Biochimica et Biophysica Acta, 1030 (1990) 258–268
Elsevier

BBAMEM 75072

Substantial glucose leakage from liposomes on filters and upon molecular-sieve chromatography in determinations of reconstituted glucose-transport activity and liposome volumes

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(Received 19 June 1990)

Key words: Glucose leakage; Glucose transport; Liposome; Membrane filtration; Molecular-sieve chromatography; Proteoliposome

Transport-protein activities are often determined by procedures that involve isolation of liposomes containing the transported radioactive solute. We determined the activity of the human red cell glucose transporter in liposomes and, by similar procedures, internal volumes of liposomes. For these purposes, we isolated freeze-thawed liposomes loaded with [^{14}C]glucose, either by filtration on cellulose-nitrate and cellulose-acetate filters, or by chromatography on Sephadex. The interaction of liposomes with filters caused substantial leakage of [^{14}C]glucose. About half of the internal [^{14}C]glucose was released on the filters from glucose-transporter liposomes with inhibited transport. Chromatography at high flow rate provided higher and more accurate values than did the filtration procedure. Leakage corrections could be made by use of flow-cell scintillation elution profiles. The ratios between the corrected chromatographic volume values and the filtration values were 1.4–3.0 for liposomes without protein, 2.4–4.0 for glucose-transporter liposomes and 3.6–7.9 for liposomes with several human red cell integral membrane proteins. The D-glucose equilibrium exchange with glucose-transporter liposomes at 50 mM D-glucose was 2.0 nmol D-glucose per μg transporter per second as determined by use of chromatography at high flow rate. The filtration procedure gave only 0.6 nmol $\cdot \mu g^{-1} \cdot s^{-1}$ due to the [^{14}C]glucose leakage. In our experiments, the chromatographic procedure thus proved superior.

Introduction

Liposomes (phospholipid vesicles) with transport proteins incorporated in their bilayers are used to determine the activities of these proteins. For instance, a protein is allowed to transport its specific solute in radioactively labelled form into the liposomes, which are then separated from the free radioactive solute, often by filtration on microporous membrane filters. The radioactivity on the filter and in the filtrate is determined [1–4]. Filtration procedures have also been used for vesicles of biological membranes [5–7], for mitochondria [8] and for whole cells [9,10]. Accurate transport activity values can only be obtained if the liposomes, etc., are quantitatively retained on the filter without leakage of the tracer. Despite the common use of filtration procedures very few data about retention and leakage are available: Kasahara and Hinkle separated small, sonicated glucose-transporter liposomes from glucose and found that filtration showed 'more than 80%' of the D-glucose uptake observed when molecular-sieve chromatography was used for the separation [1]. Kimmich studied the transport of 3-*O*-methylglucose into isolated intestinal epithelial cells. Filtration of the cells gave somewhat higher transport activity values than did centrifugation [10]. Hopfer et al. found that the sucrase activity of brush border membrane vesicles was completely retained on filters [5]. Robinson et al. filtered freeze-thawed, sonicated egg lecithin liposomes with reconstituted adipocyte glucose-transport activity and achieved up to 85% retention of the phospholipids [3].

Molecular-sieve chromatography has been used as an alternative method for the separation of liposomes from free tracer [1, 11–14]. The purpose of our work was to compare the amounts of liposome-trapped tracer ([^{14}C]glucose) recovered by filtration and by chromatography. Reconstituted glucose-transport activity and the internal volumes of liposomes were estimated by use of both methods. The values differed considerably owing to different extents of tracer leakage from the lipo-

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somes, partly or mainly caused by liposome–filter or liposome–gel-bead interactions. Interactions between liposomes and various surfaces deserve further studies.

Materials and Methods

Materials. Human red cell concentrate (stored 4–5 weeks) was supplied by the Blood Bank at the University Hospital, Uppsala, Sweden. Egg-yolk phospholipids were prepared as described in Ref. 14. Octyl glucoside was obtained from Sigma, St. Louis, MO. Sephadex G-50 M was purchased from Pharmacia LKB Biotechnology, Uppsala and DEAE-cellulose (DE52) from Whatman, Maidstone, Kent, U.K. D-[U-^{14}C]Glucose (10.0 GBq/mmol) and L-[1-^{14}C]glucose (2.04 GBq/mmol) were bought from Amersham, Little Chalfont, Bucks., U.K. Scintillation liquids were obtained from Zinsser Analytic, Maidenhead, Berks., U.K. (Quickszint 361) and from Radiomatic Instruments, Tampa, FL (Flo-Scint III). All other chemicals were of analytical grade. Microporous membrane filters were bought from Schleicher & Schuell, Dassel, F.R.G. (BA83, cellulose nitrate, 0.2-μm pore size, in the following denoted filter Type 1; and BA85, cellulose nitrate, 0.45-μm pore size; filter Type 2) and from Sartorius, Göttingen, F.R.G. (SM 11107, cellulose acetate, 0.2-μm pore size; filter Type 3).

Solutions. 'Buffer D' contained 10 mM Na-phosphate (pH 7.2), 200 mM NaCl and 50 mM D-glucose. The ionic strength of this buffer is suitable for reconstitution with cholate-solubilized lipids (unpublished data). 'Stop solution' was buffer D, brought to room temperature and supplemented with 2 mM $HgCl_2$ (glucose-transport inhibitor) on the day of use. 'Tris buffer' contained 10 mM Tris-HCl (pH 7.2), 200 mM NaCl and 50 mM D-glucose.

Integral membrane proteins and glucose transporter from red cells. Human red cell membranes were depleted of peripheral proteins, frozen and stored as described earlier [15].

The integral membrane proteins and lipids were partly solubilized with 75 mM octyl glucoside at 8 mg protein/ml [14]. The solubilized material (denoted 'integral membrane proteins') was either used without purification, or the glucose transporter was purified by chromatography on DEAE-cellulose in 50 mM octyl glucoside [14]. Part of the membrane lipids were co-eluted with the transporter [14,16].

