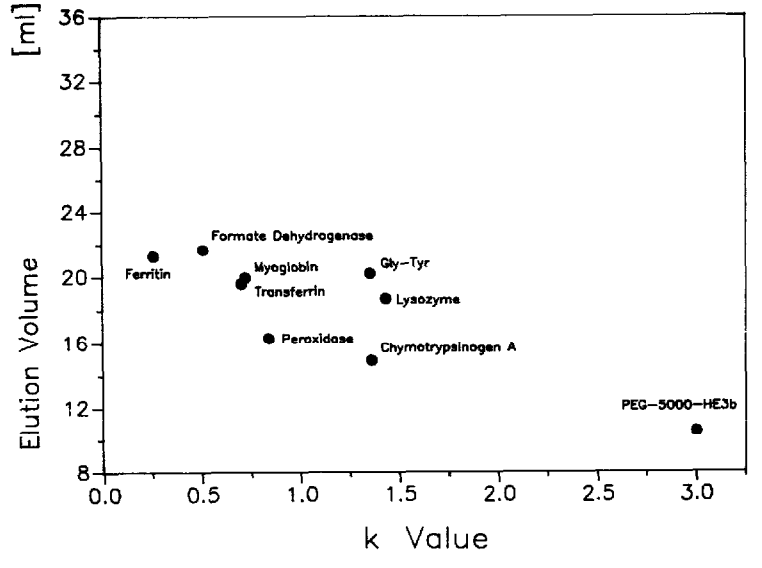

Fig. 2. Relationship between partition coefficient and elution volume of a set of standard proteins the dipeptide glycyl-L-tyrosine and a PEG-5000 triazine dye derivative in a LiParGel 750 column (30 × 1 cm I.D.). The system composition was 2.7% PEG 20000, 4.5% dextran Pl 500, 75 m*M* KBr and 10 m*M* phosphate buffer (pH 7). The temperature was 30°C and the flow-rate 1 ml/min.

TABLE I

MOLECULAR PROPERTIES OF MODEL PROTEINS USED FOR CALIBRATION, REPRODUCIBILITY OF ELUTION VOLUME BY PARTITION CHROMATOGRAPHY

The isoelectric points (IEP) in the range 3–9 were determined by isoelectric focusing using a Pharmacia Phast-gel pH gradient of 3–9. The IEP of chymotrypsinogen A and lysozyme were taken from the literature [8]. The partition coefficient k and relative standard deviation of the elution volume ($n = 10$) were determined in a phase system composed of 5.4% PEG 6000–9% dextran Vc 40, 100 mM NaCl and 50 mM phosphate (pH 7.5). The column (30 × 0.5 cm I.D.) had V_s = 1.2 ml and V_m = 2.3 ml and was operated at 23°C.

Protein	MW (dalton)	IEP	k	Standard deviation of elution volume (%)
Chymotrypsinogen A	23 000	9.5	1.0	4.3
Ferritin	440 000	7.0	0.38	6.1
Formate dehydrogenase	76 000	5.0	0.29	5.2
Lysozyme	14 600	11.0	0.76	2.8
Myoglobin	17 600	7.0	0.65	4.8
Peroxidase	40 000	8.7	0.89	3.1
Transferrin	80 000	5.6	0.43	10.0

Gel filtration of proteins in the absence of liquid liquid partition

We started the experiments using LiParGel 750 in a gel bed of 30 × 1 cm. Using different pairs of proteins for calibration we could not obtain constant values of V_s and V_m. To compare the elution behaviour of the column with the theoretical curve in Fig. 1 we chose a set of standard proteins with k values in the range 0.2–3 and applied them

