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Immobilization of phospholipid vesicles and protein-lipid vesicles containing red cell membrane proteins on octyl derivatives of large-pore gels

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For improved immobilization of phospholipid vesicles and protein-lipid vesicles (cf. Sandberg, M., Lundahl, P., Greijer, E. and Belew, M. (1987) Biochim. Biophys. Acta 924, 185–192) and for chromatographic experiments with vesicles containing membrane protein, we have prepared octyl sulfide derivatives of the large-pore gels Sephacryl S-1000 and Sepharose 2B with ligand concentrations up to 14 and 5 μmol/ml gel, respectively. The Sephacryl derivatives allowed higher flow rates, gave higher rates of adsorption and showed equally high or higher capacities than the Sepharose adsorbents. 'Small', 'medium' and 'large' vesicles of radii approx. 20, 50 and 100 nm showed distribution coefficients on Sephacryl S-1000 of 0.7, 0.5 and 0.05, respectively and could be immobilized on octyl sulfide-Sephacryl S-1000 in amounts corresponding to 110, 40 and 20 μmol of phospholipids per ml gel, respectively. 'Small' vesicles became absorbed onto this gel at a rate of 1.5 μmol of phospholipids per min per ml gel until 60 μmol of phospholipids had become immobilized, whereas the initial adsorption rate was about 0.4 $\mu mol \cdot min^{-1} \cdot ml^{-1}$ on octyl sulfide-Sepharose 4B (see reference above) and on octyl sulfide-Sepharose 2B. Lower ligand concentrations gave lower capacities for 'small' vesicles. When vesicles entrapping calcein were immobilized on octyl sulfide-Sephacryl S-1000 some calcein was released during the adsorption process. For 'small' and 'medium' vesicles, respectively, the leakage was 75 and 25% at a ligand concentration of 14 μmol/ml but only 3 and 2% at 5 μmol/ml. The internal volumes of immobilized 'small' and 'medium' vesicles were estimated at 0.97 and 2.9 μl per μmol of phospholipid by determination of entrapped calcein, which could indicate vesicle radii 20 and 50 nm, respectively. The total volumes of immobilized 'medium' lipid vesicles and 'medium' protein-lipid vesicles containing integral membrane proteins from human red cells, were estimated at 2.9 and 2.0 μl/μmol, respectively, by chromatography of D- and L-[^{14}C]glucose and calcein on the octyl sulfide-Sephacryl S-1000 column before and after immobilization. These volumes are roughly consistent with the internal volume of the vesicles. A zone of D-glucose eluted 90 μl later than a zone of L-glucose on a 4- or 5-ml column of octyl sulfide-Sephacryl S-1000 with immobilized 'medium' protein-lipid vesicles containing the glucose transporter from human red cells, probably since part of the internal vesicle volume was accessible to the D-glucose but not to the L-glucose. This indicates that the glucose transporter was active in the immobilized vesicles. This chromatographic effect of transport across the lipid bilayer persisted for at least two days. The procedure may be termed transport retention chromatography.

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Introduction

Phospholipid vesicles as well as protein-lipid vesicles can be immobilized on hydrophobic gel beads [1]. Such procedures might be useful for the concentration of vesicles and in studies of the binding of lipid membranes to hydrophobic structures, the permeability of lipid bilayers, the function of membrane proteins, for instance, those which transport ions and metabolites, and the function of membrane-bound enzymes. The chromatographic properties of immobilized vesicles might prove useful and immobilized vesicles with membrane-bound enzymes may be of biotechnical interest. The experiments reported in Ref. 1 were done mainly with alkyl sulfide derivatives of Sepharose 4B. The latter adsorbents showed high capacities for small lipid vesicles but not for large ones. The rate of adsorption of small vesicles was relatively low. In the present work we show that similar absorbents can be synthesized using the large-pore gel matrix Sephacryl S-1000 ™, an allyldextran gel cross-linked with *N,N'*-methylenebisacrylamide. The properties of octyl sulfide-Sephacryl S-1000 and octyl sulfide-Sepharose 2B have been compared over a range of ligand concentrations. These gels showed higher capacities for large vesicles and higher immobilization rates than were achieved with octyl sulfide-Sepharose 4B. The purpose of this work is to show that octyl sulfide-Sephacryl S-1000 shows favourable properties for adsorption of phospholipid vesicles; that vesicles of large total internal volume can be immobilized in a few hours with little leakage of the entrapped contents and, finally, that the activity of an integral membrane protein, the glucose transporter from human red cells, can be demonstrated in immobilized protein-lipid vesicles. The retention analyses by Bobinski and Stein [2] and by Bonsall and Hunt [3] may similarly be based on transport effects.

Materials and Methods

Chemicals

Calcein was bought from Sigma (St. Louis, MO, U.S.A.). Other chemicals were as described in Ref. 1.

Gels

Sephadex G-50 M, Sepharose 2B and 4B and Sephacryl S-1000 SF, lot HM 24928, were obtained from Pharmacia (Uppsala, Sweden).

Phospholipid solution

A solution of 0.25 M egg yolk phospholipids (70% phosphatidylcholin, 21% phosphatidylethanolamine) in 0.26 M sodium cholate, 0.2 M NaCl, 2 mM dithioerythritol, 20 mM Na_2EDTA and 11 mM D-glucose (pH 8.4) was prepared as described in Ref. 1.

Phospholipid vesicles

Egg yolk phospholipid vesicles were prepared at room temperature by chromatographic removal of the cholate from the phospholipid solution. The eluent was (A) 0.5 M $(NH_4)_2SO_4$/1 mM Tris-HCl (pH 8.0) or (B) 0.2 M NaCl/5 mM Tris-HCl (pH 7.1) as in Ref. 1, unless otherwise stated. A mixture of 4 ml of phospholipid solution and 12 ml of eluent was applied to a 390-ml column (20 × 5 cm) of Sephadex G-50 M at a flow rate of 9 ml/min. Vesicles were eluted and were fractionated on a Sepharose 4B column (see Results, Fig. 1A).

Synthesis of adsorbents

Octyl sulfide-Sepharose 2B. Octyl sulfide-Sepharose 2B was prepared by coupling 1-octanethiol to the gel beads that had been activated with 1,4-butanediol diglycidyl ether, essentially as described in Refs. 4 and 1.

Octyl sulfide-Sephacryl S-1000. The Sephacryl S-1000 gel was washed on a Büchner funnel with 50 ml of (a) 25% ethanol, (b) 50% ethanol and (c) water, per g of suction-dried gel. 40 mg of sodium borohydride was dissolved in a mixture of 20 ml of 0.6 M NaOH and 10 ml of 1,4-butanediol diglycidyl ether. This solution was immediately added to 20 g of washed, suction-dried gel (equivalent to 20 ml of packed gel) and the suspension was agitated gently at room temperature for 18 h. The activated gel was washed with 5 × 300 ml of water, suction-dried and transferred to a 250-ml round-bottomed flask. 200 mg of sodium borohydride dissolved in 15 ml of 1 M NaOH was added to the washed gel. Finally, 50 to 5500 μmol of octyl mercaptan, freshly dissolved in 15 ml of

96% ethanol, was added and the slurry was agitated gently for 20 h at room temperature. The octyl sulfide-Sephacryl S-1000 was washed with 300 ml of water followed by 50 ml of 96% ethanol. This washing procedure was repeated three to five times until the mercaptan odor had essentially disappeared. Finally, the gel was washed with 3×300 ml water. Around 19 g of gel was recovered. The gel was stored at 6 °C with 1 mg NaN_3/ml.

