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Supports for liquid–liquid partition chromatography in aqueous two-phase systems: a comparison of Superdex and LiParGel

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ABSTRACT

The ability of Superdex 200, a gel filtration matrix consisting of dextran-grafted beads, to act as a support for liquid–liquid partition chromatography (LLPC) in aqueous polyethylene glycol (PEG)–dextran two-phase systems was examined. The gel adsorbed the dextran-rich bottom phase readily and retained it during elution with the PEG-rich top phase. In contrast to LiParGel 650, a matrix designed for LLPC, the entire Superdex matrix seemed to form an immobilized stationary phase. Ideal partitioning of proteins was observed only for molecules partitioning towards the stationary phase on Superdex and for those favouring the mobile phase on LiParGel. Hence, the choice of matrix depends on the separation problem at hand.

INTRODUCTION

Partitioning in aqueous two-phase systems has been used for many years to separate and isolate cells, organelles and macromolecules. The method may also provide information about conformational changes ocurring upon interactions between molecules [1], as the distribution of a molecule in these systems depends on its conformation and general surface properties [2–5]. By optimizing the composition of the phases, separation may be achieved in only a few steps. However, finding such optimum systems may pose difficulties and in most instances multiple extractions are required in order to obtain an adequate separation. As this procedure is tedious, various forms of automated counter-current extraction have been developed [6–8]. The time required for the analyses may be further reduced by employing column chromatography. A column chromatographic approach would also increase the plate number and, hence, the sensitivity of the method.

An early attempt to immobilize one of the phases was made by merely soaking agarose beads in the bottom phase of the most thoroughly studied system formed by polyethylene glycol (PEG) and dextran, using the top phase as a mobile phase [9]. Since then, several materials have been studied and rejected as supports for liquid–liquid partition chromatography in aqueous two-phase systems (LLPC) [10]. In the end, hydrophilic vinyl particles grafted with polyacrylamide were found to be able to adsorb the dextran-rich bottom phase of PEG–dextran systems in amounts sufficient for partitioning [10,11]. LLPC on this matrix, LiParGel, has been shown to be a powerful tool for the qualitative structural analysis of, for instance, immunoglobulins [12].

However, as the bottom phase is adsorbed mainly as microdroplets inside the pores of the LiParGel particles [10], the volume of stationary phase available for partitioning of a protein is dependent on its

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ability to enter the pores. Hence, we were interested in finding an alternative support for LLPC in PEG–dextran two-phase systems which might be more evenly coated than LiParGel. The idea of a matrix resembling one of the phases, *i.e.*, an immobilized stationary phase, was appealing in order to avoid bleeding of the columns. Hubert *et al.* [13] have reported successful fractionations by LLPC on a matrix to which polyethylene oxide was bound covalently using a dextran-containing mobile phase. However, as was pointed out, the viscosity of dextran solutions may become a considerable drawback. This problem would be circumvented by binding the dextran to the support and eluting the columns with the PEG-rich phase, which is less cumbersome to handle.

Superdex was recently developed for size-exclusion chromatography. The matrix consists of macroporous agarose beads grafted with dextran. The mobility of these chains gives the gel the character of a dextran solution bound to the agarose support [14]. Hence Superdex particles may be expected to provide an immobilized stationary phase for LLPC in PEG–dextran systems.

In this work we compared the properties of Superdex 200 with those of LiParGel 650 with respect to their ability to adsorb the dextran-rich bottom phase of aqueous PEG–dextran two-phase systems and the influence of the supports on the elution of proteins in these LLPC columns.

EXPERIMENTAL

Materials

Dextran T 500 (M_r = 500 000) was supplied by Pharmacia LKB Biotechnology (Uppsala, Sweden). Polyethylene glycol 8000 (PEG) (M_r = 6000–7500) was obtained from Union Carbide (New York, USA). LiParGel 650 was a gift from Merck (Darmstadt, Germany) and Superdex 200 prep grade was a gift from Pharmacia LKB Biotechnology. Horseradish peroxidase was obtained from Merck and whale skeletal muscle myoglobin from Sigma (St. Louis, MO, USA). Human albumin, rabbit aldolase, bovine catalase and bovine thyroglobulin were produced by Pharmacia LKB Biotechnology. Human transferrin was obtained from Sigma. Human immunoglobulin G (IgG) (Sandoglobulin) was obtained from Sandoz (Basel, Switzerland). Rabbit IgG (rabbit anti-human albumin) was supplied by Dakopatts (Glostrup, Denmark).

