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Reconstitution of Glucose-Transporting Vesicles from Erythrocyte Membranes Disaggregated in Detergent

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With the eventual aim of purifying a membrane transport system by using reconstitution of transport activity as an assay, I showed that if, after the erythrocyte membrane is solubilized in deoxycholate, the detergent is removed, membrane vesicles re-form which retain glucose-transport activity. They take up and release D-glucose in preference to L-glucose and the uptake and release are sensitive to Hg^{2+} and phloretin. Release of tracer D-glucose is competitively inhibited by transported sugars inside the vesicles and increased by unlabelled D-glucose in the outside medium. Uptake of tracer is increased so much by preloading vesicles with unlabelled transported sugars that the tracer is probably concentrated against a gradient. When the membrane is solubilized, two proteins that span the membrane can be separated, suggesting that it will be possible to fractionate the membrane before reconstitution.

The eventual aim of this work is to purify a membrane transport system by using reconstitution of its transport activity as an assay. The membrane would be solubilized by detergent, its components fractionated and the fractions assayed for transport activity by reincorporating them into closed lipid vesicles in the absence of detergent and measuring transport across the vesicle membrane. The glucose-transport system of the human erythrocyte was chosen for pilot experiments, as it can easily be identified in a reconstituted system by its stereospecificity.

As a first step I have shown that the erythrocyte membrane can be sufficiently disaggregated by solubilization in detergent to allow fractionation of integral membrane proteins, and that, after solubilization, by removing the detergent membrane vesicles can be re-formed which retain at least part of the glucose-transport activity of the original membrane.

Materials and Methods

D-[1-^{3}H]Glucose (500mCi/mmol) and L-[1-^{14}C]-glucose (3.0mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. They were free from impurities running ahead of the included-volume peak on Sephadex G-25. Sodium deoxycholate, from Maybridge Chemical Co., Tintagel, Cornwall, U.K., was almost colourless as a 10% (w/v) solution. Phloretin was from Fluorochem, Glossop, Derbyshire, U.K.

Erythrocyte membranes

Outdated packed cells from a blood bank were washed in 150mM-NaCl, then lysed and washed once as described by Fairbanks *et al.* (1971) in 30vol. of 5mM-Tris/HCl (pH8.6 at 0°C). Some haemoglobin remained (see the protein assay in 'Analytical Methods'). The membranes were stored overnight at 4°C with 0.02% (w/v) NaN_3.

Solubilization and reconstitution

Meissner & Fleischer's (1974) procedure for sarcoplasmic-reticulum membranes was followed, with slight modifications. No attempt was made to optimize reconstitution by varying the method. Membranes were solubilized by stirring for 30min on ice at about 3.9 A_{280} units of protein/ml (defined below) with 0.35% (w/v) sodium deoxycholate in solubilization buffer [0.5M-KCl/0.25M-sucrose/100mM-glucose/1.5mM-$MgCl_2$/1mM-EDTA/40mM-Tris/HCl (pH8.0 at 0°C)/0.02% NaN_3] and centrifuged at 102000$g_{av.}$ for 40min to remove insoluble material. Care was taken in decanting the supernatant to include all lipid at the top of the tube and avoid contamination with pellet. About three-quarters of the protein was solubilized. To reconstitute vesicles the supernatant was dialysed for 15–18h in 8/32 Visking tubing against 100vol. of dialysis buffer, which was the same as the solubilization buffer except for the substitution of 7.5mM-Hepes* (pH7.25 at 20°C) for Tris. For glucose-free vesicles, glucose-free buffer was substituted after 5h. The dialysis buffer was initially at 4–10°C, but was allowed to warm up to room temperature (18–22°C) (Meissner &

* Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; SDS, sodium dodecyl sulphate.

Fleischer, 1974). The dialysed material was diluted 6-fold with dialysis buffer and centrifuged at 78000$g_{av.}$ for 80min, and the vesicle pellet was resuspended with gentle homogenization in 0.33vol. of dialysis buffer per volume dialysed at room temperature. Preparations did not deteriorate significantly over 2 days at 4°C.