Ligand concentration. The amounts of bound octanethiol in dry gel samples were determined by automated sulfur analyses at the Department of Chemistry, Uppsala University, Uppsala, according to the method described by Gustafsson [5,6]. Briefly, the samples were subjected to Schöniger flask combustion; the sulfite and the sulfur dioxide were converted to sulfate; the sulfate was reduced to sulfide by hydriodic and hypophosphorous acid in acetic acid solution; the sulfide was reacted with *p*-aminodimethylaniline and ferric iron to form methylene blue, which was determined spectrophotometrically. Before analyses, the gel samples were washed with water, freeze-dried and further dried at 10–20 mmHg with $CaCl_2$ for at least two days and with $Mg(ClO_4)_2$ for a week. Sepharose 2B, which had been packed in a 10×1.0 cm column at 5.5 ml/h, showed a dry weight of only 10.2 mg/ml, which is equivalent to 98 ml/g. Sephacryl S-1000, which had been packed in a column of the same size at 20 ml/h, had a dry weight of 63 mg/ml, equivalent to 16 ml/g. The Sephacryl S-1000 contained 430 μmol sulfur per g dry gel, when the coupling reactions had been performed without addition of mercaptan. This represents the background content of sulfur in the diglycidyl ether derivative of the gel.

Immobilization

The adsorbents were packed into columns of 1.0 cm diameter to a volume of 1.0 ml (bed height 1.3 cm) or 5 ml (bed height 6.4 cm). The same eluent was used in the adsorbent column as for the preparation and purification of the lipid vesicles. The samples were applied at a flow rate of 5.5 to 6 ml/h or, in some experiments with Sephacryl S-1000, 20 to 21 ml/h. The phospholipid concentrations of samples and effluents were determined according to the method of Bartlett [7], with a minor modification [1]. The amount of vesicles adsorbed, in μmol of phospholipids per ml gel, was calculated and plotted as a function of time. The final calculated value was always compared with the value obtained by elution of the adsorbed vesicles with 50 mM cholate (cf. Ref. 1). The two values agreed within ±5%.

Volume of medium vesicles

The elution volumes of D-[^{14}C]glucose and calcein on octyl sulfide-Sephacryl S-1000 were determined before and after immobilization of medium vesicles (Fig. 8 below). The fraction masses and volumes were accurately determined by weighing. These measurements gave an estimate of the sum of the total volumes of the immobilized vesicles. For determination of the internal volume of immobilized vesicles, the vesicles were prepared in the presence of 25 mM calcein and purified on Sepharose 4B. These vesicles were immobilized and then eluted with 50 mM cholate. The calcein was released and determined fluorometrically. A standard curve was determined. The amount of eluted phospholipids was determined by phosphorus analysis.

Leakage of calcein upon immobilization of vesicles

A 0.5-ml sample of phospholipid solution was mixed with 0.5 ml eluent B and 1.0 ml of 50 mM calcein in eluent B. The mixture was applied to a 50-ml Sephadex G-50 column (16×2 cm) equilibrated with eluent B containing 25 mM calcein. Vesicles were eluted from the Sephadex column with the same calcein solution and were fractionated on a 60-ml Sepharose 4B column (40×1.4 cm) in eluent B into medium and small vesicles (cf. Fig. 1A below). Free calcein was removed by this procedure. The vesicles were applied on octyl sulfide-Sephacryl S-1000 with the ligand concentration 6 or 14 μmol/ml. Immobilization conditions were chosen such that all applied vesicles were immobilized. The percentage of calcein that was released upon the adsorption of the vesicles was measured by fluorometry with an Aminco SPF-500 Corrected Spectra Spectrofluorometer, American Instrument Company, Silver Spring, MD, U.S.A., at the excitation and emission wavelengths 491 nm and 519 nm, respectively. Cuvettes of 1-cm light paths were used. The

samples were diluted to a calcein concentration of below 0.1 μM. The content of calcein in the vesicles before immobilization, was determined after release of the calcein by addition of 750 mM cholate to a final concentration of 10–25 mM, as required. The calcein leakage from the immobilized vesicles was also determined for different periods of time from 3 to 20 h by similar measurements.

Preparation and immobilization of protein-lipid vesicles with glucose transporter for chromatography of D- and L-glucose

Integral membrane proteins were prepared from human red cells as in Ref. 8 and solubilized with 60 mM octyl glucoside in 50 mM Tris-HCl (pH 7.1) at a protein concentration of 4 g/l for 20 min at 2°C. Non-solubilized material was removed by centrifugation at 160000 × *g* for 1 h at 2°C. All subsequent operations were done at 22°C. To prepare protein-lipid vesicles 4 ml of phospholipid solution was mixed with 12 ml of solubilized membrane components and was applied to a 390-ml column (20 × 5 cm) of Sephadex G-50 at 9 ml/min in an eluent (C) containing 0.4 M NaCl, 100 μM D-glucose and 5 mM Tris-HCl (pH 7.1) (cf. Fig. 2). A 45-ml sample of the vesicles was fractionated on Sepharose 4B in eluent C as illustrated in Fig. 1A. A 55-ml fraction, corresponding to medium vesicles, was collected, diluted with eluent C to 110 ml and immobilized at 10 ml/h on a 5-ml (6.4 × 1 cm) or a 3.8-ml (4.8 × 1 cm) octyl sulfide-Sephacryl S-1000 column with a ligand concentration of 6.3 μmol/ml. The solubilization, vesicle preparation and Sepharose fractionation were done on the first day of the experiment and immobilization was done overnight. After each day of experiments at room temperature the column was stored at 6°C overnight. All pH values were measured at 22°C.

Results

1. Vesicles and adsorbents

Fractionation of phospholipid vesicles. Vesicles formed in eluent (A), 0.5 M $(NH_4)_2SO_4$/1 mM Tris-HCl (pH 8.0), eluted at the void volume of a Sepharose 4B column and were denoted 'large'. Vesicles prepared in eluent (B), 0.2 M NaCl/5 mM Tris-HCl (pH 7.1), separated into 'medium' and 'small' vesicles on the Sepharose column. The distribution coefficient, K_d, was 0.05, 0.52 and 0.71 for the large, medium and small vesicles, respectively, on Sephacryl S-1000 and the size distributions were broad (Fig. 1B). The K_d-values indicate average radii of approx. 100 nm for the large vesicles and approx. 26 nm for the medium vesicles (calibration data from Ref. 9–11). The internal volume of immobilized medium vesicles, determined as described below, corresponded to an average radius of 50 nm, i.e. higher than the value above. The internal volume of small vesicles corresponded to a radius of 20 nm (cf. Table II). The large, medium and small vesicles that were collected after chromatography on Sepharose 4B