The two-phase system

All experiments were performed in a 4.4% (w/w) PEG 8000–6.2% (w/w) dextran T 500 two-phase system at pH 7.0. The compositions of the phases are given in Fig. 1. Two compositions of salts were used: 50 m*M* sodium phosphate–0.1 *M* NaCl–0.1 *M* glycine (the 0.1 *M* glycine system) and 10 m*M* sodium phosphate–0.1 *M* NaCl–0.2 *M* glycine (the 0.2 *M* glycine system). The concentrations of dextran in the top and bottom phases were not affected by the changes in the salt content as determined polarimetrically. Titration of the phases with 1 *M* HCl and/or 1 *M* NaOH showed that glycine was equally distributed between the phases.

Determination of partition properties

The partition properties of the proteins were determined as their partition coefficients in batch experiments. A 4-g amount of the two-phase system described above was thoroughly mixed with 4 mg of protein and allowed to separate at 20°C overnight. The partition coefficient, K_{batch}, was defined in accordance with the notation commonly used for aqueous two-phase partitioning [2], *i.e.*,

$$K_{batch} = C_{top\ phase}/C_{bottom\ phase}$$

where $C_{top\ phase}$ and $C_{bottom\ phase}$ are the concentrations of the protein in the top and bottom phases, respectively, determined spectrophotometrically at 280 nm. The presence of polymers in the solvent did not affect the molar absorptivities of the proteins. It should be noted that no precipitates could be observed in the interphase after incubation overnight.

Interactions of proteins with the matrices

A 3-mg amount of albumin or 2 mg of IgG dissolved in 0.9% NaCl were mixed with 1 ml of gel (LiParGel or Superdex) for 1 h at room temperature. The amount of protein remaining in/on the matrix was determined by measuring the concentration of the protein in the supernatant spectrophotometrically at 280 nm (ε_{IgG} = 1.3 ml mg^{-1} cm^{-1}, $\varepsilon_{albumin}$ = 0.6 ml mg^{-1} cm^{-1}).

Preparation of LLPC columns

The preparation of the LLPC columns is shown

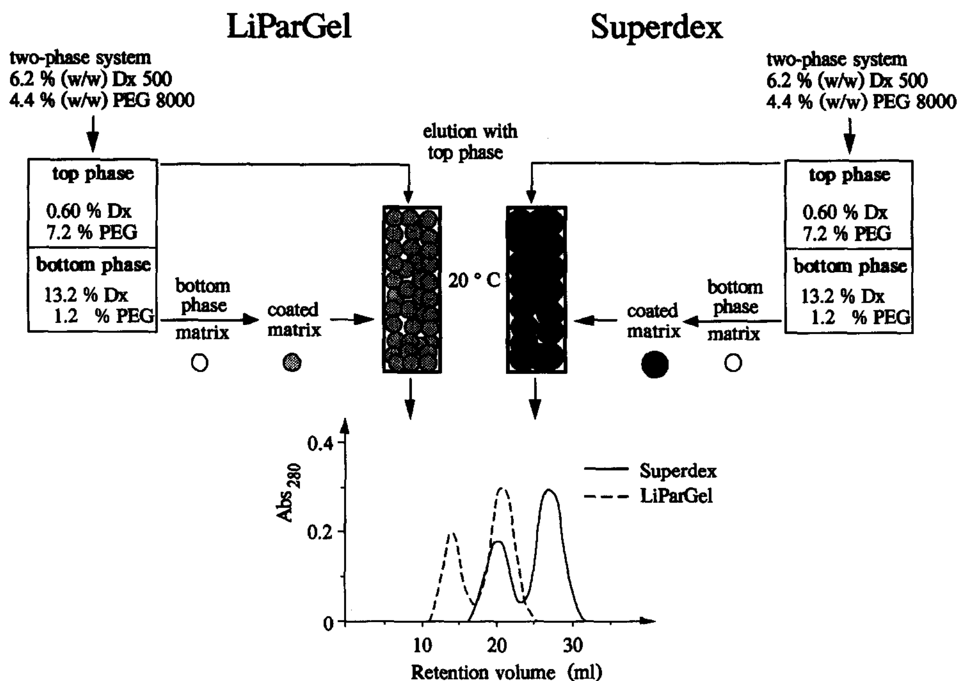

Fig. 1. Schematic diagram of the preparation of LLPC columns. The chromatograms for the references, peroxidase and myoglobin, on (solid line) Superdex and (dashed line) LiParGel, respectively, obtained in the 0.1 *M* glycine system are shown.