Analytical methods

Lipid phosphorus was determined approximately by assaying total phosphorus (Meissner & Fleischer, 1974) by the method of Bartlett (1959) after charring and digestion with $HClO_4$ (beware: these digests occasionally explode violently), as 88–100% of the phosphorus was extractable with chloroform/methanol (Bligh & Dyer, 1959). Erythrocyte membrane protein was determined as A_{280} units/ml by heating a sample at 80°C for 5min in 1% (w/v) SDS, measuring A_{280} and correcting (about 20%) for haemoglobin by subtracting $2.2 \times A_{540}$.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was run in the phosphate (pH7.1) buffer system of Shapiro *et al.* (1967) in 5% or 8% (w/v) polyacrylamide gels polymerized, stained and scanned as described by Fairbanks *et al.* (1971). Samples were heated at 80°C for 10min with 1% (w/v) SDS and dialysed overnight against the sample buffer containing 1% SDS (Shapiro *et al.*, 1967) to remove K^+ before loading.

Liquid-scintillation counting

Aqueous samples (1ml) were counted for radioactivity with 10ml of 4g of butyl-PBD [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole; from CIBA, Duxford, Cambs., U.K.)/litre in toluene/Triton X-100 (Sigma, London S.W.6, U.K.) (2:1, v/v). The external-standard channels-ratio method was used to correct for quenching, calibrating with internal standards. The variation in the ratio of ^3H to ^{14}C counts due to variation in quenching was less than 5% for a given column. Samples were counted for 10min. Efficiencies were around 50% for ^{14}C and 18% for ^3H.

Results and Discussion

Demonstration that the solubilization permits fractionation of integral membrane proteins

For reconstitution to mean anything, it was necessary to define the state of disaggregation of the membrane before reconstitution. For reconstitution to be used as an assay during purification, the disaggregation in detergent would have to allow separation of proteins tightly embedded in the bilayer, such as the 100000-dalton protein, band 3 (Steck, 1974), and the sialoglycoprotein, which both span the erythrocyte membrane (Steck, 1974; Mueller & Morrison, 1974).

Fig. 1 shows the result of centrifuging erythrocyte membranes, solubilized in deoxycholate as described in the Materials and Methods section, into a detergent-free sucrose density gradient. The sialoglycoprotein and the 100000-dalton protein sediment to different positions in the gradient, showing that the solubilization allows fractionation by very gentle means, and suggesting that they are dissociated in the detergent.

One slight possibility that this experiment is not able to rule out is that a small proportion of the integral membrane proteins is not disaggregated by the detergent and it is only these proteins that recover their activity on reconstitution.

Reconstitution of vesicles with glucose-transport activity

When deoxycholate was removed from the solubilized membranes, vesicles were formed, as shown by their ability to trap sugars. Preparations were frozen and thawed with L-[^{14}C]glucose, incubated at 37°C, on the assumption that this would help to seal vesicles as it does 'ghosts' (Bodemann & Passow, 1972), and run down a Sephadex G-50 column in unlabelled buffer. Label appeared in the void volume