Liposomes and proteoliposomes. Egg-yolk phospholipids, liposomes and proteoliposomes were prepared essentially as described in Ref. 14. Cholate-solubilized egg-yolk phospholipids (250 mM lipids; approx. 70% phosphatidylcholine and 21% phosphatidylethanolamine) were mixed with an equal volume of buffer D; or an octyl-glucoside solution of glucose transporter (0.6 mg/ml) or of integral membrane proteins (3 mg/ml, including approx. 0.7 mg glucose transporter/ml [14]). 300-μl aliquots of the mixtures were applied on a 1 × 12 cm Sephadex G-50 M column in buffer D at a flow rate of 3.0 ml/min. The elution was monitored at 280 nm (Pharmacia UV-2 monitor, 1 mm light path). The liposomes formed had an average diameter of approx. 40 nm and a specific internal volume of about 1 μl/μmol lipid [17]. The front half (1 ml, about 19 μmol of phospholipids) of the liposome fraction was collected from several runs and combined. For internal-volume determinations 19 volumes of the liposome suspension was mixed with one volume of D-[^{14}C]glucose in buffer D to 37 Bq/μl. For transport assays, an equivalent dilution was made with buffer D. These procedures were done at room temperature. The final liposome suspension was divided into 1-ml portions which were frozen for 10 min in ethanol/solid CO_2 in polystyrene Ellerman tubes (9 × 55 mm) and stored at −25°C or, for transport assays, at −70°C. On the day of use, a number of portions were thawed for 15 min in a 25°C waterbath, vigorously homogenized with a vortex mixer for 4 s, combined, and left for at least 30 min (cf. Ref. 14). Small unilamellar liposomes or liposome fragments fuse into larger liposomes upon freeze-thawing. The internal volume increases and added radioactive tracer becomes evenly distributed [18–22]. The internal volumes were not exactly reproducible. Each set of experiments for comparing the results from filtration and from chromatography was therefore done with a single batch of freshly thawed, homogenized and combined 1-ml liposome portions.

Phospholipids were determined as phosphorus according to the method of Bartlett [23] in special experiments for which liposomes and proteoliposomes were prepared and used in Tris buffer instead of buffer D. The membrane filters could be digested with the H_2SO_4 and H_2O_2 used in this method. The filter materials did not interfere with the phosphorus analyses.

The freeze-thawed liposomes and proteoliposomes are both usually referred to as 'liposomes' below.

Filtration. A microporous membrane filter of 20 mm diameter was mounted in a filter holder (Fig. 1) made of polymethylmethacrylate (Perspex). The filter was sealed by a 1.7-mm thick rubber O-ring (14 mm inner diameter) which was compressed to a thickness of 1.0 mm (cf. Refs. 10 and 24). The size of the filtration chamber was 14 mm (i.d.) × 55 mm and the effective filter area was 1.5 cm^2. The tubing from a peristaltic pump was connected to the 100-μl space below the porous polypropylene support disc (Fig. 1). The mounted filter was washed with 1 ml of stop solution, the filtration chamber was emptied, the filter holder was disconnected from the pump and the tubings were emptied. For internal-volume experiments, an aliquot of radioactive liposome suspension (7.5 μl with 0.135 μmol of phospholipids; or 15 μl) was pipetted into an polypro-

pylene microtube and gently mixed with 1 ml of stop solution (or Tris buffer with 2 mM $HgCl_2$). For transport activity measurements, a 7.5-μl aliquot of non-radioactive glucose-transporter liposome suspension was incubated with radioactive D- or L-glucose at 21–24 °C before addition of stop solution. In both cases the stop solution was room-temperatured. Ice-cold stop solution possibly makes the liposomes brittle (cf. Ref. 25). One ml of diluted radioactive liposome suspension was transferred to the filtration chamber. The pump was started and run just until the liquid meniscus reached the filter, since drawing air through the filter lowered the retention of liposome-trapped tracer on the filter. One ml of stop solution was added to the remaining liquid (0.2–0.3 ml) for rinsing and one ml of the liquid was sucked through the filter. This rinsing procedure was repeated one or three times. Finally, the pump was run just until the liquid disappeared from the filter. The filter was removed and all the filtrate, including that in the tubings, was collected. The total time for filtration and 2 × 1-ml rinsing was about 2 min at a flow rate of 2.5 ml/min. Flow rates of 0.5 and 5 ml/min were also used.

Three ml of Quickszint 361 was added to each filter in a 7-ml vial which was rotated end-over-end for 30 min. This dissolved the cellulose-nitrate filters and rendered the cellulose-acetate filters transparent. An 0.2-ml aliquot of the filtrate was mixed with 3 ml of Quickszint 361. Scintillations were counted in a LS 2800 detector (Beckman Scientific Instruments, Irvine, CA). Filters and liposomes in the vials did not affect the counting efficiency.

Flow-cell scintillation detection. A flow-cell scintillation detector (A-300 Flo-One Beta, Radiomatic Instruments, Tampa, FL) was used to monitor the chromatographic separations. The detector stream splitter was set at 50%. Half the eluent flow of 2 ml/min was thus mixed with a scintillation-liquid (Flo-Scint III) flow of 5 ml/min. The split frequency (the frequency of diverting the flow between the detection cell and the column eluent outlet) was 1 Hz, and the energy window was set at 1–400 keV. The detection cell was a 0.5-ml flat spiral of Teflon tubing. The residence time (the time a small volume element spends in the cell) was thus 0.5 ml : 6 ml/min = 5 s. This time was taken into account by the computer program when the detector signal was recorded as cpm values for each 10-s update time interval [26]. The cpm values were automatically summed between every minimum of the elution profile, giving values that correspond to the amount of tracer in each peak. A background subtraction (56 cpm) was done such that the average residual background added for each 10-s sampling time interval was less than 20 cpm.

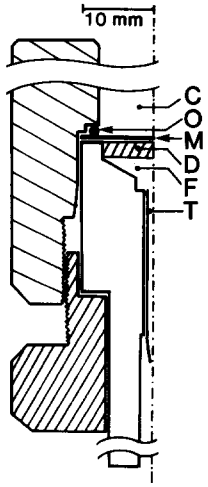

Fig. 1. Half cross-section of the filter holder used. (C) Cylindrical filtration chamber; (O) O-ring; (M) microporous membrane filter; (D) porous polypropylene support disc; (F) funnel; (T) tubing connected to a peristaltic pump. The height of the funnel F is exaggerated in the drawing.

