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# THE GLUCOSE TRANSPORT ACTIVITY OF HUMAN ERYTHROCYTE MEMBRANES

## RECONSTITUTION IN PHOSPHOLIPID LIPOSOMES AND FRACTIONATION BY MOLECULAR SIEVE AND ION EXCHANGE CHROMATOGRAPHY

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## Summary

Human erythrocyte membranes, at a protein concentration of 1—2 g/l, were solubilized with 0.12 M cholate in the presence of 0.06 M phospholipid (egg yolk phospholipids or phosphatidylcholine). More than 40% of the protein was solubilized. Cholate was removed by molecular sieve chromatography, whereby liposomes formed. These liposomes exchanged D-glucose faster than L-glucose. The recovery of glucose transport activity in the reconstituted system was estimated to be higher than 16%.

The liposomes were heterogeneous in size, as shown by molecular sieve chromatography on Sepharose 4B, and small liposomes predominated. In liposomes formed with phosphatidylcholine, the distribution of glucose transport activity did not parallel the distribution of protein or phospholipid, and the activity was found mainly in the smallest liposomes. The proteins were incorporated mainly into the liposomes that eluted at the lowest ionic strength upon ion exchange chromatography.

The glucose transport activity separated into three main peaks upon ion exchange chromatography of egg yolk phospholipid liposomes. The activity eluted at low ionic strength. The liposomes contained proteins mainly from the 3- and 4.5-regions (nomenclature according to Steck, T.L. (1974) J. Cell Biol. 62, 1—19). The activity peaks were highest in the first part of the chromatogram. The protein distribution did not coincide with the variation in activity over each peak. Therefore, it cannot be excluded that a minor component not

seen in the electrophoretic analyses might be responsible for the glucose transport activity.

## Introduction

Several attempts have been made to identify and isolate the glucose transport protein of human erythrocytes. One approach has been to study the glucose transport of membrane vesicles after release of peripheral proteins [1,2]. Labeling techniques have also been used [3—7]. Another approach has been to solubilize the membrane proteins with detergents, and to reconstitute the glucose transport system [8—12]. Fractionation has been performed either before [8—11] or after reconstitution [12]. Some of the results [1,4,10,11] suggest that a band 3 protein (nomenclature according to Steck [13]) is responsible for the transport of D-glucose, while other results indicate that one or more proteins of the 4.5-region are involved in the transport process [5—9,12]. It has also been suggested that the proteins of the 4.5-region are proteolytic degradation products of a band 3 protein, and that at least one of these products is capable of transport [11].

We have reconstituted the glucose transport system with an excess of phospholipids such that only a fraction of the liposomes contained protein, and such that the probability was low for the incorporation of more than one protein molecule or protein complex per liposome. The glucose transport protein might therefore be purified after the reconstitution by isolation of active liposomes (cf. Ref. 12). Furthermore, it seemed reasonable that the transport protein should be more stable in the lipid bilayer than in detergent solution. In the present work we describe the fractionation of the liposomes by molecular sieve and ion exchange chromatography. These procedures gave information on the incorporation of proteins into liposomes, and active liposomes were enriched.

## Materials and Methods

*Membranes.* Human blood (fresh or recently outdated) was obtained from the blood bank of the University Hospital, Uppsala. The erythrocytes were washed three times by centrifugation in isotonic phosphate buffer, pH 7.4. The washed cells were lysed by the addition of 2 vols. of water, and the membranes were isolated by molecular sieve chromatography on Sepharose 4B in 5 mM phosphate buffer, pH 7.4 [14]. In some cases membranes were prepared according to the method of Dodge et al. [15]. Sodium azide was added to a concentration of 3 mM.

*Cholic acid.* Cholic acid, 100 g (puriss., Fluka AG, Buchs SG, Switzerland), was solubilized in 0.8 l of water by the addition of 12 M NaOH, filtered, mixed with 3.2 l of acetone, and precipitated with 4 l of 3 M HCl.

*Phospholipids.* Egg yolk phospholipids containing about 70% of phosphatidylcholine and 20% of phosphatidylethanolamine were prepared from fresh egg yolks [16]. Phosphatidylcholine was purified from this material on basic aluminium oxide [17]. The egg yolk phospholipid or the phosphatidylcholine

was dissolved in chloroform, dried at low pressure in a rotatory evaporator at 40°C for 2 h, and stored at −20°C.

*Radioactive glucose and chromatographic materials.* D-[U-$^{14}$C]Glucose and L-[1-$^{14}$C]glucose, both about 230 Ci/mol, were from Amersham, U.K. Sephadex G-50 medium, Sepharose 4B and prepacked 1.5 × 5 cm Sephadex G-25 columns were from Pharmacia, Sweden. DEAE-cellulose, type DE-52, was from Whatman, U.K. Basic aluminium oxide, Woelm, activity grade I, was from ICN Pharmaceuticals, F.R.G.