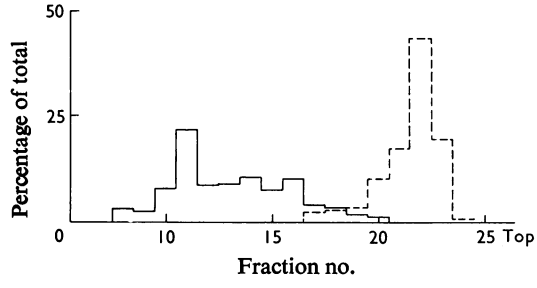
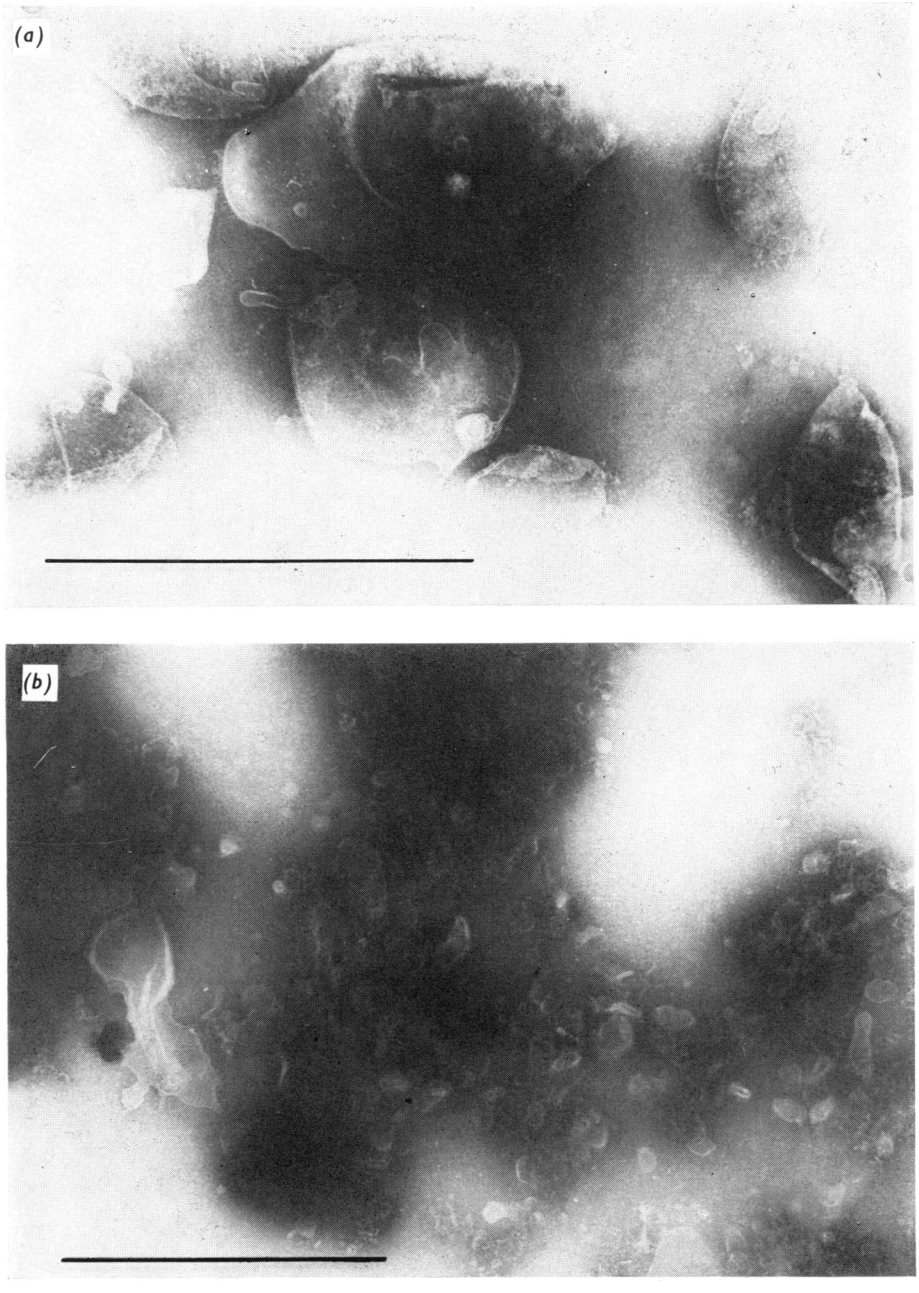


Fig. 1. *Demonstration that the erythrocyte sialoglycoprotein can be separated from the* 100000*-dalton protein when solubilized under particular conditions*

Solubilized membranes (1.5ml) were layered on a linear 15–50% (w/v) sucrose gradient (11.5ml) in 0.5M-KCl/1.5mM-$MgCl_2$/1mM-EDTA/40mM-Tris/HCl (pH8.0 at 0°C) in a Spinco type SW 40 rotor and centrifuged at 2°C for 18h at 40000rev./min (202000g; $r_{av.}$ 11.3cm). Fractions (0.5ml) were collected and 150μl samples taken for SDS/polyacrylamide-gel electrophoresis on 8% gels. Ordinate shows the amount of each protein determined by densitometry of stained gels, expressed as percentage of the total of that protein recovered. ----, Sialoglycoprotein, periodate/Schiff stain; ——, 100000-dalton protein, Coomassie-Blue stain.