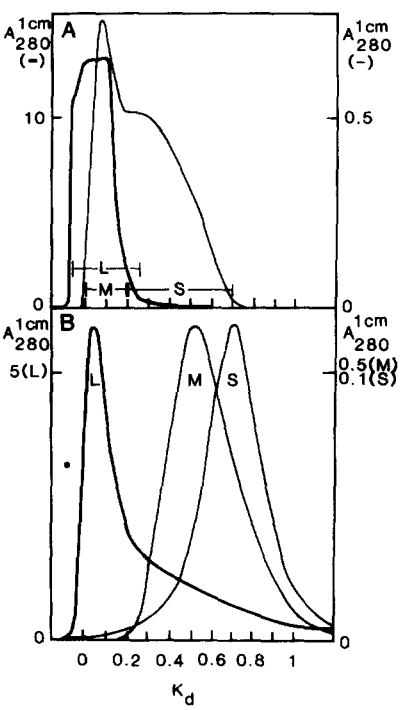

Fig. 1. Molecular-sieve chromatography of phospholipid vesicles. (A) Chromatography on Sepharose 4B of vesicles prepared in eluent A, ionic strength 1.5 M, sample volume 58 ml (thick line) or in eluent B, ionic strength 0.2 M, sample volume 38 ml (thin line). Column dimensions: 60 × 3 cm (420 ml). Eluent: The same as in the vesicles preparation. Flow rate: 30 ml/h. Denotation of fractions: L, large vesicles; M, medium vesicles; S, small vesicles. (B) Chromatography on Sephacryl S-1000 of fractions L, M and S in Fig. 1A, except that the medium vesicles (M) were prepared in eluent B with 25 mM calcein. Column dimensions: 60 × 1 cm. Eluents: The same as in the Sepharose 4B chromatography. Flow rate: 30 ml/h. Sample volume: 1 ml.

as indicated in Fig. 1A were used throughout the work unless otherwise stated. Vesicles of other size distributions could be prepared by varying the ionic strength of the eluent used with the Sephadex G-50 column, as shown by analyses on Sephacryl S-1000 (Fig. 2). The same eluent was used for the analysis as for the preparation. The yield of medium vesicles was increased in the preparation of protein-lipid vesicles containing integral membrane proteins from human red cells by use of an eluent C with 0.4 M NaCl.

Octyl sulfide concentrations of the synthesized adsorbents. The maximal amounts of bound octanethiol which we achieved were 220 μmol per g dry gel for the Sephacryl gel and 530 for the Sepharose (Fig. 3). It seems likely that Sephacryl contains fewer hydroxyl groups per gram of dry gel than does Sepharose, since Sephacryl is a co-polymer of allyldextran and *N,N'*-methylenebisacrylamide, whereas Sepharose is a non-cross-linked agarose gel. However, the maximal ligand concentration in μmol per ml packed gel was 14 for octyl sulfide-Sephacryl S-1000 and lower, 5.4, for octyl sulfide-Sepharose 2B (cf. Fig. 3). Ligand concentrations below are given in μmol per ml packed gel (μmol/ml).

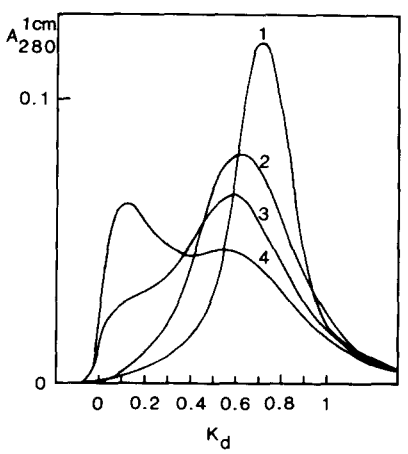

Fig. 2. Chromatography on Sephacryl S-1000 as in Fig. 1B of vesicles prepared on Sephadex G-50 in 0.2, 0.4, 0.5 and 1.0 M NaCl with 5 mM Tris-HCl (pH 8.0) (graphs 1, 2, 3 and 4, respectively). The eluents were the same as for the vesicle preparations. Each graph represents the third of three consecutive analyses. The first analysis after equilibration of the column with a given eluent showed a higher K_d value than the subsequent ones. The vesicles were prepared essentially as described in Ref. 1, except for the NaCl concentration.

2. Immobilization technique

Three aspects of vesicle immobilization were studied, (i) the capacity of the adsorbent, (ii) the rate of adsorption and (iii) the efficiency of adsorption or the ratio between the amounts of adsorbed and applied material. Egg yolk phospholipid vesicles (40–120 ml) were applied on a 1 ml column of the large-pore gels octyl sulfide-Sepharose 2B (open symbols in Figs. 3–7) and octyl sulfide-Sephacryl S-1000 (filled symbols). The ligand concentration, the flow rate, the vesicle concentration and the size of the vesicles were varied.

Immobilization of small vesicles. (i) Capacity. Octyl sulfide derivatives of Sepharose 2B and of Sephacryl S-1000 showed a capacity for immobilization of small vesicles of 110–120 μmol of phospholipids per ml adsorbent, at the ligand concentrations 2.4 and 14 μmol/ml, respectively (Fig. 4B). Both of these ligand concentrations are equivalent to a ligand density of approx. 230 μmol/g. For octyl sulfide-Sephacryl S-1000 of lower ligand concentration, 6.3 μmol/ml, the capacity for small vesicles was lower: 52 μmol of phospholipids per ml (Fig. 6D below). The capacity for immobilization of small vesicles increased with the ligand

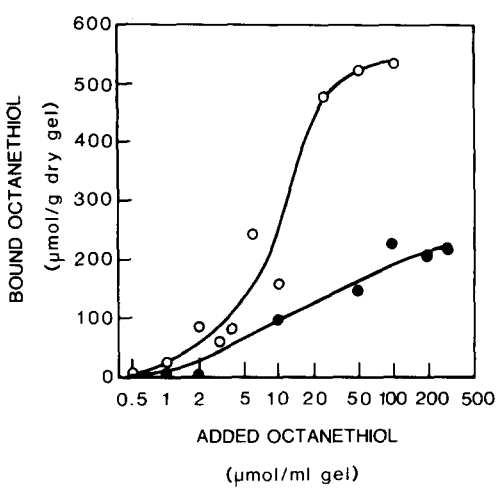

Fig. 3. The amounts of bound octanethiol upon synthesis of octyl sulfide-Sepharose 2B (○) and octyl sulfide-Sephacryl S-1000 (●) as a function of the amount of 1-octanethiol added to the activated gel. The amount of bound octanethiol in μmol per g dry gel was determined by sulfur analysis. 100 μmol/g corresponds to 1.02 μmol/ml for Sepharose 2B and to 6.3 μmol/ml for Sephacryl S-1000. The amount of added octanethiol is expressed in μmol per ml packed gel.

density for octyl sulfide-Sepharose 2B as well as for octyl sulfide-Sephacryl S-1000 (Fig. 5).

(ii) Rate. The initial rate of adsorption on octyl sulfide-Sephacryl S-1000 was 1.5 μmol of phospholipids per min per ml gel at a flow rate of 21 ml/h (data from Fig. 4B). This rate was constant until the adsorbent had become half-saturated. Approx. 60 μmol of phospholipids in the form of small vesicles could be immobilized in 40 min per ml gel (Fig. 4B). A lower flow rate resulted in more efficient but slower adsorption (Fig. 4A,B). The Sepharose 2B derivatives can not be operated at as high flow rates as the Sephacryl adsorbents and showed much lower adsorption rates, for instance 0.4 $\mu mol \cdot min^{-1} \cdot ml^{-1}$ at the ligand concentration 2.4 μmol/ml (Fig. 4B), similar to earlier results for octyl sulfide-Sepharose 4B [1]. Even at the same flow rate the adsorption rate was lower for octyl sulfide-Sepharose 2B than for octyl sulfide-Sephacryl S-1000 (Fig. 4B). The lowest rate of adsorption in this series of experiments was obtained with octyl sulfide-Sepharose 2B at the highest ligand concentration, 5.4 μmol/ml (530 μmol/g) (Fig. 4B).