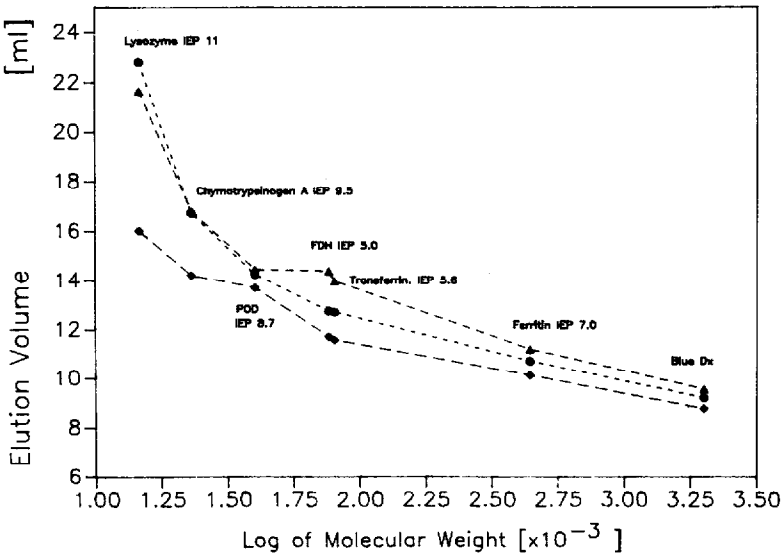

Fig. 3. Elution volume of a set of standard proteins during gel filtration on LiParGel 750 omitting the phase-forming polymers (30 × 1 cm I.D. column). The column was equilibrated against aqueous buffer (50 mM) of various pH values. The elution volume is plotted as a function of the molecular weight of the substances. Dextran blue was injected as an internal standard to determine the void volume of the column at the different pH values. The flow-rate was 1 ml/min. pH: ● = 7; ▲ = 4; ◆ = 10.

to the column. Fig. 2 shows the elution volume in the column in relation to k. The data do not follow the ideal hyperbolic function shown in Fig. 1, and it was also not possible to determine V_s and V_m unequivocally from these results. The reproducibility of the partition chromatography was fairly good, as can be judged by the standard deviation of the elution volume reported in Table I.

To establish whether the support material has any influence on the elution behaviour of the standard proteins, we prepared a column of LiParGel 750 but omitted the phase-forming polymers. The column was equilibrated against aqueous buffer of various pH and gel filtration carried out. The results are shown in Fig. 3. The elution volumes are plotted as a function of the molecular weight of the proteins. Data for the isoelectric points and the molecular weight of the standard proteins are included in Table I. Dextran blue with a molecular weight of $2 \cdot 10^6$ dalton was injected as an internal standard to determine the void volume of the column at the different pH values.

Considering the different isoelectric points, the observed shift in elution volume with pH may be interpreted as follows. The gel matrix is carrying negatively charged residues which interact with positively charged proteins. With decreasing pH any positively charged protein is more and more retained by the gel matrix and the elution volumes are increased.

The dipeptide glycyltyrosine, also injected at the different pH values, could not be eluted from the gel matrix under the conditions employed, suggesting that in addition hydrophobic interactions or π-electron effects between the tyrosine residue and the gel matrix might also play a role.

Effects of size exclusion on partition chromatography

Neglecting the basic protein lysozyme strongly affected by the matrix and the dipeptide, we used a computer fit to determine the desired values of V_s and V_m in the experimental column using the partition coefficient k and the corresponding elution volumes:

$$Y = \frac{B_2}{X} + B_1$$

where Y = elution volume, X = partition coefficient, B_1 = volume of the stationary phase and B_2 = volume of the mobile phase. The resulting graph obtained from the computer fit is shown in Fig. 4. Closer inspection reveals that ferritin is eluted significantly earlier than calculated. To establish whether mass transfer limitations due to diffusion or lack of equilibrium in partition are responsible for the unusual elution behaviour of the large molecule ferritin (MW $4.4 \cdot 10^5$ dalton), we reduced the flow-rate stepwise to one tenth of the original value. The elution volume of ferritin, however, remained constant (Fig. 5). This result led to the conclusion that the premature elution is a consequence of size exclusion effects of the matric LiParGel 750. Apparently the available volume of the stationary phase V_s is not a constant for all molecules, reflecting a broader pore size distribution typical of the polymeric resin. Large proteins cannot penetrate into the total dextran phase retained by the matrix and therefore are eluted earlier. This effect is necessarily more pronounced if dextran of higher molecular weight is used in the stationary phase, which may block access to the limiting pores for larger analytes.

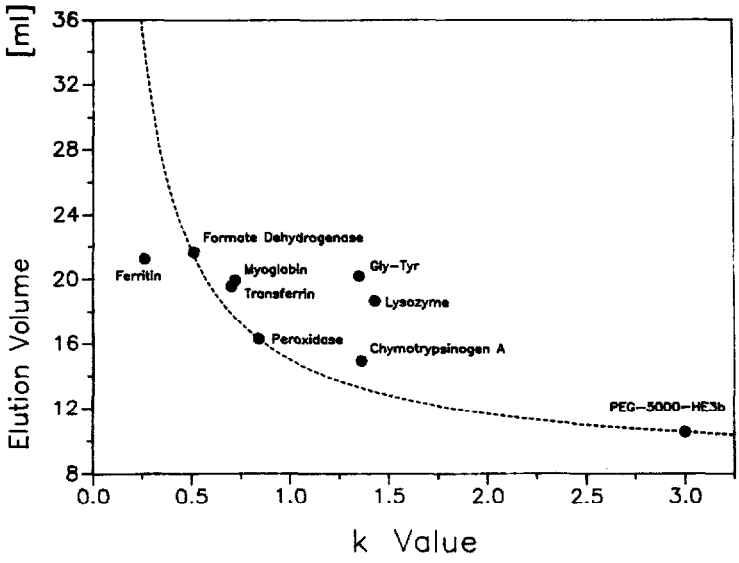

Fig. 4. Experimental results in Fig. 2 (●) replotted together with the computer-derived hyperbola (dashed line) illustrating the ideal elution curve of the column. (V_s = 7.2 ml, V_m = 8.37 ml). The values for glycyl-L-tyrosine and lysozyme were omitted in calculating V_s and V_m.