Molecular-sieve chromatography. Liposomes with trapped tracer ([^{14}C]glucose) were separated from external tracer on a 1 × 50 cm Sephadex G-50 M column, usually at a flow rate of 2 ml/min (2.5 cm/min), which gave a retention time of about 7.5 min for the liposomes. The sample volume was 0.3 ml (5.4 μmol of phospholipids). Flow-cell scintillation monitoring allowed estimates of the tracer leakage during the separation. The gel bed adsorbed (or trapped) some liposomes. Before each series of experiments therefore either repeated 'blank' runs were made, or 1 ml of liposome suspension was circulated through the column overnight via a 1-ml reservoir, to saturate adsorption sites. Equilibration and elution were done with stop solution unless otherwise stated. When flow rates below 2 ml/min were used while the liposomes migrated through the column, the flow rate was increased to 2 ml/min immediately before the start of detection and the elution of liposomes, to obtain constant detection conditions. The counting efficiency was not affected by the presence of liposomes.

Internal volumes of liposomes. The internal volumes were expressed as the amounts of liposome-trapped tracer in percent of the amount of tracer present during freezing and thawing; or in μl per ml suspension. Determinations were done at room temperature by chromatography and by filtration with aliquots of liposomes and proteoliposomes (see above).

Glucose transport (equilibrium exchange). Glucose-transporter liposomes were thawed, homogenized and combined as described above. Transport experiments were done at room temperature (21–24°C). The liposomes were isolated either by filtration or by chromatography after incubation with tracer. The stereospecific equilibrium exchange of D-glucose was determined as the difference between the amounts of internalized D- and L-glucose in the liposomes (percent of the amount of tracer added), and was finally ex-

pressed as glucose uptake per protein amount. The protein concentration in the liposome suspension was determined by automated total amino-acid analysis with hydrolysis times of 24 and 72 h. Correction by use of the known glucose-transporter amino-acid composition was made for serine due to the presence of phosphatidylserine, mainly from the red cell membranes.

Equilibrium exchange, filtration details. 7.5 μl of liposome suspension in buffer D was pipetted into an Eppendorf microtube and 5 μl was added of a D-[^{14}C]glucose solution in buffer D with a total glucose concentration of exactly 50 mM and a radioactivity of 740 Bq/μl. After 10, 20 or 30 s, 1 ml of stop solution was added. 1 ml of the suspension was immediately filtered on a filter of Type 1 at 2.5 ml/min and rinsed with 2 × 1 ml stop solution. For zero-time incubations the stop solution was added to the liposomes before the tracer. Non-specific uptake and binding of glucose was determined in exactly the same way with L-[^{14}C]glucose. The filtration and rinsing was completed within 2 min after addition of stop solution.

Equilibrium exchange, chromatographic details. The incubation was started by mixing 200 μl of liposome suspension with 100 μl of the above D-[^{14}C]glucose or L-[^{14}C]glucose solution and terminated by the addition of 33 μl of 20 mM $HgCl_2$ in buffer D to a final Hg^{2+} concentration of 2 mM. 300 μl was immediately applied onto the 1 × 50 cm Sephadex G-50 column during 40 s. The flow rate was 2 ml/min.

Results

Liposome sizes

To estimate their relative sizes, liposomes were photographed with 1700-times final magnification in a phase-contrast microscope. The detection limit was 0.6 μm. Only unilamellar liposomes were observed. In a few cases small liposomes seemed to be present inside large ones. The arithmetic means ± S.D. of the observed diameters were 2.5 ± 1.6 μm ($n = 232$) for protein-free liposomes; 2.6 ± 0.6 μm ($n = 145$) for glucose-transporter liposomes; and 1.8 ± 0.9 μm ($n = 181$) for integral-membrane-protein liposomes. Glucose-transporter liposomes prepared in Tris buffer were the smallest: 1.0 ± 0.8 μm ($n = 208$). To relate the size differences between the liposome types to the differences in internal volumes, another mean is more relevant; namely, the cube root of the average of the cubes of the individual diameters (= the diameter of the liposome with the mean volume). This mean was 3.5; 3.5; 2.3 and 1.6 μm, respectively, for the above liposome types. The size heterogeneity of glucose-transporter liposomes is illustrated in Fig. 2A, and Fig. 2B illustrates the size relationship between the Sephadex gel beads and these liposomes. The visible liposomes were estimated (by use of the depth of focus, about 1 μm, in the microscope, the size and number of liposomes, and the lipid amount) to comprise about 10% of the phospholipid and about 50% of the total internal liposome volume.