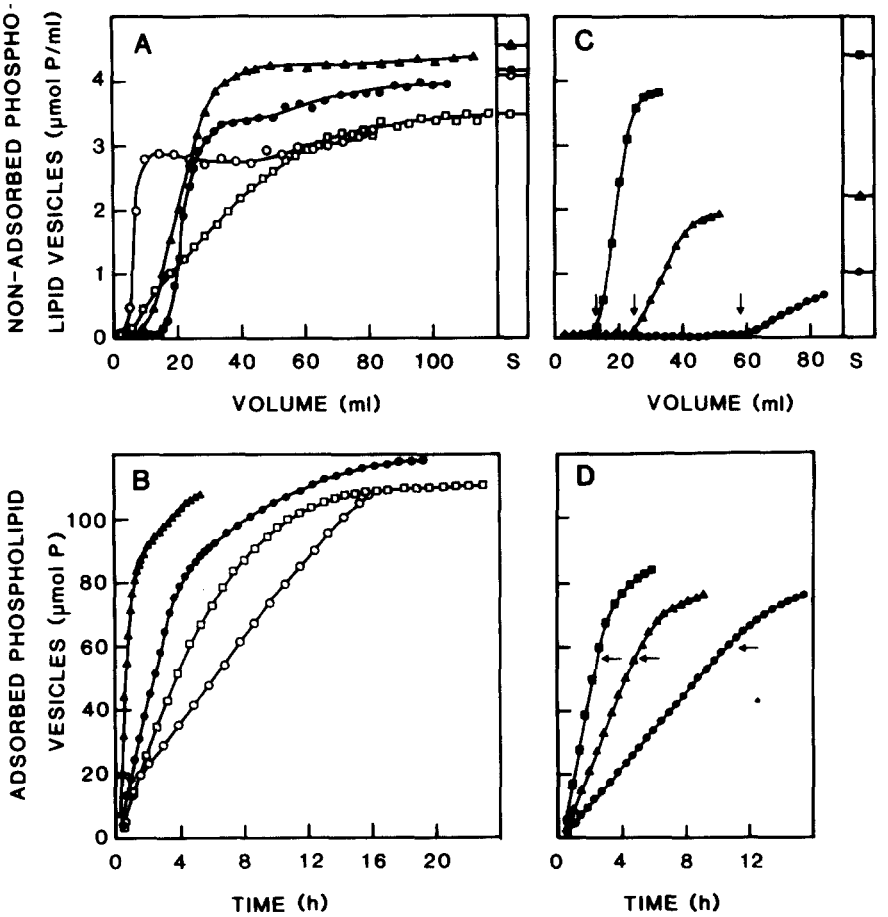

Fig. 4. Immobilization of small egg yolk phospholipid vesicles on octyl sulfide-Sepharose 2B (open symbols) and octyl sulfide-Sephacryl S-1000 (filled symbols). Volume of columns: 1.0 ml. Ligand concentrations and flow rates: (A,B): ▲, 14 μmol per ml packed gel (μmol/ml), 21 ml/h; ●, 14 μmol/ml, 5.5 ml/h; □, 2.4 μmol/ml, 5.5 ml/h; ○, 5.4 μmol/ml, 5.5 ml/h; (C,D): 14 μmol/ml, 5.5 ml/h. (A,C) Phospholipid concentration of non-adsorbed vesicles in the effluent, as a function of the volume of the applied sample. The phospholipid concentrations of the applied vesicle samples are indicated to the right (S). (B,D) Calculated amount of phospholipids in adsorbed vesicles, in μmol of phosphorus per ml packed gel, as a function of the sample application time. In (C,D) a single batch of phospholipids was prepared and applied undiluted or after appropriate dilution.

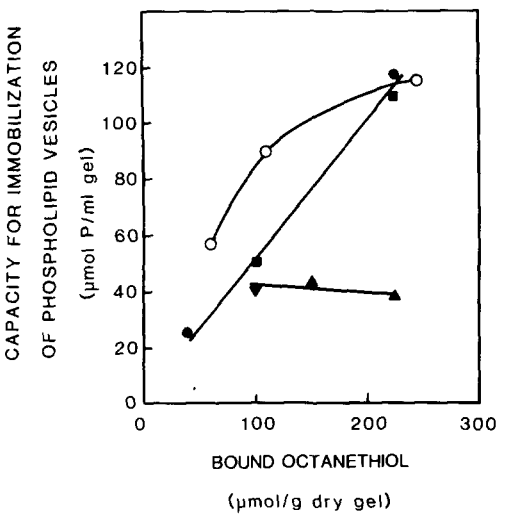

Fig. 5. Capacity for immobilization of small phospholipid vesicles on octyl sulfide-Sepharose 2B (open circles) and octyl sulfide-Sephacryl S-1000 (filled symbols), as a function of the ligand density, expressed as the amount of bound octanethiol per g of dry gel. ○, ●, Small vesicles, flow rate 5.5 ml/h; ■, small vesicles, 20 ml/h; ▲, medium vesicles, 5.5 ml/h; ▼ medium vesicles, 20 ml/h. Data from experiments illustrated in Figs. 4 and 6 and from other similar experiments.

(iii) Efficiency. Octyl sulfide-Sephacryl S-1000 of the ligand concentration 14 μmol/ml showed a vesicle adsorption that was complete at a flow rate of 5.5 ml/h, until half-saturation was reached, whereas the Sepharose 2B adsorbents were less efficient (Fig. 4A). The adsorption of vesicles in more dilute suspensions on octyl sulfide-Sephacryl S-1000 was again complete until 55–60 μmol of phospholipids had been applied, which corresponds to half-saturation, regardless of the vesicle concentration (Fig. 4C,D).

Immobilization of medium and large vesicles. (i) Capacity. Octyl sulfide-Sephacryl S-1000 showed a three times higher capacity, 38–40 μmol of phospholipids per ml, for immobilization of medium vesicles than did octyl sulfide-Sepharose 2B (Fig. 6B,D). In fact, the former gel adsorbed twice as much phospholipids in the form of large vesicles as did the latter in the form of medium vesicles (Fig. 6B). The capacity of octyl sulfide-Sephacryl S-1000 for medium vesicles was practically independent of the ligand density in the range 100–220 μmol/g (Fig. 5). The slightly lower capacity values at higher ligand densities probably reflect a slower approach toward saturation.

(ii) Rate. The initial adsorption rate for medium vesicles was approx. 0.2 μmol of phospholipids per min per ml gel, at a ligand concentration of 14 μmol/ml and a flow rate of 5.5 ml/h (Fig. 6B). At lower ligand concentration, 6.3 μmol/ml, and higher flow rate, 20 ml/h, the corresponding value was approx. 0.6 $\mu mol \cdot min^{-1} \cdot ml^{-1}$ and 25 μmol of phospholipids in the form of medium vesicles could be adsorbed in 1 h (Fig. 6D).

(iii) Efficiency. For medium vesicles the adsorption on octyl sulfide-Sephacryl S-1000 was nearly complete until half-saturation was achieved (Fig. 6). The adsorption of large vesicles on octyl sulfide-Sephacryl S-1000 showed low efficiency, and the same applies to medium vesicles on octyl sulfide-Sepharose 2B (Fig. 6A,B).