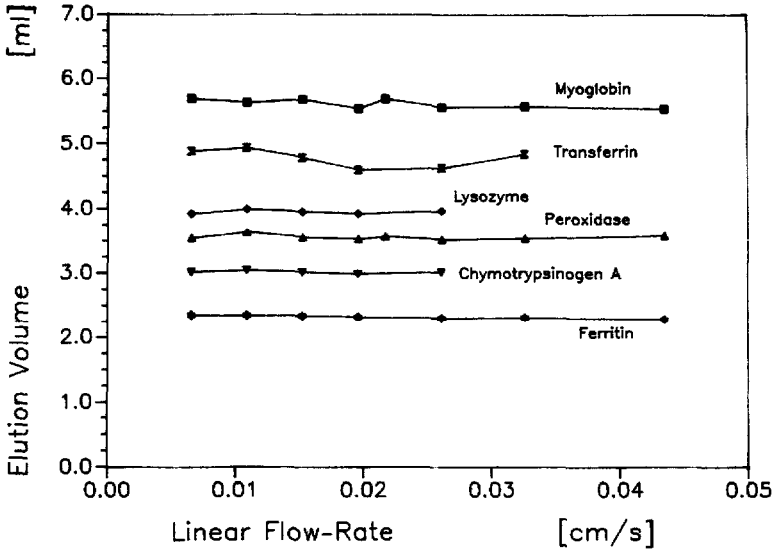

Fig. 5. Elution volume of the standard proteins from a column of LiParGel 750 as a function of the linear flow-rate. Column: 30 × 0.5 cm I.D.). System composition: 2.7% PEG 20 000, 4.5% dextran Pl 500, 75 m*M* KBr, 10 m*M* phosphate buffer (pH 7); temperature, 30°C.

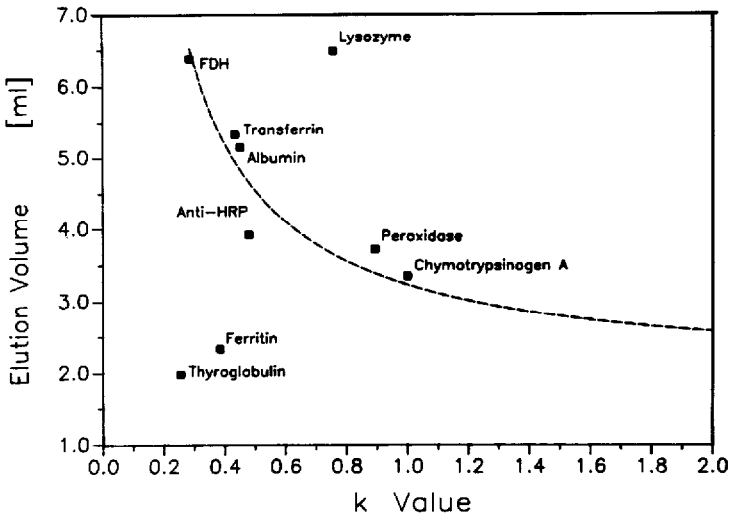

Fig. 6. Elution behaviour of the LiParGel 650. Column: 30 × 0.5 cm I.D. System composition: 5.45% PEG 6000, 9.75% dextran Vc 40, 100 m*M* NaCl, 50 m*M* phosphate buffer (pH 7.5). The temperature was 23°C and the flow-rate 0.15 ml/min. ● = Measured elution volume. The graph (dashed line) is a result of a computer fit omitting data for lysozyme, ferritin and thyroglobulin for evaluation. The calculated values for V_s and V_m are 1.31 and 1.92 ml, respectively.

To confirm the size exclusion properties of the support material, we tested LiParGel 650, which is based on the Fractogel TSK HW 65 (s) and recommended by the manufcturer for proteins smaller than $8 \cdot 10^4$ dalton. We also injected two other large protein molecules: a monoclonal antibody (MW $1.5 \cdot 10^5$ dalton) and thyroglobulin (MW $6.69 \cdot 10^5$ dalton). To exclude any influence of the phase composition on the results, we equilibrated the LiParGel 650 with a phase system of PEG 6000 and dextran V_c 40 (MW $4 \cdot 10^4$). Again a computer fit was used to correlate the data obtained. The results are presented in Fig. 6.