schematically in Fig. 1. The two-phase system, prepared as described previously [15], was equilibrated at 20°C for 72 h and the clear phases were separated. The matrix, LiParGel 650 or Superdex 200 prep grade, was allowed to equilibrate with the dextran-rich bottom phase (stationary phase) at room temperature. Excess of bottom phase was removed by rinsing the coated matrix with the PEG-rich top phase (mobile phase). The coated matrix was suspended in 3–4 volumes of mobile phase and poured into a thermostated (20°C) glass column (35 × 1 cm I.D.) with a filling reservoir. The columns were packed at a flow-rate of 0.2–0.6 ml min^{-1} and equilibrated with about 3 volumes of the mobile phase at a flow-rate of 0.2 ml min^{-1} until the eluates were almost clear. Samples were applied in 1.0 ml of mobile phase with the addition of about 5 mg of glycine. Eluates were monitored continuously at 280 nm.

In order to examine the ability of Superdex to act as a stationary phase in itself, uncoated Superdex 200 was packed in a column and equilibrated with 7.2% PEG–50 m*M* sodium phosphate–0.1 *M* NaCl–0.1 *M* glycine (pH 7.0) (i.e., a solution corresponding to the top phase of the 0.1 *M* glycine system but without any dextran). Samples were applied to the column as described above.

Calculations

The parameters of the LLPC columns were determined as described earlier [10] using peroxidase and myoglobin as references. The distribution of the references between the phases in the LLPC column was assumed to be identical with their partitioning in batch, *i.e.*, the inverted value of K_{batch} equalled K_C for the references, where K_C is the ratio of the concentration of a molecule in the stationary (dextan-rich) phase to that in the mobile (PEG-rich) phase:

$$K_C = C_{stationary\ phase}/C_{mobile\ phase}$$

The volumes of the stationary and mobile phases, V_S and V_M, were calculated from the retention volumes for the references, V_R, according to

$$V_R = V_M + K_C V_S \quad (1)$$

The standard deviation of the retention volumes for the two references was less than 5%. The volumes of stationary and mobile phases were averaged for all columns used, accounting for the number of runs on each column, with a variation less than 6% of the column volume.

The plate number, N, was calculated from the peak width at half-height (w_h) of the myoglobin peak according to

$$N = 5.54(V_R/w_h)^2 \quad (2)$$

The resolution, R_s, of the peroxidase and myoglobin peaks was calculated as

$$R_s = (\sqrt{N}/4)[k/(1 + k)](\alpha - 1) \quad (3)$$

where k is the capacity factor and α is the ratio of the partition coefficients of the references ($\alpha = K_{batch,\ peroxidase}/K_{batch,\ myoglobin}$).

In order to facilitate the comparison of chromatograms from columns with different parameters, the retention volume of each component was expressed as K_C using eqn. 1. In cases where the protein was fractionated into more than one component by LLPC, K_C was calculated from the average retention volumes for all components.

RESULTS

The properties of Superdex as a support for liquid–liquid partition chromatography (LLPC) was compared with those of LiParGel in a salt-containing aqueous two-phase system formed by polyethylene glycol 8000 (PEG) and dextran T 500. The two compositions of salts used are referred to as 0.1 M glycine and 0.2 M glycine as described under Experimental. The changes in the salt content did not affect either the volume ratio of the two-phase system or the composition of the phases, as indicated by a constant concentration of dextran. Titration of the phases showed that glycine was equally distributed. However, the distribution of the proteins tended to shift towards the dextran-rich phase in the 0.2 M glycine system (Table I).