Effect of pore size on immobilization capacity. The gel filtration results (Fig. 1B) show that octyl sulfide-Sephacryl S-1000 could immobilize large vesicles only in a small fraction of the gel bead volume and medium and small vesicles in more than half of this volume. This limits the capacity (Fig. 7). The K_d values (Fig. 1B) and the volume fraction 0.74 occupied by closely packed monodisperse spheres lead to the estimate that small and medium vesicles can occupy a volume of at most $710 \times 0.74 = 525$ and $520 \times 0.74 = 385$ μl, respectively, per ml gel. The capacities 110 and 40 μmol of phospholipids per ml gel, the internal volumes 0.97 and 2.9 μl per μmol of phospholipids (see Table II), the outer radii 20 and 50 nm for small and medium vesicles, respectively, and a thickness of the lipid bilayer of 4 nm gives estimates of the total volume of 208 and 149 μl per ml gel, i.e., 40 and 39%, respectively, of the theoretical maximum for small as well as medium vesicles (cf. Ref. 1).

Octyl sulfide-Sepharose 2B showed lower capacity for medium vesicles, but not for small ones, than did octyl sulfide-Sephacryl S-1000 (Fig. 7).

3. *Volumes of immobilized vesicles*

Estimates by chromatography of glucose and calcein. In the previous work on immobilization of vesicles [1] the volume of immobilized vesicles was not experimentally determined. We have now estimated the volume of immobilized medium vesicles by chromatography of glucose before and after adsorption of the vesicles on octyl sulfide-

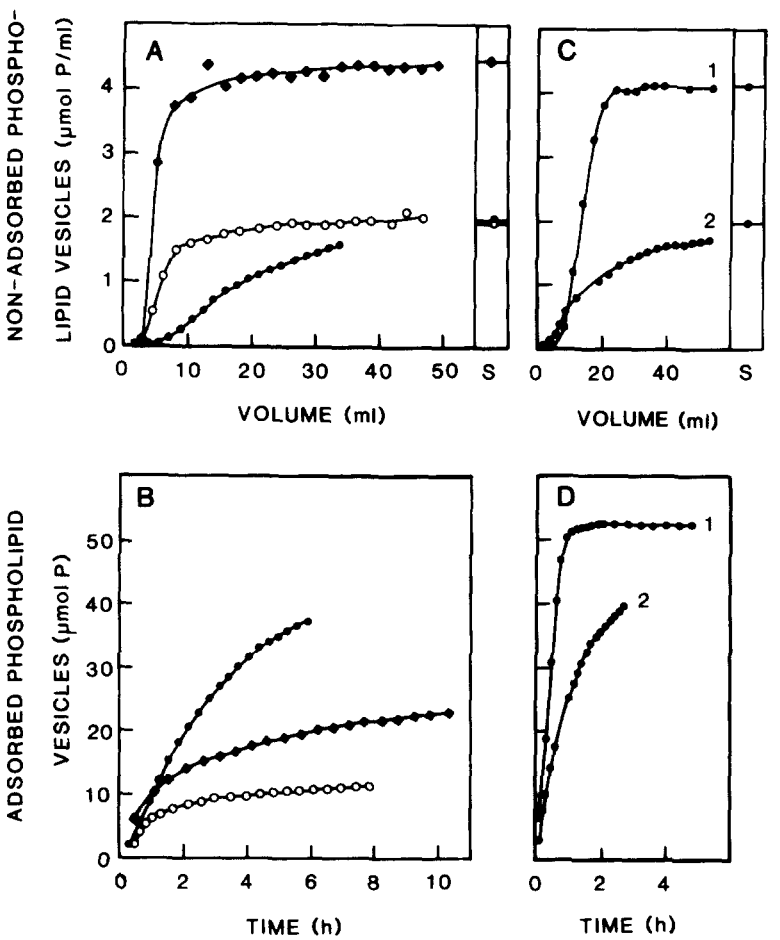

Fig. 6. (A,B) Immobilization of medium (○, ●) and large (◆) vesicles on octyl sulfide-Sepharose 2B (open circles) and octyl sulfide-Sephacryl S-1000 (filled symbols). Ligand concentration: ○, 5.4 μmol/ml; ●, ◆ 14 μmol/ml. Flow rate: 5.5 ml/h. (C,D) Immobilization of small (graph 1) and medium (graph 2) phospholipid vesicles on octyl sulfide-Sephacryl S-1000. Ligand concentration: 6.3 μmol/ml. Flow rate: 20 ml/h. (A,C) Eluted vesicles and (B,D) adsorbed vesicles as in Fig. 4. Volume of columns: 1.0 ml. For comparison of panels C and D, note that graph 1 in panel C has been shortened by five points.

Sephacryl S-1000 columns. For instance, on a column containing medium vesicles with a phospholipid content of 128 μmol, glucose eluted 360 μl earlier in the presence of the vesicles than before the immobilization (Fig. 8A). A slightly larger difference, 390 μl, was found when the vesicle volume was probed with calcein (Fig. 8B). For unknown reasons, glucose eluted somewhat later from the vesicle column in the presence of calcein than in the absence of this probe (Fig. 8B). The average 375 μl corresponds to the volume of the immobilized vesicles, i.e., the sum of the internal volumes and the volumes of the lipid bilayers. We assume that the leakage of glucose and calcein across the lipid bilayer is negligible (cf. Table I below) and that the probes are not retarded by interaction with the vesicle surfaces. Otherwise the method will underestimate the vesicle volumes. The average specific volume for medium vesicles was 2.9 μl per μmol of phospholipid (data above). Similar measurements were done with L-glucose for protein-lipid vesicles (proteoliposomes) prepared with integral membrane proteins from human red cells. The values were somewhat lower in these cases (Table II, below) possibly due to some glucose leakage related to the protein content of these vesicles, for instance through pores of protein aggregates.

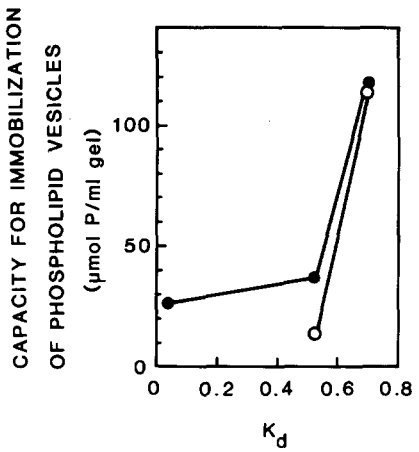

Fig. 7. Capacity for immobilization of phospholipid vesicles on octyl sulfide-Sepharose 2B (○) and octyl sulfide-Sepharyl S-1000 (●) as a function of the distribution coefficient K_d of the vesicles on Sephacryl S-1000. Ligand concentration: 2.4 and 14 μmol/ml for Sepharose and Sephacryl, respectively. Data from experiments illustrated in Figs. 1 and 4–6 and from other similar experiments.