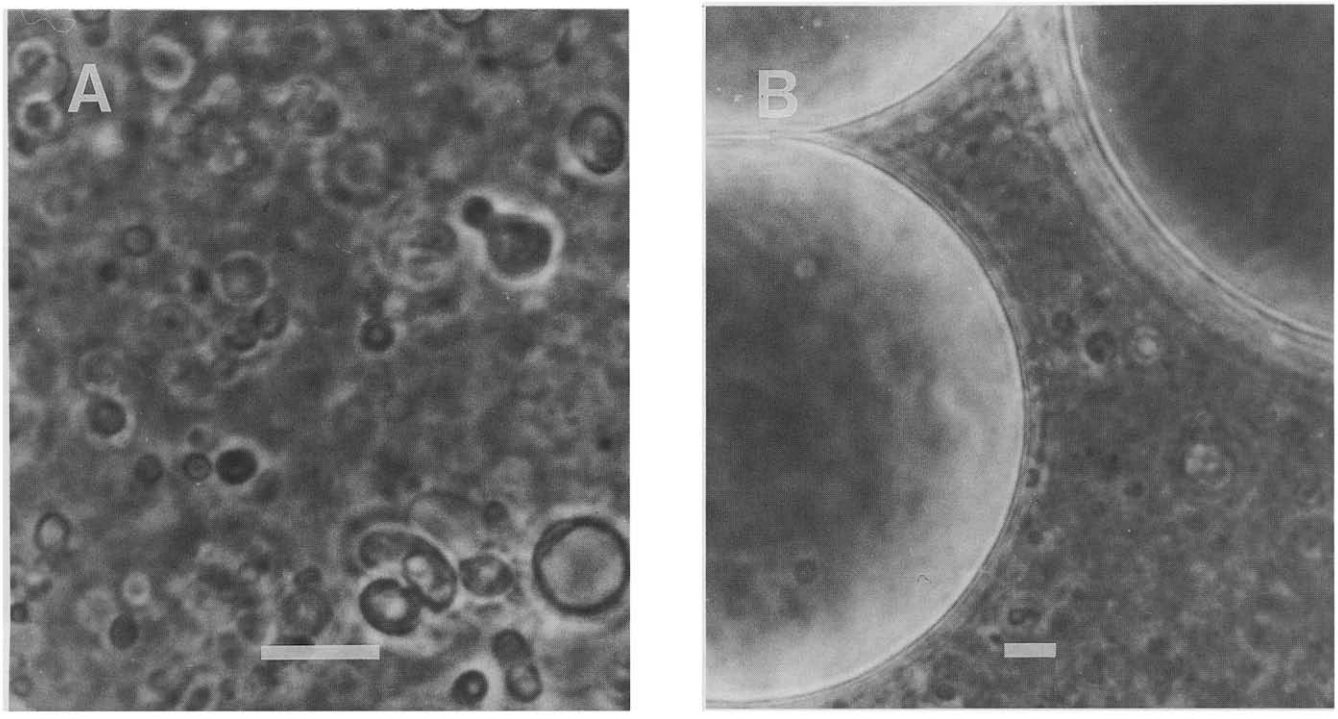

Fig. 2. Phase-contrast micrographs of (A) freeze-thawed glucose-transporter liposomes prepared in buffer D and (B) Sephadex G-50 M gel beads together with such liposomes. The bars correspond to 10 μm. Detection limit 0.6 μm.

Values of liposome internal volumes obtained by filtration

The highest internal-volume values obtained by filtration were 144 μl per ml liposome suspension (14.4%) for protein-free liposomes; 7.9% for glucose-transporter liposomes; and 2.4% for integral-membrane-protein liposomes (Table I). The cellulose-acetate filter (Type 3) gave slightly higher values, i.e., caused somewhat less leakage, than did cellulose-nitrate filters (Types 1 and 2). A 7.5-μl aliquot of liposomes gave a higher value than did a 15-μl aliquot. Rinsing with four instead of two 1-ml portions led to somewhat lower 'retention of counts' on the filters. With decreasing filtration flow rate the retained radioactivity increased considerably for protein-free liposomes and slightly for glucose-transporter liposomes. It decreased for integral-membrane-protein liposomes (Table I).

Chromatography: Interpeak tracer and leakage correction

Chromatographic separation between liposomes and external tracer is illustrated in Figs. 3, 4 and 6, below. The chromatograms show an above-background radioactivity between the liposome peak and the external-tracer peak ('interpeak tracer' , Fig. 3, a-b). This interpeak tracer is part of the originally liposome-trapped amount of tracer (see Discussion). A correction should therefore be made which should include an extrapolation of the interpeak tracer level into the external-tracer peak, for instance, as illustrated in Fig. 3. The external-tracer peak is much broader than the initial sample zone and thus hides some of the tracer derived from the liposomes. The cpm value of the tracer in the area below the dotted line a-b-c-d in Fig. 3 (here denoted the 'correction term') shall be added to the cpm value of the tracer in the liposome peak. The lowest interpeak count level represents the leakage just before the liposomes leave the column, when the difference between the internal and external concentrations of tracer is smallest. Since the interpeak tracer profile shows a positive slope (most clearly seen in Figs. 3 and 6 B,C), whereas the extrapolation is done by the horizontal line b-c, the correction term is not overestimated.

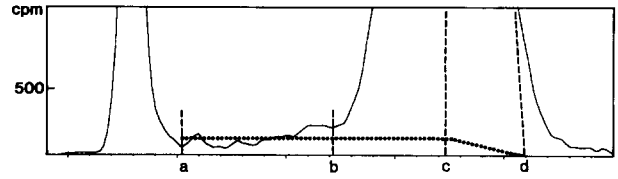

Fig. 3. Detail of the flow scintillation chromatogram in Fig. 4A illustrating separation on a 1×50 cm Sephadex G-50 M column of glucose-transporter liposomes in stop solution with trapped tracer (first large peak) and free tracer (second large peak). The elution profile allows an approximate estimate of tracer loss from the liposomes: The dotted line between a and b is drawn such that the area below it is equal to the area below the elution profile between a and b, which represents some of the tracer released from the liposomes ('interpeak tracer'). The sum of the interpeak cpm values, the extrapolated cpm values (b to c) and the additional estimated values (c to d) are represented by the area below the dotted line from a to d. This sum is denoted the 'correction term'. In detail, the average cpm level in the region a-b was extrapolated to the center c of the external-tracer peak. To the right of c the line was further drawn to a point d at the crossing of the baseline with the tangent (hatched line) through the inflection point at the right side of the external-tracer peak.

TABLE I

Internal-volume estimates

Method	Flow rate (ml/min)	Internal-volume values (percent of suspension volume) [a]		
		Protein-free liposomes	Glucose-transporter liposomes	Integral-membrane-protein liposomes
Filtration [b]	0.5	14.2 (10.1)	6.5 (4.7)	1.4 (1.1)
(Type 1	2.5	9.5 (8.1)	5.9 (4.9)	2.4 (2.0)
filters)	5.0	8.0 (6.9)	5.7 (4.9)	2.3 (2.2)
Filtration [b]	0.5	14.4 (10.4)	7.1 (5.0)	
(Type 2	2.5	8.7 (7.4)	6.2 (5.2)	
filters)	5.0	7.5 (6.9)	5.6 (5.6)	
Filtration [b]	0.5	14.1 (10.2)	7.9 (5.7)	
(Type 3	2.5	10.1 (8.0)	7.3 (6.7)	
filters)	5.0	8.3 (7.0)	6.8 (6.7)	
Chroma-	2.0	18.9±0.2% [c]	13.4±0.4% [c]	3.9±0.2% [c]
tography	2.0	20.7±0.2% [d]	18.7±0.3% [d]	8.7±0.1% [d]

[a] See Methods. Averages of duplicate determinations.
[b] Average standard error: 0.14. Standard deviation of this error: 0.16. 7.5-μl aliquots of liposome suspensions. Values within parenthesis: 15-μl aliquots. Rinsing with 2×1 ml.
[c] Without correction (cf. Fig. 3).
[d] With correction (cf. Fig. 3).