*Solubilization of membrane proteins.* A cholate/phospholipid solution was first prepared. Reprecipitated cholic acid and egg yolk phospholipids (or phosphatidylcholine) were mixed with water to concentrations of 0.25 M and about 0.13 M (100 g/l), respectively. For solubilization of the cholic acid, NaOH was added to a pH of 8.2. The phospholipids were dissolved by stirring for 6—15 h at room temperature. D-Glucose was added to a concentration of 11 mM, NaCl to 308 mM, $MgCl_2$ to 0.40 mM, $CaCl_2$ to 0.28 mM and 2-mercaptoethanol to 14 mM, i.e., twice the concentrations of solution A (below). Finally the pH was adjusted to 8.2.

A membrane suspension with a protein concentration of 2—4 g/l was mixed 1 : 1 with the above cholate/phospholipid solution, the pH was readjusted to 8.2, and this mixture was vigorously stirred for 10—19 h at 2°C. The non-solubilized material was pelleted by centrifugation at 130 000 × *g* for 1 h at 2°C.

*Reconstitution of the D-glucose transport system.* Liposomes were prepared by molecular sieve chromatography either in a 9-ml or in a 450-ml column, at room temperature. The 9-ml column, 1.5 × 5 cm, contained Sephadex G-25 equilibrated with 5.5 mM D-glucose, 154 mM NaCl, 0.20 mM $MgCl_2$, 0.14 mM $CaCl_2$ and 7 mM 2-mercaptoethanol (solution A), pH 6.7. A sample of 200 μl of the solubilized membrane protein was applied to the column and eluted with solution A. The liposomes were collected in an opalescent fraction of 1.3 ml with a phospholipid concentration of 7—8 g/l. The 450-ml column, 5 × 23 cm, contained Sephadex G-50 equilibrated with solution A. A 20-ml sample of the solubilized membrane protein was applied at a flow rate of 0.7 l/h. Liposomes were collected according to ultraviolet-monitor recording. Cholate, with a micellar molecular weight of about 2000 [18], eluted well separated from the liposomes (cf. Ref. 19).

*Molecular sieve chromatography.* Liposomes prepared in a Sephadex G-50 column as described above were applied at a flow rate of 120 ml/h to a 5 × 53 cm Sepharose 4B column (void volume 330 ml, total volume 1000 ml) at 22°C. The column was equilibrated with 2.0 mM Tris-HCl (pH 7.4), 100 mM glycerol, 5.5 mM D-glucose, 0.20 mM $MgCl_2$, 0.14 mM $CaCl_2$ and 3 mM 2-mercaptoethanol (solution C, ionic strength 2 mM). This low ionic strength was suitable for ion exchange experiments. In one case (Fig. 4a) solution B (below) was used.

*Ion exchange chromatography.* The liposomes were freed of NaCl either during the Sepharose 4B chromatography in solution C (above), or on Sephadex G-50 (5 × 23 cm column). In the latter case the eluant was solution B (ionic strength 5 mM), which contains 10 mM Tris-HCl, 7 mM 2-mercaptoethanol and the other components as in solution C. After removal of the NaCl

the liposomes were applied to a DEAE-cellulose column equilibrated with solution B or C at 6°C, and non-adsorbed material was eluted with 200 ml of the starting buffer at a flow rate of 25 ml/h. The adsorbed liposomes were eluted by a linear gradient of NaCl. The increase in NaCl concentration per l of eluant was chosen between 0.1 and 1.5 M/l. The NaCl concentration in the eluate was determined by measurements of electric conductivity. A new DEAE-cellulose column was used for each experiment.

*Stereospecific D-glucose transport activity.* A sample of liposomes (1.0—1.3 ml) was frozen (1 h or more at −20°C), slowly thawed (0.5 h at room temperature) and homogenized with a Vortex mixer (3 s). After 2 min, 5 μl (0.25 μCi) of $^{14}$C-labelled D- or L-glucose were mixed with 200 μl of the liposome suspension and the mixture was incubated for 120 s at room temperature (22°C).

For some dilute samples (Fig. 4 and Fig. 5a, upper curve) egg yolk phospholipid liposomes in solution A were added before freezing (0.25 ml liposomes to 0.75 ml sample) to obtain a lipid concentration greater than 5 g/l. A phospholipid concentration of 7—8 g/l gave an inner volume greater than 10% of the total volume.

The incubation mixture contained 5.4 mM D-glucose, 0.20 mM $MgCl_2$, 0.14 mM $CaCl_2$ and 7 mM 2-mercaptoethanol, and, in addition, either 150 mM NaCl, pH 6.7 (Table I and Fig. 3) or 0—70 mM NaCl, 2—10 mM Tris-HCl, pH 7.4, and 75—100 mM glycerol (Figs. 1, 4 and 5).

The incubation was ended by adding 200 μl of ice cold solution A, pH 6.7, including 0.1 mM phloretin and 1% ethanol. Sephadex G-25 columns (1.5 × 5 cm) were equilibrated with the same solution, at 8°C. The samples were kept at 2°C for 2 min before application to the columns. Two pairs of samples were analyzed in parallel. Fractions of 0.45 ml were collected. Finally, the free radioactive glucose was collected in a 5.9 ml fraction out of which 0.45 ml was taken. Scintillation fluid was added, and the radioactivity was measured.

