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Solubilization, Reconstitution, and Attempted Affinity Chromatography of the Sugar Transporter of the Erythrocyte Membrane

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Reconstitution of the sugar transport system of human erythrocytes into artificial liposomes was achieved by freezing, thawing, and sonicating preformed phospholipid vesicles in the presence of intact ghosts, protein-depleted ghosts, or detergent-treated ghosts. D-glucose equilibrium exchange activities and affinity constants in the range of the reported erythrocyte values were reached in the best experiments. Whereas the extraction of peripheral membrane proteins did not depress the transport function crucially after reconstituting these protein-depleted ghosts, the selective solubilization of integral membrane proteins by a variety of nonionic detergents resulted in an uncontrollable, continuously increasing inactivation of the carrier. However, Emulphogene BC-720 extracts could be prepared in which the glucose transporter retained activity for days at 4°C. These extracts were applied to affinity chromatography matrices of phloretin-Agarose, prepared by coupling phloretinyl-3′-benzylamine (PBA) to CH-Sepharose 4B and to Affigel 202. Although the solubilized sugar transporter appeared to be selectively adsorbed to both PBA matrices, it could not be eluted by specific counter ligands or gentle eluants in a biologically active form. However, chaotropic agents could be used to elute intrinsic proteins, including bands 3 and 4.5, from the Affigel affinity medium.

Key words: human erythrocyte, glucose transport, membrane transport, reconstitution, membrane protein solubilization, afffinity chromatography, phloretin derivatives

The combined difficulties of handling intrinsic (ie, hydrophobic-hydrophilic) membrane proteins and of efficiently reconstituting transport systems are proving to be a very serious hurdle in the understanding of structure-function relationships in membrane transport systems.

In the following we present procedures to solubilize the intrinsic proteins of the human erythrocyte membrane, in particular its monosaccharide transporter. Our methods lead to excellent yields of solubilized transporter which can be stored for up

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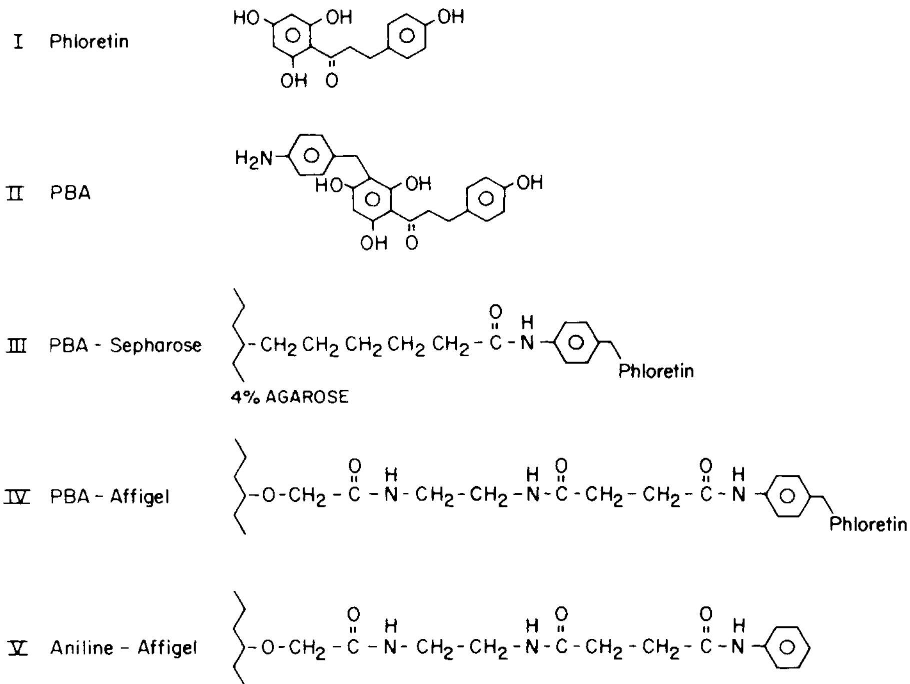

Fig. 1. Structure of phloretin, PBA, and the affinity matrices.

to 4 days at 4°C if the solubilizing detergent (Emulphogene BC-720) is kept at less than the 0.1% level. The technique offers alternatives and advantages over the existing procedures using Triton X-100 [1,2], deoxycholate [3], cholate [4,5], or octyl glucoside [6].