In order to examine the ability of the dextran-grafted Superdex particles to form a two-phase system with a PEG-containing mobile phase, albumin, human IgG (HIgG) and the two references, peroxidase and myoglobin, were applied one by one on a Superdex column equilibrated with a solution corresponding to the top phase of the 0.1 M glycine system but without any dextran. Although the separation was poor, the retention volumes for the four proteins differed slightly (Fig. 2). However, the retention volumes could not be correlated either to the partition properties of the proteins or to their sizes (Table I), in spite of Superdex being a gel filtration matrix. The volume of stationary phase per unit column volume, calculated from eqn. 1, was only 0.06 ml ml^{-1}. Using the complete top phase of the equilibrated two-phase system as a mobile phase did not improve the resolution, indicating

TABLE I

SIZE, NET CHARGE AND PARTITIONING PROPERTIES OF THE PROTEINS STUDIED, TAKEN AS THE RELATIVE MOLECULAR MASS, THE ISOELECTRIC POINT (IEP) AND THE PARTITION COEFFICIENTS DETERMINED IN BATCH EXPERIMENTS, RESPECTIVELY

Protein	M_r	IEP	K_{batch}[a]	
			0.1 M glycine	0.2 M glycine
Thyroglobulin	670 000	4.5	0.92	0.70
Catalase	230 000	5.4	0.69	0.40
Aldolase	160 000	6.1	0.48	0.32
RIgG	160 000	5.8	1.3	1.1
HIgG	160 000	6.8	0.66	0.67
Transferrin (–Fe)	80 000	6.2	0.46	0.27
Albumin	70 000	4.9	0.32	0.21
Peroxidase	40 000	7.2	1.2	1.1
Myoglobin	17 000	7.0	0.61	0.56

[a] $K_{batch} = C_{top\ phase}/C_{bottom\ phase}$.

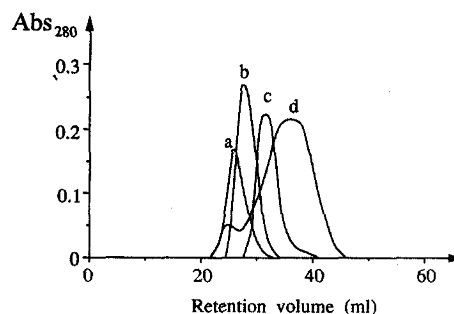

Fig. 2. Chromatogram for (a) peroxidase, (b) myoglobin, (c) albumin and (d) HIgG (0.8–1.1 mg) obtained on Superdex 200 in 7.2% PEG 8000–50 mM sodium phosphate–0.1 M NaCl–0.1 M glycine (pH 7.0).

TABLE II

PARAMETERS OF THE COLUMNS USED

V_C = column volume (ml); V_S = volume of the stationary phase (ml); V_M = volume of the mobile phase (ml); N = plate number per metre; R_S = resolution of peroxidase and myoglobin; n = number of runs (number of columns) for which the values are averaged.

Column	System	V_S/V_C (ml ml^{-1})	V_M/V_C (ml ml^{-1})	N (m^{-1})	R_s	n
Superdex 200	0.1 *M* glycine	0.44 ± 0.01	0.71 ± 0.02	1700 ± 300	1.9 ± 0.2	4(2)
	0.2 *M* glycine	0.28 ± 0.04	0.81 ± 0.06	1800 ± 300	1.2 ± 0.2	18(7)
LiParGel 650	0.1 *M* glycine	0.37 ± 0.03	0.46 ± 0.03	1000 ± 200	1.5 ± 0.2	20(10)
	0.2 *M* glycine	0.32 ± 0.01	0.40 ± 0.01	1080 ± 150	1.7 ± 0.2	6(3)

that the amount of dextran on the Superdex particles was not large enough to form a two-phase system with the PEG-containing mobile phase. Thus, in order to obtain sufficient amounts of the two phases to allow a separation based on partitioning in this system, the Superdex particles were coated with the bottom phase of the PEG–dextran two-phase system. The preparation of LLPC column is depicted schematically in Fig. 1.

The parameters of the Superdex LLPC columns were calculated as described under Experimental and compared with those of LiParGel columns (Table II). The variation in the volumes of the stationary and mobile phases in a column, averaged for the columns used in this study, was less than 6% of the column volume for either of the matrices.