The stereospecific D-glucose transport activity was expressed as D-[$^{14}$C]glucose uptake minus L-[$^{14}$C]glucose uptake, in percent of total radioactivity. This difference is denoted D-L. The L-glucose uptake represents the sum of nonspecific uptake at time zero (Fig. 3) and leakage, and varied for instance with the protein concentration. The standard error for a given batch of liposomes was found to be approx. 0.02 × (D-L) + 0.03, i.e., D-L values with standard errors were, for instance, 0.00 ± 0.03, 1.00 ± 0.05, or 5.00 ± 0.13, in percent of the total radioactivity. The factor 0.02 derives mainly from the imprecision in the time of incubation, and the term 0.03 is the error at zero activity.

*Sodium dodecyl sulphate acrylamide gel electrophoresis.* Discontinuous electrophoresis in 0.3 × 10 × 10 cm gel slabs was performed according to the method of Neville [20], except that the membranes and the liposome samples (about 10 μg of protein and about 500 μg of lipids) were solubilized with an equal volume of a solution containing 0.14 M sodium dodecyl sulphate, 0.6 M sucrose, 0.010 M EDTA, 1.4 M 2-mercaptoethanol, 0.082 M Tris and 0.080 M boric acid, pH 8.64.

The gels were stained for protein with Coomassie brilliant blue R-250 and for carbohydrate with periodic acid-Schiff's reagent, in both cases essentially according to the method of Fairbanks et al. [21].

*Free zone electrophoresis.* This was performed according to the method of Hjertén [22] in a 3 × 300 mm quartz tube which rotated at 40 rev./min to stabilize against convection.

*Chemical analyses.* Sialic acid was determined according to the method of Warren [23]. Phosphate was determined with the Fiske-Subbarow reagent as described by Bartlett [24]. Membrane protein was determined by absorbance measurements after sodium dodecyl sulphate solubilization [25]. For dilute liposome samples, amino acids were determined after alkaline hydrolysis (2.5 M NaOH, 3 h, 110°C) and reaction with ninhydrin. The absorbance was measured at 570 nm. A molar extinction coefficient of 18 900 $M^{-1}$ was used for the calculation of the amino acid concentration. All pH values were measured at 25°C.

## Results

*Solubilization.* The cholate/egg yolk phospholipid solution (see Materials and Methods) solubilized from 40% of the erythrocyte membrane protein in 10 h, up to 65% in 18 h. The selectivity was low. The sialoglycoproteins were partially solubilized (Fig. 7). The concentration of egg yolk phospholipids used in the solubilization step affected the D-glucose transport activity, but not the glucose leakage (Table I). A concentration of egg yolk phospholipids of 50 g/l gave a high activity and was chosen for the subsequent experiments. The same concentration was chosen for experiments with phosphatidylcholine. Cholate/phosphatidylcholine solubilized about 90% of the membrane protein in 16 h, with some selectivity: spectrin and band 5 were solubilized to a relatively low degree. The stereospecific D-glucose transport activity was 2- to 3-fold higher after solubilization and reconstitution with egg yolk phospholipids rather than with purified phosphatidylcholine, using the same amount of membranes, in spite of the fact that the degree of solubilization was lower in the first case (data not shown).

*Reconstitution.* The glucose transport system was reconstituted into liposomes by chromatographic removal of cholate from the cholate/phospholipid-solubilized membrane components. The stereospecific D-glucose transport

TABLE I

EFFECT OF PHOSPHOLIPID CONCENTRATION IN THE SOLUBILIZATION OF THE STEREOSPECIFIC D-GLUCOSE TRANSPORT SYSTEM OF HUMAN ERYTHROCYTE MEMBRANES

Egg yolk phospholipids at the concentrations given in the table were solubilized in 0.12 M cholate containing other components as indicated in Materials and Methods, and erythrocyte membranes were added to a protein concentration of 2.3 g/l. This membrane suspension was stirred for 19 h at 2°C in one experiment, and for 2 h at 22°C in another experiment. After centrifugation, additional egg yolk phospholipids were solubilized in the supernatant (2 h, 22°C) to a total concentration of 50 g/l. Liposomes were formed upon removal of cholate by molecular sieve chromatography on a 9-ml Sephadex G-25 column, at pH 8.2. After freeze-thawing, the stereospecific D-glucose transport activity was determined (see Materials and Methods). The tabulated values represent the means from two experiments with different membrane and phospholipid preparations. The standard error was about 0.2 for the stereospecific D-glucose transport and about 0.4 for the L-glucose uptake.

| Phospholipid concentration (g/l) | 0 | 20 | 50 |
|---|---|---|---|
| L-Glucose uptake (%) | 1.8 | 1.7 | 1.3 |
| Stereospecific D-glucose transport (%) | 0.1 | 1.1 | 1.3 |

activity was found mainly in one peak (Fig. 1, fraction I). The liposomes of fraction I were heterogeneous in size. For the case of phosphatidylcholine liposomes, this is illustrated in Fig. 2b. Fraction I (Fig. 2a) contained spectrin, band 3, band 4.2, protein from the 4.5-region, and sialoglycoproteins (Fig. 7, samples 2 and 4). The material in fraction II showed an additional peak in Sepharose 4B chromatography (Fig. 2c), and was not used.