Of the variety of methods available to attempt isolation of the sugar transporter, affinity chromatography would seem to be the simplest and most efficient. We reasoned that since phloretin (Fig. 1) at micromolar levels is a potent competitive inhibitor of erythrocyte sugar transport [7–9], it could serve as ligand on an insoluble affinity chromatography matrix. This view was supported by early work in which a model protein, also possessing a glucose-phloretin receptor site (mutarotase), could be purified on a phloretin-agarose matrix by specific elution with D-glucose, even in the presence of detergent [10]. To prepare an insolulble phloretin matrix, phloretin-3'-benzylamine (PBA) was synthesized (Fig. 1) and coupled to commercially available agarose derivatives. PBA is even more potent than phloretin as a strictly competitive inhibitor of glucose influx in human erythrocytes with almost 10^4 times the affinity for the carrier site as glucose [9]. The benzylamine group on phloretin's A ring allows the easy attachment of the compound to insoluble polymers while preserving the features of the ligand required for transport inhibitory properties. Although adsorption of solubilized transporter to both matrices was saturable and somewhat selective, no elution condition was found that delivered undenatured carrier or purified membrane protein. We nevertheless wish to report our results as a potentially valuable approach to deal with the known difficulties of membrane protein isolation.

MATERIALS AND METHODS

Materials

[^{3}H]- and [^{14}C]-labeled glucose isomers were purchased from NEN (Boston). The detergents were from Sigma except SDS (Serva), Zwittergent 314 (Calbiochem), Tween 20/60 (Atlas), and Aminoxyd WS 35 (Th. Goldschmidt, Essen, BRD). Emulphogene BC-720 (polyoxyethylene-10-tridecylether) was obtained from either Sigma or GAF Corporation. Bio Beads SM-2 and Affi-Gel 202 are available from Bio-Rad and CH-Sepharose 4B was purchased from Pharmacia Fine Chemicals.

Analytical Methods

SDS-PAGE was performed in a Laemmli system [11] as described elsewhere [12]. Protein was determined using the Peterson modification [13] of the Lowry assay with or without the trichloroacetate deoxycholate (TCA-DOC) precipitation step; the Bradford assay [14] was also sometimes used. Total phospholipid phosphate was measured by the method of Chen et al [15] and cholesterol in total lipid extracts by the method of Zlatkis and Zak [16].

Extraction of Proteins From Erythrocyte Membranes

Ghosts were prepared following the method of Dodge et al [17] and were used immediately or stored at −20°C. Extrinsic proteins were extracted with 0.5 M NaCl, 0.1 mM EDTA, dilute NaOH (pH 12), or 20 mM lithium-3,5-diiodo-salicylate (LIS) and we obtained protein and transporter recovery results similar to those previously reported [18–20]. Intrinsic proteins were solubilized as shown in Table II. Detergents were removed from the samples by adsorption on Bio Beads SM-2 [21] (1 gm wet Beads per 20 mg detergent) or by exhaustive dialysis in the case of the bile salts and Emulphogene (against dilute ethanol).

Emulphogene BC-720 was used to solubilize the glucose transporter in the affinity chromatography experiments. Charged oxidation products [22] which were present in the filtered commercially available material were removed using an ISCO Electrophoretic concentrator with 1 mM EDTA and 10 mM EDTA in the sample and electrode compartments, respectively. The purified detergent was collected as the immobile component and stored at 4°C in EDTA.