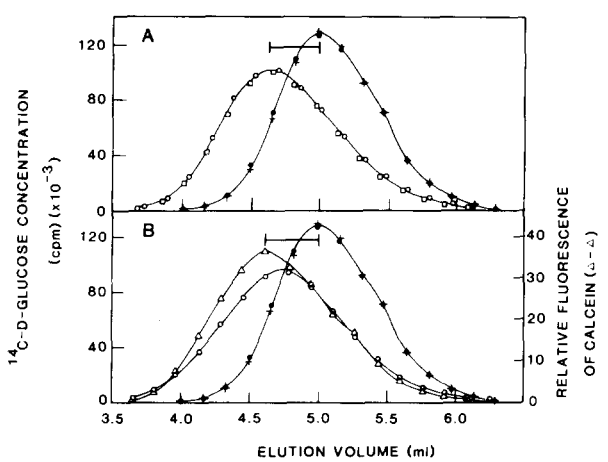

Fig. 8. Chromatographic determination of the total volume of immobilized medium phospholipid vesicles. (A) Chromatography of D-[^{14}C]glucose on octyl sulfide-Sephacryl S-1000 before (●, +) and after (□, ○) immobilization of an amount of medium vesicles that corresponded to 128 μmol of phospholipids. Column dimensions: 6.4×1 cm (5 ml). Ligand concentration: 6.3 μmol/ml. Flow rate: 20 ml/h. Eluent: B with 5 mM glucose. Sample volume: 100 μl. Two experiments of each type are shown to illustrate the reproducibility. (B) Chromatography of 100 μM calcein (△) mixed with D-[^{14}C]glucose (○) after the immobilization. Conditions as in (A), except that 1 μM calcein was included in the eluent. The elution profile for chromatography of glucose before the immobilization (●, +) is shown again for comparison.

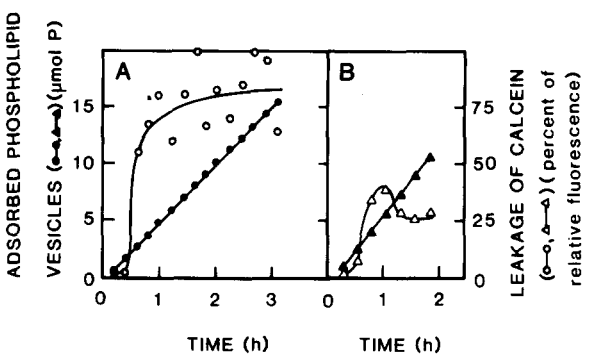

Fig. 9. Leakage of calcein from (A) small and (B) medium vesicles during immobilization on octyl sulfide-Sephacryl S-1000 at the ligand concentration 14 μmol/ml. Flow rate: 5.5 ml/h. Column volume: 1.0 ml. Sample concentration and volume: (A) 0.91 mM phospholipid, 17 ml; (B) 1.17 mM phospholipid, 9 ml. Phosphorus determination showed that all vesicles of the applied samples were adsorbed. ●, ▲, Amount of phospholipids in the adsorbed vesicles; ○, △, leakage of calcein expressed as the relative fluorescence of calcein in the eluent in percent of the relative fluorescence of the calcein in a cholate-solubilized aliquot of the applied vesicle sample.

Estimates by determination of entrapped calcein. When vesicles containing calcein were immobilized on octyl sulfide-Sephacryl S-1000 with a ligand concentration of 14 μmol per ml packed gel, as much as 75% of the calcein present in small vesicles was released during the adsorption process. The corresponding value for medium vesicles

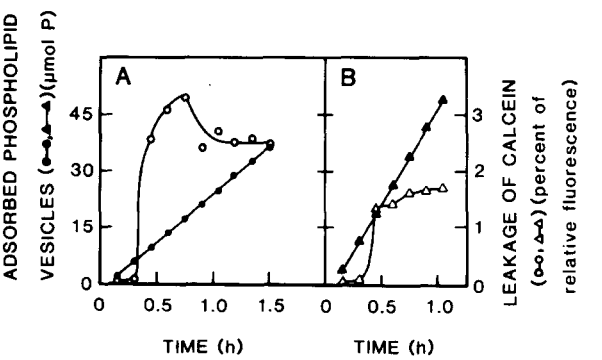

Fig. 10. Leakage of calcein from (A) small and (B) medium vesicles during immobilization on octyl sulfide-Sephacryl S-1000 at low ligand concentration, 6.3 μmol/ml. Flow rate: 20 ml/h. Column volume: 5 ml. Phospholipid concentration and volume of the applied samples: (A) 1.28 mM, 28 ml; (B) 2.50 mM, 20 ml. All vesicles of the applied samples were adsorbed. Symbols as in Fig. 9.

TABLE I

LEAKAGE OF CALCEIN FROM PHOSPHOLIPID VESICLES AFTER IMMOBILIZATION

Phospholipid vesicles containing calcein were immobilized on 1-ml columns of octyl sulfide-Sephacryl S-1000 at a flow rate of 20 ml/h and the calcein leaking out from the immobilized vesicles during elution with buffer B was determined by fluorometry.

Vesicle size	Ligand concn. (μmol/ml)	Adsorbed phospholipids (μmol)	Time of elution (h)	Leakage of calcein	
				%	$\%\cdot h^{-1}$
Small	14	74	19	11	0.6
Medium	14	55	23	8	0.3
Small	6.3	38	2.5	0.6	0.2
Medium	6.3	56	3.5	0.2	0.06

was also high, 25% (Fig. 9). At lower ligand concentration, 6.3 μmol/ml, only about 3 and 1.5% leaked out of small and medium vesicles, respectively (Fig. 10). Similarly, the slow leakage of calcein from the immobilized vesicles that occurred after the adsorption process was lower at a ligand concentration of 6.3 μmol/ml than it was at 14 μmol/ml, and lower for medium vesicles than for small ones (Table I). In the most favourable case, medium vesicles immobilized on octyl sulfide-Sephacryl S-1000 with the ligand concentration 6.3 μmol/ml, we considered the leakage (1.5%) during the adsorption (Fig. 10B) and after the immobilization ($0.06\%\cdot h^{-1}$) (Table I) as negligible and measured the amount of calcein that was released when the immobilized vesicles were solubilized with cholate to determine the internal specific volume of the immobilized vesicles. The results are shown in Table II. The internal specific volumes for small and medium vesicles correspond to vesicle radii of 20 and 50 nm, respectively, according to the theoretical graph reported by Enoch and Strittmatter [12]. The measurements did not reveal any difference between the internal and the total specific volume of medium vesicles (Table II), possibly due to insufficient precision. The latter volume is expected to be 28% larger than the former one.

4. Transport retention chromatography

When a protein that transports a component A across a membrane has been incorporated into the lipid bilayer of immobilized vesicles, the elution volume for A on the protein-lipid vesicles column will be larger than that for a component B that does not enter the internal volume of the vesicles. The chromatographic procedure may be termed 'transport retention chromatography'. We have

TABLE II

VOLUMES OF IMMOBILIZED PHOSPHOLIPID AND PROTEIN-LIPID VESICLES PER AMOUNT OF PHOSPHOLIPID

The internal volume of immobilized phospholipid vesicles was measured by fluorometric determination of entrapped calcein after elution with cholate. The total vesicle volume of immobilized phospholipid and protein-lipid vesicles was estimated by chromatography of D- or L-[^{14}C]glucose before and after the immobilization. The adsorbent was octyl sulfide-Sephacryl S-1000 of ligand concentration 6.3 μmol/ml (Expts. 1, 2 and 4) or octyl sulfide-Sepharose 2B of ligand concentration 2.4 μmol/ml (Expt. 3). n.d., not determined.