*Time course of the uptake of D-glucose.* Liposomes were prepared from egg yolk phospholipids in the presence of membrane components. Samples of the liposomes were incubated for a series of times with D- and L-glucose. The stereospecific uptake of D-glucose was rapid for about 2 min, whereas the leakage was slow (about 0.1% per min), as illustrated in Fig. 3. The curve for the stereospecific D-glucose transport starts from zero at time zero, since the value for this transport is the difference between the uptake of D-glucose and of L-glucose. On the other hand, the L-glucose uptake, extrapolated to time zero, shows a blank value. Another experiment (time interval 0.3—8 min) gave essentially the same result. An incubation time of 120 s was used in all subsequent experiments. The stereospecific uptake of D-glucose in 120 s was proportional to the concentration of solubilized protein up to 1 g/l (data not shown).

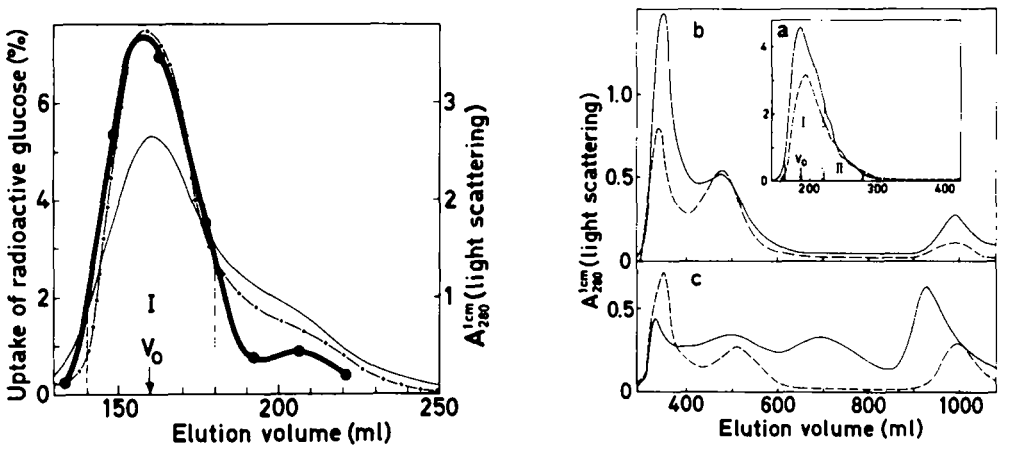


Fig. 1. Preparation of liposomes from egg yolk phospholipids and erythrocyte membranes in cholate, by molecular sieve chromatography. A 20-ml sample of solubilized membranes containing 1.0 g of phospholipids, 8 mg of erythrocyte membrane protein and 1.0 g of cholate was applied to a 450-ml Sephadex G-50 column and eluted with solution A (see Materials and Methods) at pH 8.2. When the liposomes started to elute, the eluant was continuously applied to another 450-ml Sephadex G-50 column for the purpose of lowering the ionic strength. This column was equilibrated with solution B. •———• (thin line) and ———, apparent absorbance at 280 nm (mainly due to light scattering) for the first and second chromatography, respectively; •———• (thick line), stereospecific D-glucose transport activity, measured after freeze-thawing of samples from the second chromatography. Fraction I was analyzed by ion exchange chromatography (Fig. 5a, upper curves, and Fig. 5b).

Fig. 2. Fractionation of liposomes by molecular sieve chromatography on Sepharose 4B. Liposomes were prepared from phosphatidylcholine with and without erythrocyte membranes on a 450-ml Sephadex G-50 column, as described in Materials and Methods, at pH 6.7 (a). Samples of 40 ml of fraction I and II were applied to 1 1 Sepharose 4B columns and eluted with solution C (b and c, respectively). ———, liposomes with protein; - - - - - -, liposomes without protein. The glucose transport activity in the experiment illustrated in b (full line) is shown in Fig. 4b.

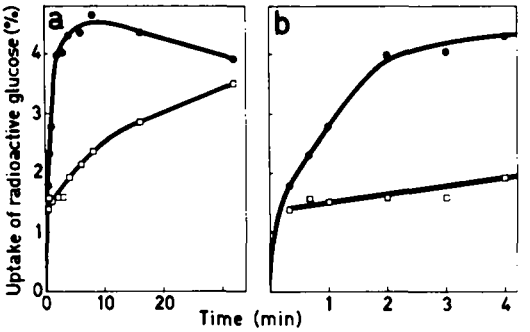


Fig. 3. Time course of the uptake of radioactive glucose into liposomes. Liposomes were prepared from egg yolk phospholipids and erythrocyte membranes (1.1 g of solubilized protein per l) on a 9-ml Sephadex G-25 column. The liposomes were frozen and thawed and incubated with D-[$^{14}$C]- and L-[$^{14}$C]glucose for various times (see Materials and Methods). The values for glucose uptake in the time interval 0—4 min in a are shown on an expanded time scale in b. ●———●, stereospecific D-glucose transport activity; □———□, L-[$^{14}$C]glucose uptake.