When we lowered the flow rate and thus increased the time for tracer outleakage from the liposomes on the column, the interpeak tracer level remained essentially the same, which shows that the diffusional leakage and, for proteoliposomes, the residual glucose transport activity, were low (Fig. 4). However, the area of the peak of liposome-entrapped tracer decreased with decreasing flow rate, although the total detected radioactivity remained constant (Fig. 4). This decrease was thus not caused by increased adsorption of liposomes that remained intact. Neither was it caused by increased retardation of liposomes, since only a very small fraction of the liposomes appeared in the inter-peak region and within the external-tracer peak at all flow rates, as shown by UV-monitoring. In Tris buffer, the phospholipid recovery was high and nearly independent of flow rate (Fig. 5), although the gel at the top of the column gradually turned whiter from run to run, in Tris buffer as well as in buffer D. The flow rate effect was not eliminated by our correction, which underestimates the part of the leakage represented by tracer hidden in the peak of free glucose: the corrected value of trapped tracer at 0.25 ml/min (Fig. 4C) was only half of that at 2 ml/min (Fig. 4A). At low flow rates the tracer leakage hidden within the external-tracer peak is thus high. At 2 ml/min it is much lower, although our corrected amount of trapped tracer is probably slightly too low even at this flow rate. The corrected values are thus lower limits for the true values.

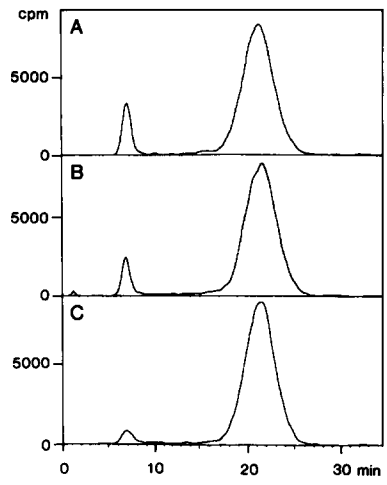

Fig. 4. Flow-scintillation chromatograms from separations on a 1×50 cm Sephadex G-50 M column in stop solution. Estimates of internal volumes for freeze-thawed glucose-transporter liposomes at a flow rate of (A) 2 ml/min; (B) 1 ml/min; and (C) 0.25 ml/min. The corrected internal-volume values were (A) 15.0%, (B) 12.8% and (C) 7.7%. (The corresponding values before correction were 10.2, 7.3 and 3.5%.) The total cpm sum over the whole chromatogram was equal in B and C and 2% higher in A. In (B) and (C) the flow rate was increased to 2 ml/min when the liposomes were about to leave the column and the detection was started. The retention time for the liposome peak was about 7 min in A and the time from the sample application to the increase of the flow rate was 12 min in B and 40 min in C. The liposome peaks are aligned for clarity and the time scale refers to (A).

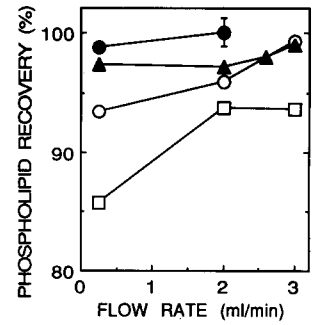

Fig. 5. Phospholipid recovery versus flow rate upon chromatography of liposomes on a 1×50 cm Sephadex G-50 M column. Tris buffer (with 2 mM $HgCl_2$) was used for the liposome preparation and in the Sephadex chromatography to allow phospholipid determinations by phosphorus analyses. The eluate was collected from just ahead of the liposome peak up to 15 ml in excess of the column volume, and the total phosphorus content was determined. The amounts are expressed as percentages of the phosphorus amounts obtained when the liposome samples (0.3 ml) were applied with the column application device and collected before entering the column. ○, protein-free liposomes; □, glucose-transporter liposomes; ▲, integral-membrane-protein liposomes (all freeze-thawed); and ●, small protein-free liposomes (not freeze-thawed). Single determinations, except at 2 ml/min, where duplicate or triplicate determinations were made. The symbols cover the corresponding error bars (±S.E.). At 3 ml/min the gel bed was slightly compressed.

The amount of interpeak tracer (the leakage) increased relative to the amount of tracer in the liposome peak as the liposome sizes and internal volumes decreased and their protein contents increased (Fig. 6). The correction term in percent of the corrected value was 9% for protein-free liposomes, 28% for glucose-transporter liposomes and 55% for integral-membrane-protein liposomes (data from Table I).

Diffusional leakage

The interpeak counts and the correction term depend partly on diffusional leakage. To roughly estimate what fraction of the correction term is due to the diffusional leakage, we determined this type of leakage from free tracer-loaded liposomes with inhibited glucose transporter by delayed filtrations. Stop solution was added (1 ml to 7.5 or 15 μl of liposome suspension) and filtration was made as described above. A 2-min delay of the start of the filtration lowered the retention of radioactivity on the filter by about 5%, and a 7.5-min delay lowered it by about 19% (not illustrated). At a chromatographic elution time for glucose-transporter liposomes of 7.5 min (flow rate 2 ml/min) the (underestimated) correction term was about 30% (experiments illustrated in Figs. 4A and 6B) of the corrected value for trapped radioactivity. Thus, in this case, about two thirds (19/30) of the leakage was diffusional.

For protein-free liposomes the diffusional leakage in 7.5 min was only about 3%, whereas the correction term

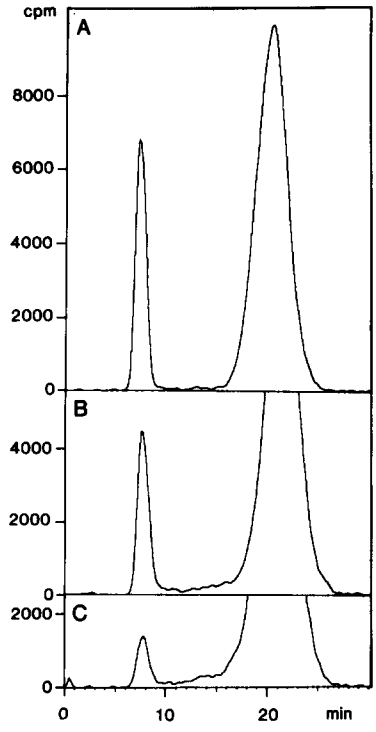

Fig. 6. Flow-scintillation chromatograms from separation on a 1×50 cm Sephadex G-50 M column in stop solution at a flow rate of 2 ml/min. Estimates of internal volumes of (A) protein-free liposomes; (B) glucose-transporter liposomes; and (C) integral-membrane-protein liposomes (all freeze-thawed). The corrected internal volumes were 20.5, 18.4, and 8.7%, respectively. (The corresponding values before correction were 18.7, 13.0 and 3.8%, respectively.) Cf. Table I. The total cpm sums over the whole chromatograms were equal in A and B and 13% higher in C.

was about 9% (data from Table I) of the corrected value.