The volume of stationary phase adsorbed on the Superdex per unit column volume was smaller in the 0.2 *M* than in the 0.1 *M* glycine system. The changes were even more pronounced at slightly elevated pH (data not shown), indicating the involvement of ionic interactions in the coating of this matrix. It was also of interest that the total volume of the two phases equalled the column volume, indicating an extremely small volume of the support. In contrast to Superdex, the coating of LiParGel was only slightly affected by changes in the concentration of salts (Table II). Although the volume ratio, *i.e.*, the ratio of the stationary to the mobile phase, was constant, the total volume of the phases in the LiParGel columns was slightly smaller in the 0.2 *M* glycine system. This may indicate a swelling of the matrix in the presence of higher concentrations of salts.

Proteins were generally found to have a larger retention volume on the Superdex columns than on LiParGel, which is exemplified by the chromatogram for the two references in Fig. 1. The resolution of the two reference proteins decreased with decreasing volume of stationary phase on Superdex, in spite of a constant plate number, whereas the reverse tendency was observed on LiParGel (Table II). As the flow-rate was similar in all experiments, the larger plate number in the Superdex columns indicated a larger surface area of stationary phase available for partitioning on these particles.

Fig. 3 shows the chromatogram for albumin, HIgG and rabbit IgG (RIgG) obtained by LLPC on

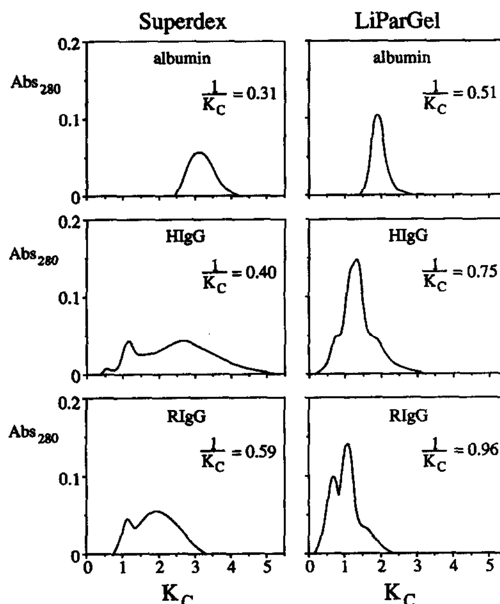

Fig. 3. Chromatograms for albumin, HIgG and RIgG (0.6–1.0 mg) obtained on Superdex and LiParGel in the 0.1 *M* glycine system. The average retention volume for each protein is given as an inverted value of K_C, which may be compared with K_{batch}.

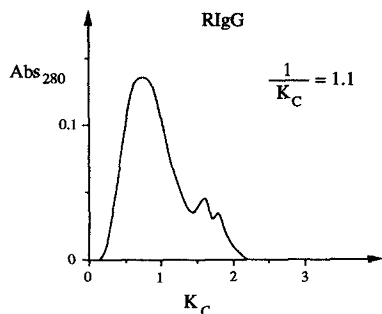

Fig. 4. Chromatogram for RIgG obtained on LiParGel in the 0.2 *M* glycine system. The average retention volume is expressed as $1/K_C$.

Superdex and LiParGel in the 0.1 *M* glycine system. The retention volumes were expressed as K_C (see Experimental) in order to facilitate the comparison of chromatograms obtained from columns with different parameters. Human albumin was eluted as a single peak on both matrices. IgG was fractionated into three poorly resolved components on LiParGel whereas Superdex gave a two-peak pattern, the peak with the largest retention volume being very broad. The elution profiles obtained in the 0.2 *M* glycine system were similar to those obtained in the 0.1 *M* system for all proteins except RIgG. On LiParGel, the characteristic three-peak pattern usually obtained for immunoglobulins on this matrix was shifted for RIgG into a pattern completely dominated by a peak with a small retention volume (Fig. 4). The difference between the elution patterns for IgGs on Superdex and those on LiParGel indicated a profound influence of the properties of the support on the results obtained by LLPC.

In order to evaluate the influence of the matrices on LLPC of proteins, the retention volumes were compared with those calculated from eqn. 1, $V_{R,\,calc.}$, using the inverted values of K_{batch} as K_C. A plot of the calculated retention volumes against the inverted values of K_{batch} is linear, the slope and the intercept of the line equalling the volumes of stationary and mobile phases, respectively. In Fig. 5, the calculated retention volumes for albumin, HIgG and RIgG are plotted together with those obtained experimentally, averaged for duplicate applications. It should be stressed that for IgG, and also for any protein that was fractionated into more than one component, all calculations refer to the average retention volume for the entire population.