*Effect of pH on the transport activity.* The stereospecific D-glucose transport in liposomes was measured in solution A at various pH values. It showed maxima at pH 6.6 and at pH 7.3 when the incubation mixture was buffered with 5 mM citrate and 5 mM phosphate, respectively. With 5 mM Tris-HCl the activity was nearly constant between pH 6.5 and 8.5.

*Fractionation by molecular sieve chromatography*

*Egg yolk phospholipids.* Liposomes prepared from egg yolk phospholipids and erythrocyte membrane components had a size distribution similar to that shown in Fig. 2b. The stereospecific D-glucose transport activity appeared as a minor peak at the void volume, and a retarded major peak that seemed to coincide with the small liposomes (not illustrated).

*Phosphatidylcholine.* When the liposomes were prepared from purified phosphatidylcholine at pH 8.2 or 6.7 (Fig. 4a and b, respectively), about half of the transport activity eluted in a peak corresponding to the smallest liposomes (elution volume above 500 ml, $K_{av} > 0.25$). In the experiment illustrated in Fig. 4b, the fraction 500—650 ml contained 60% of the glucose transport activity, and 30% of the protein, as estimated with ninhydrin after alkaline hydrolysis. The elution pattern of sialoglycoproteins showed a peak at 490 ml (not illustrated).

*Fractionation by ion exchange chromatography*

*Egg yolk phospholipids.* Liposomes prepared from egg yolk phospholipids and erythrocyte membrane components were fractionated according to their charge by ion exchange chromatography on DEAE-cellulose. Liposomes were adsorbed in buffer B (ionic strength 5 mM) and eluted with a shallow gradient of NaCl (0.1—0.2 M/l), which resulted in at least three peaks of stereospecific D-glucose transport activity, between 3 and 40 mM NaCl (Fig. 5a). This activity corresponds to at least 70% of the total activity, as judged from other experiments (not shown). The absorbance curve showed several small peaks in this interval (Fig. 5a and b). In a control experiment with protein-free liposomes,

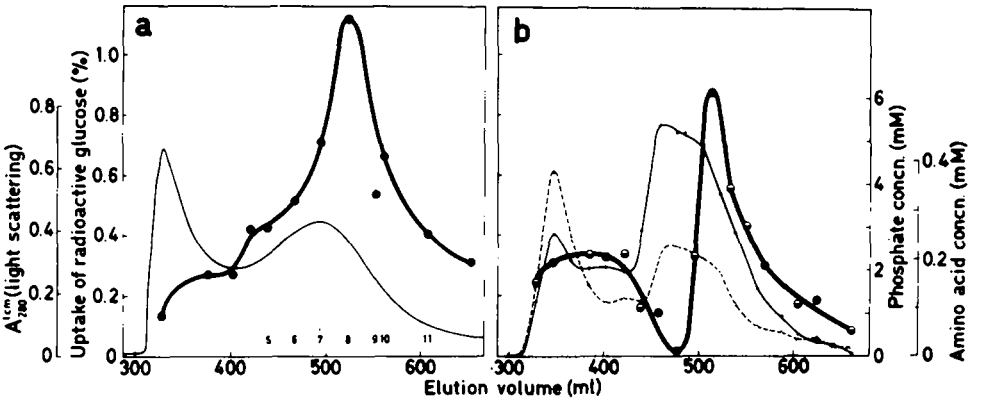


Fig. 4. Stereospecific D-glucose transport activity in liposomes fractionated by molecular sieve chromatography on Sepharose 4B. Liposomes were prepared on a 450-ml Sephadex G-50 column from phosphatidylcholine and erythrocyte membranes at pH 8.2 (a) and at pH 6.7 (b). The concentration of solubilized protein was 0.4 and 0.6 g/l, respectively. A 40-ml sample of the liposomes corresponding to fraction I (cf. Fig. 2a) was fractionated on a 1 1 Sepharose 4B column in solution B (a) and C (b). Protein-free liposomes were added before activity measurements (see Materials and Methods). ———, apparent absorbance; ●———● (thin line), phosphatidylcholine, determined as phosphate; ○- - - - -○, amino acids; ●———● (thick line), stereospecific D-glucose transport activity. ⊖, measurements performed after 3 days; values corrected by a factor of 1.2 for decrease in activity. The numbers 5—11 in a indicate the samples that were analyzed by gel electrophoresis (Fig. 7).

the absorbance curve showed only one or two small peaks in the same interval (Fig. 5c). The liposomes eluted between 0 and 150 mM NaCl without protein, and between 3 and 150 mM NaCl with protein. The amount of non-adsorbed material was less in the case of liposomes with protein (Fig. 5b and c).