Values of liposome internal volumes obtained by chromatography

The average corrected internal-volume values obtained by chromatography were 207 μl per ml suspension (20.7%) for protein-free liposomes; 18.7% for glucose-transporter liposomes and 8.7% for integral-membrane-protein liposomes (Table I). The ratios between these values are 1.0 : 0.9 : 0.4, in agreement with the ratios 1.0 : 1.0 : 0.3 among the average volumes for visible liposomes calculated from the micrograph data. We conclude that the volume ratios were similar among the smaller liposomes of the three types.

Comparison of internal-volume values obtained by chromatography and by filtration

The chromatographic method showed higher and obviously more accurate internal-volume values with the 1 × 50-cm column at a flow rate of 2 ml/min than did the filtration method (Table I). (Even without the correction term the values were considerably higher than those obtained by filtration.) The corrected chromatographic internal-volume values were 1.4–3.0, 2.4–4.0 and 3.6–7.9 times the values obtained by filtration for protein-free liposomes, glucose-transporter liposomes and integral-membrane-protein liposomes, respectively. Before correction the corresponding ratios were 1.3–2.7, 1.7–2.9 and 1.6–3.5, respectively (data from Table I). The smaller the liposomes and the more protein included in their bilayers, the larger is the discrepancy between the methods.

Leakage upon filtration

To verify that the discrepancies between the methods is due to excessive liposome leakage upon filtration, glucose-transporter liposomes prepared in buffer D and loaded with tracer upon freezing and thawing were first separated from external tracer by chromatography. (Glucose transport was inhibited). Aliquots of the eluted liposome suspension were then filtered. If a filtration could have been started immediately when the center of the liposome fraction emerged from the column (at time $t = 0$ min, Fig. 7), this filtration would have been finished at $t = 2$ min. If only diffusional leakage of glucose from the liposomes had occurred, about 95% of the tracer would have been retained on the filter at $t = 2$ min and the rest would have been found in the filtrate (see Diffusional leakage, above). However, our extrapolation of the graph in Fig. 7 to $t = 2$ min showed that only 44 ± 5% of the tracer in the liposome fraction was retained on the filter. 56 ± 5% of the tracer had thus

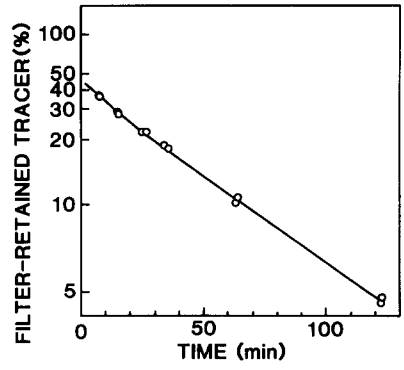

Fig. 7. Retention of the internal tracer in liposomes upon filtration. The amount of tracer on the filter in percent of the sum of the tracer on the filter and of that in the filtrate. Tracer-loaded glucose-transporter liposomes (internal-volume type of experiment, inhibited transport, 0.3-ml sample), were freed from external tracer by chromatography on a 1×50 cm Sephadex G-50 M column in stop solution at 2 ml/min. The whole liposome fraction (5 ml) was collected. An aliquot (140 μl, 0.135 μmol phospholipids) of this fraction was diluted with stop solution to a volume of 1.0 ml and immediately filtered on a Type 1 filter at 2.5 ml/min and with 2×1 ml rinsing. Further aliquots were diluted and filtered in the same way at given times. The time scale shows the time intervals between the elution of the liposome peak center from the Sephadex column and the completion of filtration and rinsing. Two experiments were done. The data are plotted in a loglog/lin diagram for non-ambiguous extrapolation to the time 2 min (44 ± 5%).

been lost due to leakage from the liposomes, to liposome rupture by shear forces, or to passage of liposomes through the filter. The phospholipid retention on the 0.2-μm cellulose-nitrate filter was 94–98% for liposomes prepared in Tris-buffer (Table II, below) and the liposomes prepared in buffer D were larger and probably even better retained. Therefore, the loss of the originally trapped tracer was probably due to release of glucose from the liposomes during filtration. Since the filtration time was about 2 min, only about 1/10 of this leakage (5 out of the 56%) was due to ordinary diffusion through the lipid bilayers. A similar amount (about 5%) of tracer may have passed the filter while entrapped in liposomes. 40–50% of the glucose entrapped in these liposomes was thus released on the filter by effects other than ordinary diffusion and passage of liposomes.

'Sidedness' of membrane filters

For Type 1 and Type 2 filters, delivered in sheets, the filter side that faced the protective sheet was placed upwards during filtration, except in some of the experiments described in this section. For Type 3 filters, the side facing downward in the manufacturers package of circular filters was normally used upwards, except in some cases here. To test the influence on the filtration of different orientation of the filters, we filtered glucose-transporter liposomes (diluted 7.5-μl aliquots) in stop solution at 2.5 ml/min. For Type 1 filters the normal orientation gave a 21% higher retention of tracer than did the reverse orientation. The opposite was true for Type 2 and Type 3 filters (−19% and −13%, respectively). The internal volume values for glucose-transporter liposomes found with Type 2 and Type 3 filters would thus have been somewhat higher if the filters had been used with the reverse orientation (cf. Table I).