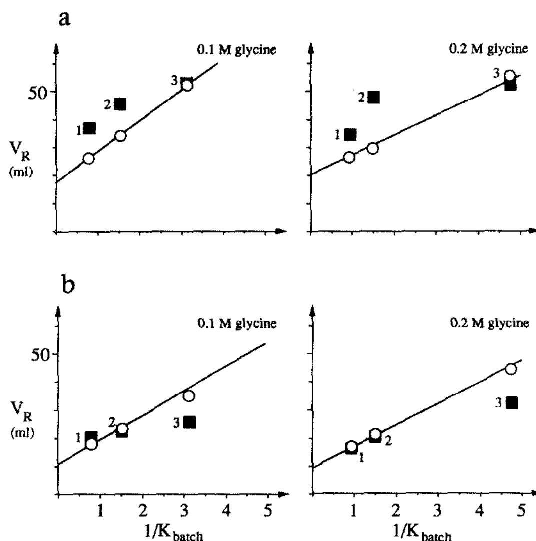

Fig. 5. Retention volumes calculated from eqn. 1 using the inverted values of K_{batch} as K_C (open symbols) as a function of the inverted values of K_{batch}, plotted together with the experimentally obtained retention volumes (closed symbols), for (a) Superdex and (b) LiParGel. The slope and the intercept of the lines equal the volumes of stationary and mobile phases, respectively, averaged for all columns used in each system referring to a column volume of 25 ml. 1 = RIgG; 2 = HIgG; 3 = albumin.

The retention volume for albumin on Superdex agreed with the calculated value whereas the IgGs were strongly retarded (Fig. 5a). In contrast, whereas the average retention volumes for IgG on LiParGel were in accordance with the theoretical values, the retention volume obtained for albumin on this matrix was considerably smaller than the calculated value (Fig. 5b). The results might be due to

TABLE III

AMOUNT OF ALBUMIN OR HUMAN IgG RETAINED IN/ON SUPERDEX 200 AND LIPARGEL 650 IN THE ABSENCE OF PHASE-FORMING POLYMERS

The proteins were added in excess to 1 ml of gel in 0.9% NaCl. The exclusion limits of the matrices are M_r 600 000 and 5 000 000, respectively.

Column	Amount of protein retained (nmol ml^{-1} gel)	
	Albumin	Human IgG
Superdex 200	8.1	11
LiParGel 650	5.1	5.9

differences in the capacity of the matrices or to an affinity of the proteins for the Superdex particles. In order to examine this point, HIgG and albumin were mixed with Superdex or LiParGel at low ionic strength (0.9% NaCl) in the absence of phase-forming polymers. Superdex was found to retain more protein than LiParGel (Table III). Furthermore, whereas LiParGel retained equimolar amounts of both proteins, the interaction of Superdex with IgG appeared to be stronger than that with albumin.

In an attempt to elucidate the properties responsible for the partitioning of proteins on the two matrices, another four proteins (aldolase, catalase, thyroglobulin, and transferrin) were applied to the columns. The salt concentrations were chosen to give the least ideal conditions in order to emphasize the deviations, *i.e.*, the 0.1 *M* glycine system on LiParGel and the 0.2 *M* system on Superdex (*cf.*, Fig. 5). The differences between the obtained retention volumes and the calculated values are shown in Fig. 6 as a function of the inverted values of K_{batch}. This difference may be considered to describe the influence of the chromatographic system on the partitioning of the molecules. The deviation from ideal elution of proteins on Superdex was not related to either the partition properties in batch of the molecules or their isoelectric points (*cf.*, Fig. 6 and Table I). However, large proteins ($M_r > 100\,000$) seemed to be retained whereas small molecules ($M_r < 100\,000$) were eluted as expected. The discrepancy between the experimentally obtained retention volume and the calculated value decreased slightly for RIgG at higher concentrations of salts whereas it increased for HIgG. Hence the unexpected elution behaviour on Superdex was not related merely to the size or shape of the protein.

On LiParGel, the retention volumes agreed fairly well with the expected values although there was a tendency for early elution of molecules partitioning towards the dextran-rich phase, *e.g.*, albumin (Fig. 6). The deviation of albumin from the calculated retention volume was larger at higher concentrations of salts and even more pronounced at pH 4.5 (data not shown), which is close to the isoelectric point of the molecule. This observation would eliminate the possibility of an electrostatic repulsion of albumin from LiParGel in the LLPC columns.