Expt.	Vesicle type	Vesicle size	Adsorbed phospholipids (μmol)	Internal specific volume (μl/μmol)	Total specific volume (μl/μmol)
1	phospholipid	small	38	0.97	n.d.
2	phospholipid	medium	56, 128	2.9	2.9 [a]
3	protein-lipid	small and medium	320	n.d.	1.4 ± 0.2 [b]
4	protein-lipid	medium	134	n.d.	2.0 [c]

[a] Data from Fig. 8.

[b] Integral red cell membrane proteins were solubilized at a protein concentration of 2 g/l. Protein-lipid vesicles were prepared in buffer B from 24 ml of protein solution mixed with 4 ml of phospholipid solution and were not fractionated on Sepharose 4B. Other details were as described in Methods. The value is an average for two experiments.

[c] Data from Fig. 11A.

incorporated integral membrane proteins from human red cells [8] into the lipid bilayer of egg yolk phospholipid vesicles of medium size, immobilized the vesicles on octyl sulfide-Sephacryl S-1000 and shown that D-[^{14}C]glucose eluted later than L-[^{14}C]glucose on the vesicle column. The difference in elution volume was approx. 90 μl, one third of the vesicle volume (Fig. 11A). Data from this experiment are included in Table II. Separate experiments (not shown) confirmed that the glucose transport activity was preserved in non-immobilized medium vesicles under conditions similar to those used in the chromatographic analyses. A control experiment with immobilized phospholipid vesicles without proteins showed the same elution volumes for D- and L-glucose (Fig. 11B). A second experiment with the membrane protein-lipid vesicles demonstrated a 95 μl-retention of D-glucose (Fig. 12A). The experiments in Figs. 11A and 12A were done two days after preparation of the vesicles. Elution volume analyses as in fig. 12A were repeated after another two days. The

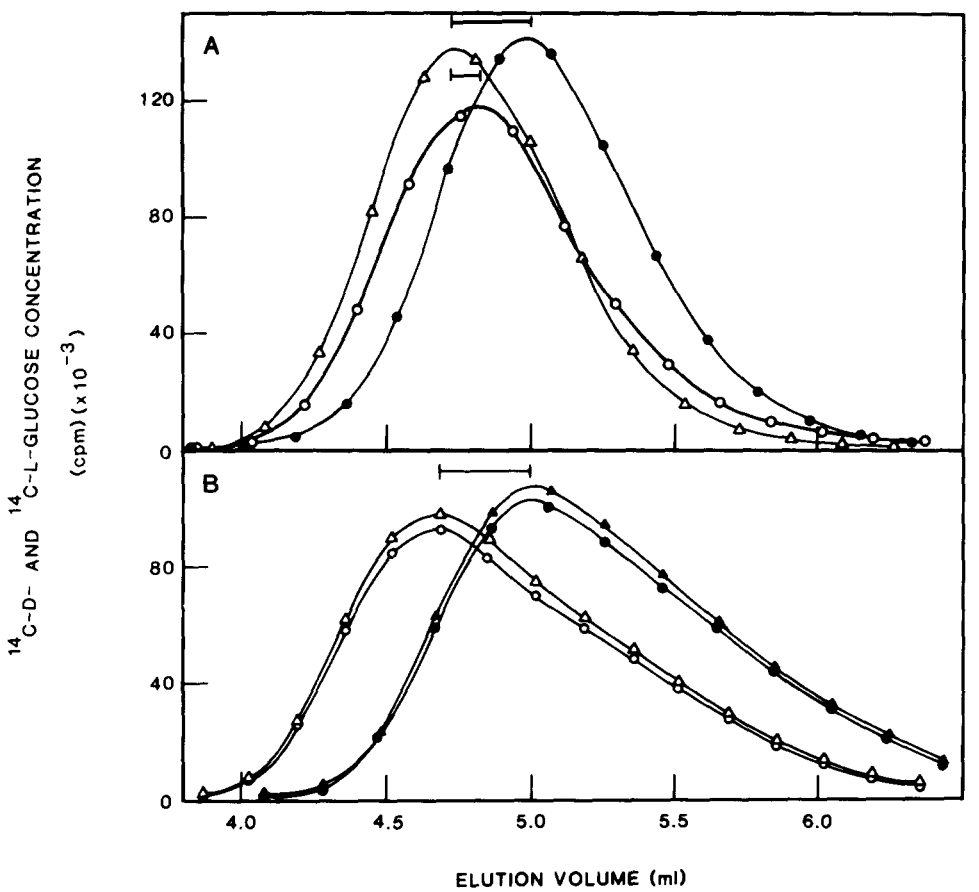

Fig. 11. Transport retention chromatography. (A) Chromatography of D-[^{14}C]glucose (○) and L-[^{14}C]glucose (△) on a column of octyl sulfide-Sephacryl S-1000 in which medium protein-lipid vesicles had been immobilized. The vesicles were composed of egg yolk phospholipids and integral membrane proteins from human red cells, including the glucose transporter (see Methods). The average number of glucose transporters per vesicle can be estimated at approx. 8 (see Discussion). The chromatographic analyses were done at room temperature about 50 h after the preparation of the vesicles. Phospholipids in adsorbed vesicles: 134 μmol. Column volume: 5 ml. Concentration of D-glucose in the vesicles and the eluent; 100 μM. Eluent: C. Flow rate: 6 ml/h. Sample volume: 100 μl. The elution profile for D-glucose before immobilization is also shown (●). The protein-lipid vesicle column shows a retention of D-glucose in relation to L-glucose of approx. 90 μl (short bar), i.e., 0.67 μl per μmol of phospholipid. The vesicle volume is approx. 270 μl (long bar). (B) Control experiment with chromatography of D-[^{14}C]glucose (○, ●) and L-[^{14}C]glucose (△, ▲) before (●, ▲) and after (○, △) immobilization of medium vesicles without proteins. The vesicle volume is approx. 310 μl (bar).