Phospholipid retention on membrane filters

Phospholipid retention on 0.2-μm cellulose-nitrate (Type 1) and cellulose-acetate (Type 3) filters was determined by phosphorus analyses after filtrations (in Tris buffer with 2 mM $HgCl_2$) of glucose-transporter liposomes prepared in Tris buffer. Rinsing was done with 2 × 1 ml. In parallel, internal-volume experiments were performed with glucose-transporter liposomes: The liposomes prepared in Tris buffer had the internal volume 3.7% as determined by chromatography, whereas the value for the liposomes prepared in buffer D was 18.7% (Table I). The difference is unexpectedly large. Another two cycles of freezing and thawing increased the internal volume of the Tris-buffer liposomes to 8.0% as determined by chromatography, whereas filtration gave a value of only 3.2% (average of all four values in Table II). The phosphorus retention on the filters with these three times freeze-thawed liposomes was 60–98% (Table II), (average 82 ± 14%) which could correspond to an internal-volume value of (0.82 ± 0.14) × 8.0% ≈ (6.6 ± 1.1)%. However, the experimental filtration value was 3.2%. This indicates a leakage upon filtration of 100 × [1–3.2/(6.6 ± 1.1)] ≈ (50 ± 8)%, which is consistent with data from Table I and Fig. 7.

Type 3 filters retain less phospholipid than do Type 1 filters (Table II), probably since (small) liposomes bind more weakly to cellulose-acetate than to cellulose-nitrate. (Large liposomes are sterically retained on both filter types.) Small liposomes represent much less of the total internal volume than of the total phospholipid content of the liposome sample and the retention values differ more than the internal-volume values. The values in Table II thus indicate that Type 3 filters let through a larger proportion of small liposomes than did Type 1 filters.

Glucose transport activity

Glucose-transport assays were performed at room temperature (21–24°C) with glucose-transporter liposomes as described in Methods. Isolation of liposomes was done either by filtration (Fig. 8A) or by chromatography (Fig. 8B,C). A single batch of liposomes, kept at room temperature, was used. The assays were started with the shortest, 10-s, incubations. The filtration assays were done within 4 h, and were started at the same time as the chromatographic assays, which were finished in 12 h. The liposome preparation was stable, since the stereospecific D-glucose uptake in 10 s was 2.60% of the added tracer at 0 h and nearly the same, 2.50%, after 6 h (chromatographic determinations). The slope of the corrected graph in Fig. 8C in the interval 0–10 s corresponds to a stereospecific glucose equilibrium exchange at 50 mM D-glucose of 2.0 ± 0.2 nmol D-glucose per μg protein per s. The non-corrected value is 1.4 ± 0.1 nmol $\cdot \mu g^{-1} \cdot s^{-1}$ (Fig. 8B), whereas filtration showed only 0.6 ± 0.1 nmol $\cdot \mu g^{-1} \cdot s^{-1}$ (Fig. 8A). These values are calculated by use of the total protein concentration

TABLE II

Retention of glucose-transporter-liposome phospholipids on filters and corresponding values of internal liposome volumes

Method	Flow rate (ml/min)	Phospholipid retention [a] (%)	Internal-volume values [b] (percent of suspension volume)
Filtration (with Type 1 filters)	0.5	98.2 ± 0.2	3.6
	5.0	94.0	3.2
Filtration (with Type 3 filters)	0.5	74.6 ± 2.8	2.8
	5.0	59.7	3.0
Chromatography	2.0	–	8.0 ± 0.5 [c]

[a] One or two determinations with 7.5-μl aliquots of liposome suspension. 2 × 1 ml rinsing.
[b] Single determinations with 7.5-μl aliquots. 2 × 1 ml rinsing.
[c] Average of two determinations with correction (cf. Fig. 3).

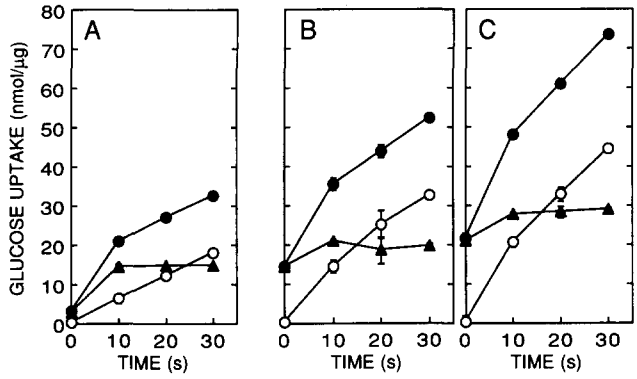

Fig. 8. Glucose uptake into liposomes with purified human red cell glucose transporter, per protein amount, versus time for incubation with [^{14}C]glucose at 50 mM glucose under equilibrium exchange conditions at room temperature and pH 7.2. ●, D-glucose uptake; ▲, L-glucose uptake; ○, stereospecific (D−L) uptake. Isolation of internalized tracer was accomplished by (A) membrane filtration on 0.2-μm nitrocellulose filters (Type 1) or by (B,C) chromatography on Sephadex G-50 M. The uptake values in panel B are not corrected, whereas those in panel C are corrected. Assays were done at least in duplicate. Error limits are ±S.E. for D- and L-uptake. For the stereospecific uptake the error limits are the square roots of the sums of the squared S.E. values for the D- and L-averages. Most error bars are covered by the diagram symbols. The D−L values at times 0 were about 0.4 nmol/μg. The protein concentration in the liposome suspension was 40±2 μg/ml. The glucose-transporter preparation contains about 4% nucleoside transporter according to data in Ref. 35.

(about 95% glucose transporter) in the liposome suspension.

As expected, the loss of [^{14}C]glucose from glucose-transporter liposomes on filtration is about the same fraction (two thirds) in the equilibrium exchange experiments (Fig. 8) as in the internal-volume determinations (Table I).

Discussion

Chromatography

The occurrence of tracer between the liposome peak and the external-tracer peak in the flow-scintillation chromatograms is due to ordinary diffusion of tracer from the liposomes through the lipid bilayer; to 'induced' leakage caused by liposome interaction with the gel beads; to retardation and adsorption of liposomes; and for proteoliposomes to a slow protein-mediated out-transport of D-glucose, even though inhibitor was present. In all of these cases the radioactive glucose between the liposome peak and the peak of free tracer was originally present in the liposomes. A correction term was therefore added to the liposomal counts (see Fig. 3 and Results). The combined effects of diffusional leakage, liposome retardation, adsorption of liposomes which remain intact and residual transport activity probably cannot explain all of the loss of tracer in the liposome peak. We propose that part of the loss is due to interactions between the liposomes and the gel bead surfaces, which affect the phospholipid bilayer and thereby cause a 'matrix-induced' leakage, or which allow shear forces from the liquid flow to disrupt the liposomes. Such interactions might also occur upon exchange of already adsorbed liposomes or phospholipids by 'incoming' liposomes, which thereby become leaky.