EXPLANATION OF PLATE 1

Negative-stain electron microscopy of vesicles

Preparations were fixed with 3% glutaraldehyde+4% formaldehyde, dialysed against water for at least 20h to remove salt and sucrose, diluted with 0.5% ammonium molybdate, 12-fold in (*a*), 2.5-fold in (*b*), and sprayed on to Formvar-coated grids. Scale represents 1 μm. The preparation in (*a*) contained 169 μg of P/ml, trapping 0.06 μl of L-glucose/μg of P in a release experiment. Preparation (*b*) contained 50 μg of P/ml, trapping 0.023 μl of L-glucose/μg of P.

with the vesicles, corresponding to 0.006–0.06 μl of buffer trapped inside the vesicles per μg of lipid P (Fig. 2 and Table 1) (preparations normally contained 60–170 μg of P/ml). This compares with 0.024 μl/μg of lipid P for sonicated liposomes of 29% (w/w) cholesterol added to 4% (w/w) phosphatidic acid in phosphatidylcholine which are 26nm in diameter (Johnson, 1973). Negative-stain electron microscopy of preparations (Plate 1) showed vesicles ranging from around 30nm in diameter, the size of sonicated liposomes, to about 500nm in diameter. Preparations with a high capacity to trap labelled L-glucose (e.g. Plate 1*a*) had a high proportion of large vesicles; preparations with low capacity (Plate 1*b*) contained mostly small vesicles. No membranous material was seen in solubilized membranes examined thus.

Stereospecific release of D-glucose from the vesicles was demonstrated in the same way by freezing and thawing the vesicles with a mixture of L-[^{14}C]glucose and D-[^{3}H]glucose (Fig. 2). The ratio of ^{3}H to ^{14}C eluted with the vesicles was lower than in the sample loaded on the column, so D-glucose had been preferentially lost from the vesicles during their passage down the column. These results are conveniently expressed (Table 1) as the apparent volumes of L-

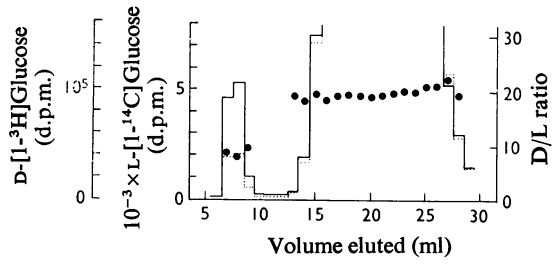


Fig. 2. *Preferential release of* D-*glucose from vesicles on Sephadex G-50 column*

Vesicles (0.35ml) were quick-frozen and thawed with 20 μCi of D-[1-^{3}H]glucose and 1 μCi of L-[1-^{14}C]-glucose, incubated for 40min at 37°C, and run on a column (1cm×30cm) of Sephadex G-50 (medium grade) in dialysis buffer at room temperature, eluted at 0.7–1.0ml/min: 1ml fractions were collected and counted for radioactivity. ·····, D-[1-^{3}H]Glucose; ——, L-[1-^{14}C]glucose; ●, D-glucose/L-glucose ratio. The peak at 7–8ml is the void-volume peak and contains the vesicles; the second larger peak is at the included volume and contains radioactivity that was not inside the vesicles. The L-glucose in the vesicle peak was equivalent to 2.1 μl of the vesicle suspension loaded on the column.

Table 1. *Selective uptake and release of* D-*glucose*

Release was inhibited by adding Hg^{2+} and phloretin to the sample and column as in uptake experiments. In the uptake experiment the vesicles were incubated with label for 2min. All columns were run at room temperature. Other experimental details were as in Fig. 2 or the text.