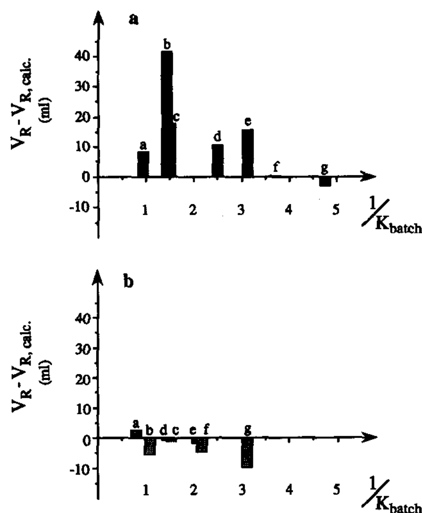

Fig. 6. Differences between the experimentally obtained retention volumes (V_R) and those calculated as described in Fig. 5 ($V_{R, calc.}$) as a function of the inverted values of K_{batch} for (a) Superdex in the 0.2 *M* glycine system and (b) LiParGel in the 0.1 *M* system. a = RIgG; b = thyroglobulin; c = HIgG; d = catalase; e = aldolase; f = transferrin; g = albumin.

DISCUSSION

We have previously used LiParGel 650 as a support for liquid–liquid partition chromatography of immunoglobulins in aqueous PEG–dextran two-phase systems at pH 7.0 [12]. The hydrophilic vinyl particles grafted with polyacrylamide are able to retain the dextran-rich bottom phase of PEG–dextran two-phase systems during elution with the PEG-rich top phase as a mobile phase [11]. Superdex has, to our knowledge, so far been used only as a matrix for size-exclusion chromatography. The gel consists of macroporous agarose beads with an average size similar to that of the LiParGel particles (30–36 μm) [11,14]. The exclusion limit is reduced by binding dextran covalently to the surface of the beads and also to the walls of the pores. As these dextran chains retain a large degree of mobility, Superdex may be regarded as a solution of dextran immobilized to the agarose support [14]. Thus, resembling the bottom phase of PEG–dextran systems closely, Superdex would be expected to form a two-phase system with a PEG-containing mobile phase, *i.e.*, to constitute an immobilized stationary phase for LLPC. This expectation was supported by the fact that the elution of proteins on Superdex in a salt-

containing solution of PEG was not related to their sizes (Fig. 2). However, the amount of dextran on the particles seemed to be insufficient to give an adequate resolution of proteins upon LLPC in the two-phase system used.

The coating of LiParGel is suggested to be due to the strong incompatibility of the polyacrylamide on the particles with PEG in the mobile phase rather than to an attraction of the dextran-rich phase to the matrix [10]. However, the volume of the stationary phase adsorbed on the matrix was found to be smaller at pH 4.5 than at neutral pH, indicating the involvement of electrostatic forces between the stationary phase and the LiParGel particles. This is in accordance with the observation of negative charges on LiParGel by others [16].

The stationary phase is considered to be adsorbed on LiParGel mainly as microdroplets inside the pores [10]. The exclusion limit for globular proteins on LiParGel being M_r 5000 (information leaflet from Merck), all proteins used in this study should be able to penetrate the pores to gain access to the stationary phase. Nevertheless, large molecules may be partially excluded as the distribution of pore sizes of supports based on organic polymers easily ranges over one order of magnitude [15]. Considering the size of the phase-forming polymers, the accessibility of a molecule to the inside of the particles may be further reduced by the polymers in the stationary phase blocking the pores. However, the deviation from ideal elution observed on LiParGel could not be ascribed merely to exclusion phenomena (*cf.*, Fig. 6 and Table I).

Coating Superdex, we found that the matrix readily adsorbed the dextran-rich bottom phase of our two-phase system. This may seem surprising as dextran has been shown to elute well on Superdex in ordinary gel filtration buffers [14]. However, in the presence of PEG, the formation of a two-phase system would be expected as outlined above. The adsorption of the bottom phase may be due simply to mixing of dextran in the phases with that on the particles, which would give a phase system with a composition different from that of the original system. Hence the comparison of the observed elution behaviour on this matrix with that expected from the partitioning properties observed in batch experiments may be inaccurate. The chemical properties of the covalently bound dextran chains may also differ from those of free dextran in solution owing to processing during the preparation of the matrix, implying the possibility of a three-phase system formed by immobilized dextran, free dextran and PEG, or by the core of the matrix (agarose), dextran and PEG.