The leakage upon chromatography increased with decreased flow rate which increases both the diffusional leakage and the time of interaction between the liposomes and the gel beads. Furthermore, a decrease in flow rate increases the thickness of the unstirred liquid layer around the beads and decreases the hydrodynamic lifting force (the inertial force [27]). This force is small for liposomes smaller than 1 μm (cf. Refs. 27,28). For larger liposomes and at laminar flow the hydrodynamic lifting force probably removes the liposomes from the gel bead surfaces, in the same way as blood cells are removed from walls in capillaries [29]. In addition, the smaller the liposomes, the larger is their contact area with the gel bead surfaces, per phospholipid amount.

On chromatography at flow rates below 0.25 ml/min, the liposomes separated into two or three overlapping fractions and some minor ones (not illustrated). Probably small liposomes in a sample thus interact more strongly with the gel bead surfaces than do large ones.

The lowest leakage was achieved at high chromatographic flow rates (2 ml/min). At even higher flow rates the gel bed became compressed. In a 1-cm (i.d.) column the flow is probably predominantly laminar at 2 ml/min (2.5 cm/min). Novel effects may occur when secondary or turbulent flow sets in. Also in the case of 'centrifugal gel filtration' [19, 30–32] the high linear flow rate, about 2 cm/min or more [30], probably gives low matrix-induced leakage, although this may be difficult to determine.

When a 1 × 12 cm column was used for the separation of liposomes and free tracer the internal-volume values increased with increasing flow rate. The peaks overlapped with 0.1-ml samples already at a flow rate of 1 ml/min and interpeak counts could not be observed. At 2 ml/min the values were (perhaps fortuitously) about equal to the corrected values obtained with the 50-cm column. For quick measurements the short column can be used at the expense of accuracy, and fractions can be collected according to UV-monitoring as described in Ref. 14.

Filtration

A large matrix-induced leakage was probably caused by the filters when liposomes were filtered. This is analogous to the disruption of liposomes adsorbed to glass beads described by Jackson et al. [33]. Rupturing of liposomes on the filter by shear forces probably also contributes to the low retention of liposome-trapped tracer.

Cellulose-nitrate and cellulose-acetate filters gave about the same leakage. Filters made of a mixture of these materials (MF-Millipore filters) would presumably behave similarly. However, the structure of the liposome adsorption site on the gel beads or filters, etc., is sometimes critical for the degree of leakage: In recent experiments with immobilization of small liposomes in gel beads with hydrophobic ligands, the ligand density strongly influenced the degree of calcein leakage from the liposomes upon the immobilization [17].

Glucose transport activity

The value 2.0 nmol $\cdot \mu g^{-1} \cdot s^{-1}$ (Fig. 8C) obtained for the specific D-glucose transport activity is slightly higher than the value of 200 nmol per μg per 2 min (1.7 nmol $\cdot \mu g^{-1} \cdot s^{-1}$) that has recently been reported [14]. The latter value was obtained by chromatographic isolation of liposomes on a short column after 2-min incubations of glucose-transporter liposomes with very low concentrations of transporter, in order to get a low uptake of tracer and thus a more linear time-dependence than for higher concentrations of transporter. Our results indicate that a low protein content also leads to larger internal volume upon freeze-thawing than does a high protein content. A protein effect on the size of freeze-thawed membrane vesicles has been hypothesized earlier [34]. The glucose exchange 2.0 nmol $\cdot \mu g^{-1} \cdot s^{-1}$ corresponds to 40–65% of the exchange in human red cells, as estimated in the way described in Ref.14. A substantial fraction of the reconstituted glucose transporters probably reside in the lipid bilayers of small liposomes which take up only a small fraction of the transported tracer, since their internal volumes are small. Therefore the above relatively high percentage values indicate that the degree of denaturation of the transporter is low. Furthermore, after similar reconstitutions with glucose transporter, it was earlier reported, on the basis of centrifugation experiments, that only about 40% of the protein was bilayer-incorporated [2]. Our own experiments of this type did not give clear results, possibly since the smallest liposomes migrated too slowly in the centrifugal field.

Earlier equilibrium-exchange determinations by use of a filtration method gave a value of 0.5 nmol $\cdot \mu g^{-1} \cdot s^{-1}$ for human red cell glucose transporter reconstituted in freeze-thawed, sonicated soybean L-α-phosphatidylcholine liposomes, at 50 mM glucose and 23°C (value calculated from data out of Fig. 2C in Ref. 2), which is similar to our filtration value of 0.6 nmol $\cdot \mu g^{-1} \cdot s^{-1}$.

The high (and equal) levels of both D- and L-glucose liposome radioactivity at the incubation time zero in the chromatographic separations (Fig. 8B and C, cf. Ref.13) as compared to the values in the filtrations (Fig. 8A) are probably caused by glucose uptake at the top of the column upon liposome breakage and resealing or upon matrix-induced leakage, especially as the concentration of tracer after addition of stop solution was much higher in the chromatographic experiments (220 Bq/μl) than in the filtration experiments (3.7 Bq/μl). The L-glucose values are nearly constant from 10 s (Fig. 8), which reflects that the diffusional glucose leakage into the liposomes is slow, consistent with the data above. The linearity of our stereospecific uptake curve in Fig. 8A may be due to selective loss of small liposomes or of tracer from small liposomes during filtration. In these liposomes, equilibrium tracer concentration is reached or nearly reached during the incubation.

General

Good results were obtained by chromatography on a 50-cm column. A flow-cell scintillation detector greatly facilitated the evaluation of the separation. The filtration procedure is quicker and cheaper, but gives too low values. For reproducible results the filter holder must be carefully designed and properly used.

Future studies of filtration techniques and chromatographic procedures with liposomes of other lipid compositions and prepared by other reconstitution methods are needed to find out how general the effects are that we have observed. It also remains to study whether tracers other than glucose reveal similar leakage effects.

Acknowledgements

We are grateful to Eva Greijer for assistance and for preparation of lipids and integral membrane proteins. We thank David Eaker for linguistic revision. The study was supported by grants from the Swedish Natural Science Research Council, the O.E. and Edla Johansson Science Foundation, the Sven and Lilly Lawski Science Foundation and the Swedish National Board of Technical Development.

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