Lysozyme is eluted again much too late, corresponding to our previous observations. The premature elution of thyroglobulin, ferritin and the antibody follows their increasing molecular weight and verifies the assumption of a size exclusion effect of the support material. The largest molecule, thyroglobulin, shows the largest difference with regard to the calculated elution volumes and the antibody the smallest difference. We again reduced the flow-rate in this experiment to exclude mass transport problems and limitation in diffusion, and no change in the elution positions of thyroglobulin, ferritin and the antibody was observed (data not shown).

Polyacrylated LiChrospher Diol as support material

The next experiments were carried out with polyacrylated LiChrospher Diol 1000/10 as support material. The material was a research sample, based on silica beads of 10-μm diameter and selected for the large pore size of 100 nm. We equilibrated the column with the same phase system as in the experiments in Fig. 2 and chromatographed the same set of standards. Fitting was performed neglecting the experimental value for lysozyme, and the results are shown in Fig. 7.

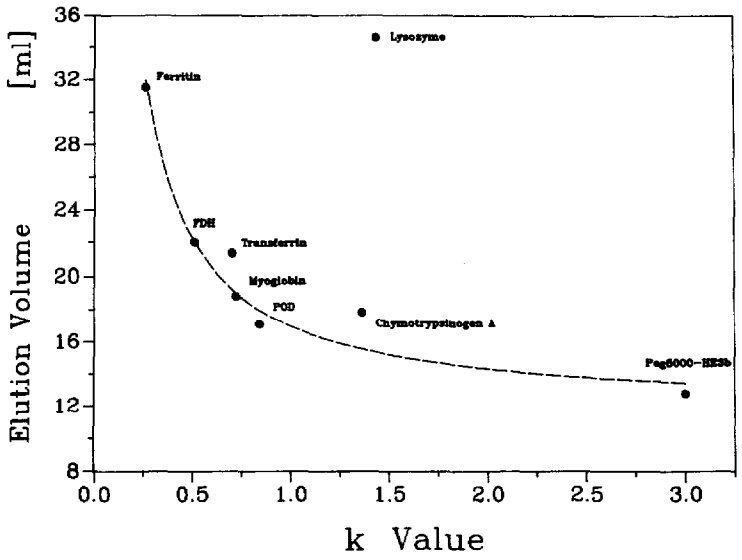

Fig. 7. Elution volume of the standard proteins and the PEG-5000 triazine dye derivative from a column of LiChrospher Diol 1000/10 (30 × 1 cm I.D.). System composition: 2.7% PEG 20 000, 4.5% dextran Pl 500, 75 m*M* KBr, 10 m*M* phosphate buffer (pH 7). The temperature was 30°C and the flow-rate 0.35 ml/min. ● = Measured elution volume. The graph (dashed line) is the result of a computer fit omitting the data for lysozyme. The calculated values for V_s and V_m are 5.75 and 10 ml, respectively.

The silica-based support shows no size exclusion properties indicated by the elution behaviour of ferritin, where the experimental elution volume is idential with the calculated value. The difference between the calculated and the experimental elution positions of lysozyme is even larger than that obtained with LiParGel. This result was not unexpected, as it was known that the starting material contained negatively charged groups. Other interactions might contribute to the observed result, but could not be differentiated.

DISCUSSION

It is obvious that in liquid–liquid partition chromatography using LiParGel 650 and 750 to retain the dextran-rich bottom phase of an aqueous phase system, the elution volume of a given substance may be a function of at least four different superimposed effects: the partition coefficient in the phase system, interactions of the analyte with hydrophobic and/or negatively charged residues and size exclusion properties of the support material. With LiChrospher Diol 1000/10, the pore size of the material seems to be sufficient to avoid size exclusion effects, at least up to molecular weights of *ca.* $5 \cdot 10^5$ dalton. The equations derived from the basic experiments of Martin and Synge [10] to calculate the volumes of stationary and mobile phase of a given column are valid only for true partitioning in the absence of other strong interactions. As the latter requirement is difficult to judge beforehand, a set of standards and a computer fit of the experimental data will yield more accurate values for V_s and V_m than calculations based on two components only.

Changing the ionic strength or pH of the phases employed will suppress interactions due to the charge [11], but the size exclusion properties can only be avoided by employing support material with larger pore sizes and to some extent also by lowering the molecular weight of the polymers forming the phase system. This conclusion does not affect the general applicability of the support material for partition chromatography. Liquid–liquid partition chromatography is well suited to solve difficult separation problems [11–16]. Some of the additional interactions described here might even be used with advantage to achieve a desired separation.

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