Type of experiment	Inhibitors	D-Glucose trapped (μl/μg of P)	L-Glucose trapped (μl/μg of P)	D-Glucose trapped / L-glucose trapped
Release	—	0.016	0.036	0.43
Release	Hg^{2+} and phloretin	0.029	0.029	1.01
Uptake	—	0.020	0.011	1.83

Table 2. *Selective release of* D-*glucose in the cold: competitive inhibition and trans-acceleration*

Vesicles prepared in glucose-free buffer were frozen and thawed with D-[1-^{3}H]glucose and L-[1-^{14}C]glucose with or without 0.4M competing sugar and run on a Sephadex G-50 column at about 2°C with or without D-glucose in the column buffer and with an extra 0.2M-KCl where needed to balance the osmoticity of the 0.4M sugar. Other details were as in Fig. 2. Two experiments, in which all measurements were made on a single preparation, are shown. Lipid concentrations in the vesicles determined as total P were 112 μg/ml for Expt. 1, 169 μg/ml for Expt. 2. Glucose trapped is expressed as (d.p.m. in vesicles/μg of P loaded)/(d.p.m./μl in sample loaded).

Competing sugar inside vesicles	D-Glucose in column buffer	Expt. 1		Expt. 2	
		L-Glucose trapped (μl/μg of P)	D-Glucose trapped / L-glucose trapped	L-Glucose trapped (μl/μg of P)	D-Glucose trapped / L-glucose trapped
—	—	0.0056	0.38	0.038	0.42
D-Glucose	—	0.0087	1.02	0.028	0.86
D-Mannose	—	0.011	1.00	0.028	0.90
D-Galactose	—	0.017	0.81	0.028	0.82
L-Glucose	—	0.011	0.43	0.025	0.51
D-Glucose	0.4M	0.011	0.61	0.039	0.73

and D-glucose trapped by the vesicles, i.e. the volume of the suspension loaded on the column that contained the L- or D-glucose eluted with the vesicles. The ratio of the apparent volume of D-glucose trapped to the apparent volume of L-glucose trapped is a measure of stereospecific release of D-glucose and is also equal to (D/L ratio in vesicles)/(D/L ratio in sample loaded). Stereospecific release was abolished by the inhibitors Hg^{2+} and phloretin (Table 1). Phloretin alone inhibited stereospecific release, Hg^{2+} alone abolished it completely.

Stereospecific uptake of D-glucose was shown by incubating vesicles at 37°C for 15 min to seal them and then at a given temperature for a given time with labelled L- and D-glucose. Incubation was stopped with the addition of Hg^{2+} to 1.5 mM, and phloretin (10 mM in ethanol) to 0.1 mM, to block transport (Karlish *et al.*, 1972), and the vesicles were run down a Sephadex G-50 column in dialysis buffer with the EDTA omitted and 0.1 mM-phloretin, 1% (v/v) ethanol and 1 μM-$HgCl_2$ added. D-Glucose was taken up in preference to L-glucose (Table 1). Preferential uptake was also abolished by addition of Hg^{2+} and phloretin before label.

The demonstration of increased, decreased and unchanged $^3H/^{14}C$ ratios in the vesicle fractions under the various conditions shown in Table 1 shows that the changes are not artifacts of impure label or counting errors, and make it unlikely that the radioisotopes are merely selectively bound. Bacterial contamination, monitored by plating on a nutrient-broth medium at room temperature, was kept low by NaN_3 and did not appear to be responsible for the trapping and transport of sugars. Parallel preparations with and without 0.01% (w/v) penicillin added to the dialysis medium, which lowered contamination at least 3-fold, gave the same results, and in general there was no correlation between the degree of contamination in different preparations (0–200 colonies from 5 μl) and their capacity to trap or take up sugars.

Competitive inhibition

To demonstrate saturability and competitive inhibition of release of labelled D-glucose, vesicles were prepared in glucose-free dialysis medium and various unlabelled sugars added with the labelled sugars (Table 2). Addition of D-glucose decreased the release of tracer, showing that release is saturable. This can also be regarded as competitive inhibition of release of labelled D-glucose by the distinct sugar unlabelled D-glucose. Release is also inhibited by the transported sugars D-mannose and to a lesser extent by D-galactose (which has a lower affinity for the transport system; Miller, 1971), but not by the non-transported sugar L-glucose.