As the dextran is bound both on the surface of the particles and on the walls inside the pores [14], the stationary phase would be more evenly distributed and more easily accessible on Superdex than that of LiParGel. The larger plate number for Superdex columns, in spite of similar flow-rates, also implies a larger surface of stationary phase available for partitioning. The total volume of phases in the Superdex columns was found to be equal to the column volume. This is in accordance with the extremely small relative volume of the matrix, *i.e.*, the volume of the support divided by the total volume of the support and the pores, reported [14]. Hence the entire support may be regarded as an immobilized stationary phase for LLPC.

In contrast to LiParGel, the degree of coating of Superdex was markedly reduced at higher concentrations of salts. In addition, the volume of the stationary phase was smaller at elevated pH (pH 8.2), indicating the involvement of ionizable groups with pK_a values in the range 7–8 in the adsorption of the stationary phase.

The influence of the supports on LLPC of a set of proteins was examined. For each matrix, the salt composition giving the least ideal results was chosen in an attempt to emphasize the deviations from ideal partitioning. It should be noted that the partitioning of a protein in PEG–dextran systems is related to the surface properties of the molecule, *e.g.*, its charge, size, shape and hydrophobicity [2–5, 12]. Hence the retention of a protein on an LLPC column is governed by a combination of these, and probably other still unknown, properties unless the experiment is designed to select a single parameter.

The retention volumes obtained on LiParGel were generally slightly smaller than those calculated and the deviation was more pronounced for molecules partitioning towards the bottom phase (Fig. 6). The differences between the experimentally obtained retention volume for a molecule and the calculated value may reflect a distortion of its conformation due to the repeated translocation over the interphase. It is conceivable that this effect would be

more pronounced for molecules favouring the dextran-rich stationary phase. However, as these molecules were found to elute earlier than expected, the non-linearity may rather be ascribed to non-equilibrium conditions in the columns due to difficulties in passing the interphase, *i.e.*, proteins are not allowed to partition properly between the phases.

On Superdex, all proteins except the smallest were eluted with a larger retention volume than that calculated (Fig. 6). The influence of the carboxyl groups present on Superdex on the retention of proteins may be neglected at ionic strengths higher than 0.2 *M* [17], which agrees with the lack of correlation between the retention of a molecule and its net charge (Fig. 6). A more plausible explanation for the retardation of large molecules emerges from the view of the phases as a network of polymers. In contrast to the situation in the LiParGel columns, this network is "immobilized" and fairly rigid in Superdex columns, restricting the free movement of large molecules. This hypothesis is supported by the extreme retention of thyroglobulin although the deviations from ideal elution could not be ascribed solely to the size of the molecules (Fig. 6). Taken together, LLPC of a molecule both on Superdex and on LiParGel is likely to reflect a combination of its surface properties, although the two matrices influence the partitioning in different ways.

The non-ideal partitioning of proteins on the two matrices might apply also to the two proteins used as references, peroxidase and myoglobin. In order to determine the parameters of a column properly, a set of standard proteins should be used, which has previously been pointed out by others [16]. Thus, the comparison of the parameters of Superdex columns with those of LiParGel columns may be deceiving. However, as the retention volume for each protein is related to those for the references run on the same column, the elution behaviour relative to the other molecules would not be affected.

Although the parameters governing the partitioning in the columns are still obscure, LLPC has turned out to be a powerful and yet simple method for aquiring qualitative information concerning the conformation of molecules and also conformational changes following interactions between molecules (unpublished observations). Here, we have shown that LLPC of proteins on LiParGel agrees fairly well with the partition properties of molecules favouring the PEG-rich phase whereas ideal elution was observed on Superdex only for molecules partitioning towards the dextran-rich phase. However, the deviations from ideal behaviour are not necessarily a disadvantage but may be exploited as an additional parameter for separation. Hence the two matrices are complementary to each other and the choice depends on the separation problem at hand.

ACKNOWLEDGEMENT

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