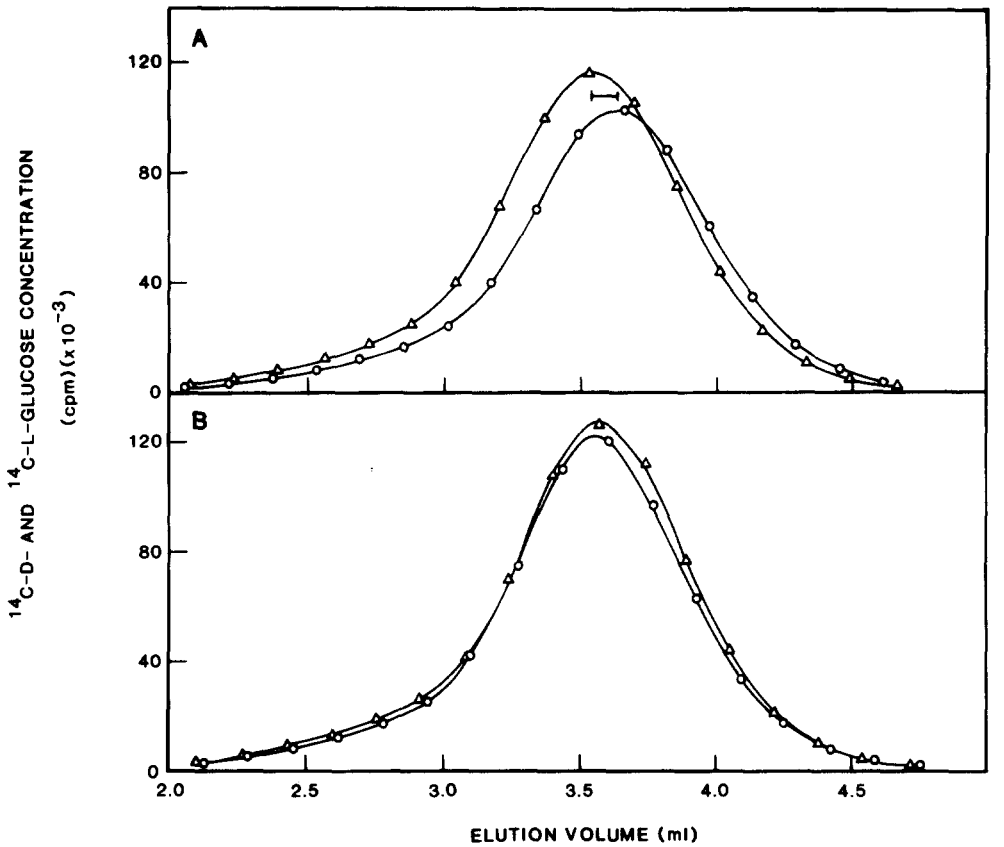

Fig. 12. Transport retention chromatography. (A) Chromatography of D-[^{14}C]glucose (○) and L-[^{14}C]glucose (△) on a column of octyl sulfide-Sephacryl S-1000 in which medium protein-lipid vesicles had been immobilized as in Fig. 11A. Phospholipids in adsorbed vesicles: 100 μmol. Column volume: 3.8 ml. All other conditions as in Fig. 11A. The column shows a retention of D-glucose in relation to L-glucose of approx. 95 μl or 0.95 μl per μmol of phospholipid. (B) An experiment as in (A), but performed 100 h after the vesicle preparation. The glucose transporter has lost its activity.

result indicated that the glucose transporter had lost its activity (Fig. 12B). As expected, a control experiment in the presence of Hg^{2+} gave the same result as in Fig. 12B.

When the retention of D-glucose in relation to L-glucose is expressed in μl per μmol of immobilized phospholipid, representative values are 0.67 (Fig. 11A) and 0.95 (Fig. 12A). The average is thus 0.81 μl/μmol. The analyses were done in the presence of 100 μM D-glucose at room temperature and at a flow rate of 6 ml/h. The retention decreased with increasing flow rate: at 10 ml/h it was two thirds and at 20 ml/h half of that at 6 ml/h. The optimal flow rate is probably below 6 ml/h.

Discussion

Octyl sulfide derivatives of the large-pore Sephacryl S-1000, which have not been described earlier, showed some favourable immobilization properties compared to octyl sulfide derivatives of Sepharose 2B. The vesicle adsorption was faster and the capacities for medium and large vesicles were higher. The capacities for immobilization of small, medium and large vesicles on octyl sulfide-Sephacryl S-1000 corresponded to 110, 40 and 20 μmol of phospholipids, respectively, per ml gel. However, the maximal internal volumes per ml gel were 110 × 0.97, 40 × 2.9 and 20 × 6.3 μl or about 110, 120 and 130 μl, respectively (data from Table

II and, for large vesicles of radius 100 nm, from Ref. 12). The increase in specific internal volume with increasing vesicle radius is counteracted by the decrease in adsorbent capacity and the total volume of the adsorbed vesicles is nearly independent of the vesicle size. Larger capacities and larger internal vesicles volumes per ml adsorbent could be achieved by use of gel beads with still larger pores than those in the Sephacryl S-1000 matrix, but to our knowledge such gels are not yet commercially available. Another approach would be to try to form lipid bilayers around gel beads, covering their surfaces.

Low ligand concentrations were used in the present work. The range was 2–14 μmol of ligand per ml packed gel. This may be insufficient to achieve high adsorption capacities for proteins, although successful protein separations still might be possible, but it was generally enough for immobilization of the lipid vesicles. At the maximal ligand concentration that we achieved for octyl sulfide-Sephacryl S-1000, 14 μmol/ml, the number of ligands exceeded the number of large vesicles in a saturated gel by a factor of 250 000 (value calculated by use of the number of lipid molecules per vesicles of radius 100 nm from Ref. 12). Since the K_d value for large vesicles was about 0.05, about 10 000 octyl groups would have been present per large vesicle in the accessible gel pores. The corresponding values for medium vesicles are about 32 000 ligands per vesicle and approx. 17 000 ligands per vesicle in the accessible volume. Naturally only a small fraction of these ligands actually interact with a vesicle, for geometrical reasons and because of the fibrillar gel structures. For small vesicles in a saturated octyl sulfide gel of the lowest ligand concentration we used, 60 μmol/g or 0.6 μmol/ml (Fig. 5), only about 100 ligands would have been present per vesicle and in this case the capacity was lower than at higher ligand density (Fig. 5). Sephacryl S-1000 showed higher maximal ligand concentration per ml packed gel than did Sepharose 2B (Fig. 3), although the ligand density per g dry gel was low. Probably the Sephacryl structure is formed of fibrils, each one of which is composed of many extensively cross-linked dextran and acrylamide polymers (cf. Ref. 13). In this way large pores can be formed in gels of high polymer content. Sepharose 2B, in which the polysaccharides interact by hydrogen bonding, contains thinner fibrils and also more narrow pores than Sephacryl S-1000 does, in spite of the low agarose concentration.

By use of protein-lipid vesicles containing membrane proteins from human red cells we succeeded in demonstrating the activity of a transport protein, the glucose transporter, in immobilized vesicles. Medium protein-lipid vesicles were prepared from 1 mmol of phospholipids and a mixture of integral membrane proteins solubilized from 48 mg of proteins and containing 5 mg of red cell glucose transporter (purification and amino acid analyses done in a separate experiment, personal communication from E. Mascher). The relative molecular mass of the transporter polypeptide is approx. 54 000. Since each medium vesicle contains about 85 000 lipid molecules we can calculate that, on the average, approx. eight glucose transporter monomers were incorporated per vesicle. If small vesicles, radius 20 nm, had been prepared, the same type of calculation indicates that, on the average, one monomer would have been incorporated per vesicle. In this case some of the vesicles would have lacked transporter. Furthermore, although the same internal volume could have been achieved for immobilized small vesicles as for immobilized medium ones, the total surface area, and therefore the leakage through the lipid bilayer, would have been larger (cf. Table I). From this point of view it might have been best to use large vesicles. Chromatographic experiments with D- and L-[^{14}C]glucose showed that the rate of transport of D-glucose by facilitated diffusion through the glucose transporters was fast in relation to the leakage of glucose through the lipid bilayers, which produced a retention of D-glucose relative to L-glucose. Similar results might be achieved with other transport systems and with immobilized cells as well as protein-lipid vesicles. Chromatography on columns of immobilized proteoliposomes may be used for investigations of the activity of transport systems under varying conditions and in the future possibly also for practical chromatographic separations. Immobilized lipid vesicles containing charged lipids or other charged amphipathic molecules might show useful ion-exchange properties, and immobilized vesicles can also be used to

anchor alkyl derivatives of molecules for affinity chromatography. Immobilization of vesicles containing hydrophobic enzymes might be convenient when such enzymes are to be used in chemical reactions. Lipid vesicles entrapping drugs may be stabilized by immobilization in small gel spheres of large pore size.

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