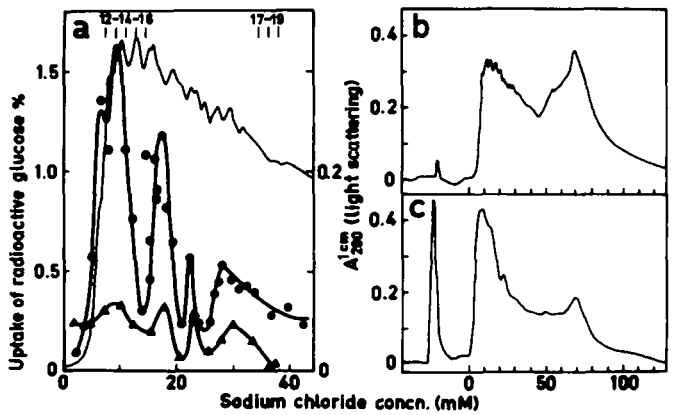


Fig. 5. Ion exchange chromatography of liposomes. Liposomes were prepared from egg yolk phospholipids and erythrocyte membranes and were desalted on a Sephadex G-50 column (see Materials and Methods). The concentration of solubilized protein was 0.4 g/l. A sample of 80 ml of the liposomes was applied on a 1.9 × 52 cm DEAE-cellulose column in solution B (ionic strength 5 mM) and eluted by a 0.22 M/l gradient of NaCl (experiment 1) and, for another batch of liposomes, by a 0.11 M/l gradient (experiment 2). Protein-free liposomes were added before activity measurements in experiment 1 (see Materials and Methods). ———, apparent absorbance (experiment 1, a—c); ●———● and ▲———▲, stereospecific D-glucose transport activity (experiments 1 and 2, respectively). b and c show the elution pattern in experiment 1 for liposomes prepared with and without protein, respectively. The numbers 12—19 in a indicate the samples from experiment 1 that were analyzed by gel electrophoresis (Fig. 7).

Steep gradients (1—1.5 M/l) did not resolve the transport activity into several peaks, but gave rise to one double peak (not shown). Sialoglycoproteins were more retarded than the activity.

*Phosphatidylcholine.* In order to prepare small liposomes of the same charge and size, liposomes were produced from purified phosphatidylcholine and fractionated on Sepharose 4B. The surface charge of most of these liposomes was low, as indicated by the fact that in solution B (ionic strength 5 mM) they did not adsorb to DEAE-cellulose. The liposomes that were adsorbed eluted in several peaks upon applying a gradient in salt concentration (not shown).

To increase the retention, the ionic strength of the starting buffer and of the liposomes was lowered to 2 mM (solution C). Liposomes containing stereospecific D-glucose transport activity eluted at the very beginning of the salt gradient, as did most of the protein (Table II). Apparently, the proteins had been incorporated mostly into the liposomes having the lowest surface charge. The elution patterns for phosphatidylcholine liposomes with and without protein were similar.

The small phosphatidylcholine liposomes were not more homogeneous than egg yolk phospholipid liposomes, as judged from ion exchange chromatography. In both cases the lipids seem to contribute more to the surface charge of the liposomes than do the incorporated proteins.

*Heterogeneity of liposomes.* Fractions from ion exchange chromatography of egg yolk phospholipid liposomes without protein, were rechromatographed to test for heterogeneity (Fig. 6). This experiment showed that the liposomes in fraction I′ differed in surface charge from those in fraction II′. The rechromatographed material eluted at a higher salt concentration than in the original chromatography. This is probably due to a displacement effect in the original chromatography where much more material was applied to the column. In the case of liposomes containing proteins, the activity eluted mainly in the region of fraction I′ plus II′.

Rechromatography of four fractions from the interval 70 to 200 mM NaCl gave rise, in each case, to several peaks (not shown). These results indicate that

TABLE II

DISTRIBUTION OF PROTEIN AFTER ION EXCHANGE CHROMATOGRAPHY OF PHOSPHATIDYLCHOLINE LIPOSOMES

Liposomes prepared from phosphatidylcholine and erythrocyte membranes were fractionated on Sepharose 4B. A sample of 90 ml of the small liposomes (the 445—650 ml fraction, Fig. 4b) was applied to a 3.2 × 70 cm DEAE-cellulose column in solution C (ionic strength 2 mM), and eluted with 0.22 M/l gradient of NaCl. Protein was estimated after alkaline hydrolysis and ninhydrin reaction (see Materials and Methods). The amount of phospholipid was estimated from the total amount of phospholipid applied (Fig. 4b), and from integration under the curve for apparent absorption (not shown). The activity was found mainly in the 14—65 mM elution interval. n.d., not determined. Values for liposomes were obtained assuming an average particle weight of $8 \cdot 10^6$ for the liposomes, and an average molecular weight of 770 for the phospholipids (cf. Ref. 29). Values for protein were obtained assuming an average molecular weight of 120 000 and 50% recovery of amino acids in the hydrolysis step.