These experiments also illustrate the variability of different individual preparations, which contrasts

Table 3. *Countertransport: enhancement of selective* D-*glucose uptake by preloading vesicles with unlabelled sugar*

Vesicles (1 ml) prepared in glucose-free buffer were frozen and thawed with or without 0.4M-unlabelled sugar, incubated at 37°C and run down a Sephadex G-50 column at about 2°C as in a release experiment but in glucose-free buffer with an extra 0.2M-KCl in the buffer where needed to balance the sugar osmoticity. The vesicle peak was collected and 2 μCi of L-[1-^{14}C]glucose+40 μCi of D-[1-^{3}H]glucose were immediately added to 0.5 ml. After 5 min incubation at about 2°C or at 37°C, Hg^{2+} and phloretin were added and the vesicles were run down a second Sephadex G-50 column as in an uptake measurement, at about 2°C, again with extra KCl in the buffer where appropriate. The two experiments and expression of results are the same as in Table 2.

Sugar preloaded into vesicles	Temperature of incubation with label (°C)	Expt. 1			Expt. 2		
		D-Glucose trapped (μl/μg of P)	L-Glucose trapped (μl/μg of P)	D-Glucose trapped / L-glucose trapped	D-Glucose trapped (μl/μg of P)	L-Glucose trapped (μl/μg of P)	D-Glucose trapped / L-glucose trapped
—	2	0.015	0.008	1.8	0.013	0.009	1.5
D-Glucose	2	0.32	0.011	28	0.14	0.020	7.1
D-Mannose	2	0.24	0.013	19	0.084	0.017	4.9
D-Galactose	2	0.08	0.011	7.1	0.056	0.013	4.3
L-Glucose	2	0.013	0.009	1.4	0.016	0.014	1.2
—	37	0.017	0.015	1.2	0.026	0.018	1.4

with the reproducibility of preparations made in parallel from a common solubilized sample in which, for example, the selectivity ratio in a release measurement (volume of D-glucose trapped)/(volume of L-glucose trapped) has a standard deviation of about 5%.

Trans effects and uphill countertransport

The most convincing evidence that glucose transport is recovered in these vesicles is the observation of *trans* effects, i.e. the acceleration of labelled sugar movement by unlabelled sugar on the other, *trans*, side of the membrane, and uphill countertransport, the accumulation of labelled sugar against a concentration gradient by coupling its transport to the flux of unlabelled sugar in the opposite direction (see, e.g., Lefevre, 1975). Table 2 shows the *trans* effect on release. Release of labelled D-glucose in the presence of 0.4M-D-glucose in the vesicles is increased by 0.4M-D-glucose in the column buffer. Table 3 shows the increase of uptake of labelled D-glucose into vesicles preloaded with unlabelled 0.4M transported sugar. Even when this unlabelled sugar is D-glucose, it is to be regarded as a distinct species from labelled D-glucose (see, e.g., Lefevre, 1975). When the labelled sugars are added to the vesicles, unlabelled sugar is flowing out. This increases the rate of uptake (*trans* effect), and the tracer taken up does not re-emerge, as the unlabelled sugar competes for exit. Preloaded D-glucose and D-mannose cause dramatic increases in uptake, D-galactose causes a more modest increase and L-glucose causes no increase. The amount of labelled D-glucose taken up into vesicles preloaded with transported sugar is much greater than the amount of L-glucose trapped in vesicles in a release measurement, and is much greater than the amount taken up when, in an uptake measurement, the sample is incubated at 37°C instead of at 0°C (Table 3), so a reasonable interpretation of the results is that the labelled D-glucose has been accumulated in the vesicles by countertransport to a higher concentration than in the outside medium. The dramatic increase in uptake in the preloaded vesicles is in any case excellent evidence for the recovery of transport activity.

Composition of the vesicles

The protein composition of the vesicles and of the solubilized membranes from which they were reconstituted, as shown by SDS/polyacrylamide-gel electrophoresis, seems to be much the same as that of 'ghosts' (Steck, 1974). The exception was protein band 6 (nomenclature of Fairbanks *et al.*, 1971), relatively little of which was recovered in the vesicles, presumably because it does not bind to erythrocyte membranes at high salt concentrations (Steck, 1974). Lipid class composition was not determined, but solubilization by deoxycholate is non-selective or slightly favours phospholipids rather than cholesterol (Kirkpatrick *et al.*, 1974).

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