| Elution interval (mM NaCl) | <14 | 14—65 | 65—133 | 133—200 | >200 |
|---|---|---|---|---|---|
| Liposomes (nmol) | 0.8 | 3.9 | 8.2 | 7.5 | 2.7 |
| Protein (nmol) | n.d. | 12.6 | 6.6 | 0.0 | n.d. |
| Molar ratio of liposomes to protein | — | 0.3 | 1.2 | — | — |

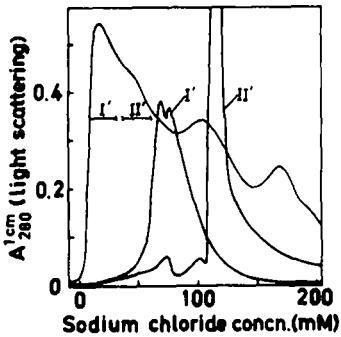


Fig. 6. Heterogeneity of protein-free liposomes as demonstrated by ion exchange chromatography. Protein-free liposomes were prepared from egg yolk phospholipids. These liposomes were fractionated and desalted by molecular sieve chromatography on Sepharose 4B (Fig. 2b, dashed line). A 80-ml sample of the small liposomes was applied to a 2.6 × 35 cm DEAE-cellulose column in solution C (ionic strength 2 mM), and was eluted by a 1.1 M/l gradient of NaCl. Fraction I' and II' were dialyzed against the starting buffer and rechromatographed separately under the same conditions as in the original experiment.

there are different kinds of binding sites for the liposomes in the DEAE-cellulose. The liposomes also eluted in several peaks from other anion exchangers (not shown).

Free zone electrophoresis of phosphatidylcholine liposomes, without protein, gave three peaks (not shown). About 80% of the material appeared in the middle peak. Similar results were obtained for egg yolk phospholipid liposomes.

*Activity and electrophoretic patterns*

To identify the protein that is responsible for the stereospecific D-glucose transport activity, the fractionated liposomes were analyzed by sodium dodecyl sulphate acrylamide gel electrophoresis, as shown in Fig. 7.

*Molecular sieve chromatography.* The small liposomes from Sepharose 4B

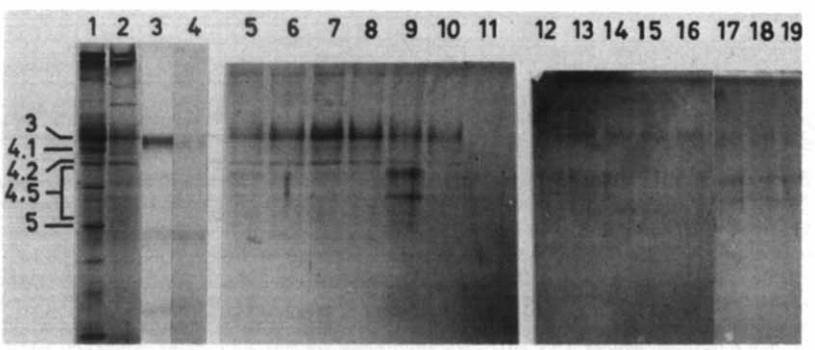


Fig. 7. Sodium dodecyl sulphate acrylamide gel electrophoresis. Samples 1 and 3, human erythrocyte membranes. Samples 2 and 4, liposomes prepared from egg yolk phospholipids and erythrocyte membranes (Fig. 1, fraction I). Samples 5—11, liposomes prepared from phosphatidylcholine and erythrocyte membranes and fractionated by molecular sieve chromatography on Sepharose 4B (Fig. 4a). Samples 12—19, liposomes prepared from egg yolk phospholipids and erythrocyte membranes and fractionated by ion exchange chromatography on DEAE-cellulose (Fig. 5a). Samples 1, 2 and 5—19 were stained for protein with Coomassie brilliant blue R-250. Samples 3 and 4 were stained for carbohydrate with periodic acid-Schiff's reagent. The protein bands are denoted according to the nomenclature of Steck [13].

chromatography (Fig. 4a) contained bands 3 and 4.2 and, in addition, small amounts of spectrin and of 4.1- and 4.5-components (Fig. 7, samples 5—11). The big liposomes that eluted at the void volume contained more of spectrin and of 4.5-components (not shown). One sample (No. 9) showed signs of proteolytic degradation, which lowered the activity and gave rise to three bands in the 4.5-region. The protein distribution over the peak of small liposomes was proportional to the lipid concentration. However, the transport activity curve is displaced toward the smallest liposomes, where the activity per protein or phospholipid unit is the highest (cf. Fig. 4b).

*Ion exchange chromatography.* Liposomes that were capable of stereospecific D-glucose transport eluted in several peaks upon DEAE-cellulose chromatography (Fig. 5a). The liposomes contained mainly proteins from the regions 3 and 4.5. The activity decreased from the first to the last of the activity peaks, whereas the amount of band 3 protein decreased only slightly (Fig. 7). The very low activity between the peaks is not clearly reflected in the protein pattern. It cannot be excluded that the component responsible for the activity is not visible in the acrylamide gels. It might be a minor component or stain poorly. The three sharp zones in the 4.5-region (mainly in samples 17 to 19) probably correspond to degradation products (cf. sample 9).

## Discussion

The purpose of this work has been to incorporate the D-glucose transport system from human erythrocyte membranes into the lipid bilayer of liposomes, and to isolate the liposomes that are active in glucose transport. The transport activity was reasonably stable at −20°C for liposomes prepared from egg yolk phospholipids. About half of the activity remained after 10 days.

*Solubilization of membranes.* For solubilization of membranes, phospholipids and cholate were used in a molar ratio of about 0.5. A molar ratio of 2 gives phospholipid/cholate mixed micelles of a particle weight of about 125 000 [26]. The particle weight of our mixed micelles is not higher, sinze the micellar size decreases with a decreasing ratio of phospholipid to cholate. Solubilization with 0.12 M cholate in the absence of phospholipid, followed by addition of phospholipid, gave a low transport activity (Table I). The phospholipid/cholate mixed micelles might promote the solubilization of the transport protein, and perhaps protect against inactivation. About half of the activity remained after 5 days at 6°C in the egg yolk phospholipid/cholate mixture. For Triton X-100-solubilized proteins the half-time for the activity is reported to be 2 h at 20°C [9] and about 20% of the activity remained after 24 h at 4°C [11].

*Preparation of liposomes.* Molecular sieve chromatography was used to remove cholate from the phospholipid/cholate solution. Some big liposomes or aggregates were formed; more when purified phosphatidylcholine was used than when egg yolk phospholipids were the starting material, and more at pH 6.7 than at 8.2. The explanation might be that the charge of the mixed micelles is lower with purified phosphatidylcholine than with egg yolk phospholipids, and lower at pH 6.7 than at 8.2, due mainly to partial protonization of the carboxyl group of the cholate, and that a weakened electrostatic repulsion between the micelles promotes the formation of big liposomes.

The average size of the small liposomes corresponds to a diameter of about 40 nm, as estimated from the $K_{av}$ values obtained by molecular sieve chromatography (cf. Ref. 19).

The process of liposome formation seems to take place in more than one step, as can be seen in the Sephadex G-50 chromatograms (Figs. 1 and 2a). Probably neither the phosphatidylcholine/cholate micelles, nor the egg yolk phospholipid/cholate micelles are homogeneous in size or composition.

*Rate of glucose transport.* The initial rate of stereospecific uptake of D-glucose was at least 1.9 μmol/min per mg of solubilized protein (or 1.2 per mg of total protein). This value is calculated from the uptake after 20 s in the experiment illustrated in Fig. 3, and represents equilibrium exchange at a D-glucose concentration of 5.4 mM.

For a $K_m$ value of 31 mM [27], and the above initial rate value of 1.2 μmol glucose/min per mg of total protein, a $V$ value of 8.1 μmol/min per mg of total protein can be calculated, assuming Michaelis-Menten kinetics. The calculated $V$ value for D-glucose transport in human red blood cells is 52 μmol/ml per mg of protein, assuming a $V$ value of 310 μmol/min per ml of cell water [27], 0.11 pl cell water per cell [17], and 0.66 pg protein per ghost [28]. The value of $V$ calculated for the reconstituted system is thus at least 16% of the corresponding value for human erythrocytes. Possibly the freeze-thawed liposomes are partly multilamellar. This might mask some of the transport activity, so that the lower limit of $V$ (16%) is underestimated.

*Transport activity and protein pattern.* The transport activity per mg of protein varies by a factor of 5 to 10 or more over regions of the chromatograms where the protein composition seems to vary only slightly. This applied both to molecular sieve chromatography of phosphatidylcholine liposomes, and to ion exchange chromatography of egg yolk phospholipid liposomes. The component responsible for the transport might not be visible in the electrophoresis gels. This component might correspond to the diffuse zone in the 4.5-region described by Kasahara and Hinkle [8]. If a protein from the band 3 region is active in glucose transport, the activity is affected or modulated by some other factors, such that the transport component is active in one population of liposomes, and inactive in another population.

*General.* The present results indicate that a mixture of cholate-solubilized phospholipids gives rise to a set of classes of liposomes upon removal of the detergent. When the formation of liposomes is performed in the presence of integral membrane proteins, the proteins do not distribute randomly among these different classes. Furthermore, the glucose transport protein, in its active state, appears to favour a subclass of liposomes of a certain size and charge(s). This suggests that analyses of the lipid composition of the various classes of liposomes, with regard to the polar groups as well as the hydrocarbon chains, might give a clue to useful improvements in the preparation of liposomes, and in the reconstitution of transport activities.

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