Preparation of (Proteo)Liposomes To Study D-Glucose Transport

Soy bean L-α-Phosphatidylcholine II S (Sigma) was washed with acetone [23] and stored as a 10% solution in $CHCl_3$ at −20°C. Liposomes were made by resuspending 40 mg of this lipid (which had been freed from the organic solvent by evaporation with N_2 and vacuum overnight) in 1 ml 100 mM KCl, 10 mM Tris-HCl, 0.02% KN_3, pH 7.5, and sonicating the sample for 10 to 15 min with a Branson Sonifier (micro tip, intensity ca 4, 50% sonication time). Contaminants were removed from the opalescent sample by centrifugation for 15 min at 27,000g. A 300 μl aliquot of these liposomes was added to fractions containing from 100- to 400 μg membrane fragments or detergent-free ghost protein extracts (solubilized membrane samples had been concentrated for 4 hr at 200,000g after Bio-Beads treatment). This mixture was adjusted to 750 μl with the KCl-Tris buffer in thick-walled conical glass centrifuge tubes and quickly frozen in dry ice/methanol. The sample was then thawed at room temperature, diluted with an additional 750 μl buffer (with the desired glucose concentration when the vesicles were being prepared for equilibrium exchange exper-

iments), and sonicated at the lowest intensity for 10 sec at room temperature. The suspension, which had become turbid after freezing, cleared during this step. This fusion of protein and lipid was originally described by Kasahara and Hinkle [1] and Pick [24].

In some reconstitution experiments, we employed liposomes produced from the total lipid of ghosts by a slight modification of the Rose and Oklander procedure [25]. The chloroform/propanol extract was handled under N_2 after adding 2% D-α-tocopherol at the first filtrate step. The dried lipid was sonicated for 1 hr with enough Krebs-Ringer phosphate (KRP) buffer to give a suspension of 15 mg lipid/ml which was treated twice in a freeze/thaw cycle with sonication and finally centrifuged at 100,000g for 15 min [26]. The upper liposome pellet was resuspended by brief sonication in a minimum volume of KRP buffer and stored under N_2 at $-70°C$ until used. RBC membrane fragments, Emulphogene solubilized fractions, or proteins from affinity column eluates could be fused with these lipid vesicles essentially as described above with the following differences: 1) the freeze/thaw cycle was repeated three times to enlarge the intraliposomal space, 2) an ultrasonic bath was used to prepare the proteoliposomes, and 3) removal of Emulphogene with Bio Beads was found to be unnecessary; in fact, the detergent up to levels of 0.05% actually increased the efficiency of active transporter insertion into liposomes without significantly increasing passive leak and represents an important advantage over other detergents.

Glucose Influx or Equilibrium Exchange Uptake

Routinely, 90 μl of (proteo)liposomes (about 500 μg lipid) were incubated for various time periods at room temperature with 10 μl solutions containing 1.25 μCi L-[^{3}H]-glucose and 0.5 μCi D-[^{14}C]-glucose. Glucose uptake was stopped by diluting the mixture with 2 ml ice-cold 150 mM NaCl, 1 mM HEPES-Tris, 0.02% KN_3, pH 7.4, and the liposomes were rapidly collected on 0.6 μm cellulose nitrate filters and analyzed for ^{3}H and ^{14}C. Short time measurements were performed with an automatic uptake apparatus (Inovativ Labor AG., 8134 Adliswil, Switzerland) as described elsewhere [27]. Specific transport activities were calculated by subtracting the L- from the D-glucose uptake values.

Preparation of the Affinity Media: PBA Sepharose

Sixty milliliters of CH-Sepharose 4B beads was suspended in 50% aqueous ethylene glycol containing 1 mmole of PBA (see Fig. 1). Eight millimoles of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (EDAC) was added and the mixture was kept at room temperature for 1.5 hr while maintaining the pH between 4.5 and 6.3. After standing for an additional 2 days at 4°C, the mixture was poured into a column and unreacted PBA was washed from the beads with 50% ethylene glycol-water. The recovery of uncoupled PBA in the column eluate was analyzed spectrophotometrically ($\epsilon_{332} = 21.0 \times 10^3\ M^{-1}\ cm^{-1}$ in 0.025 M $Na_2B_4O_7$, pH 9.3 [9]).

PBA Affigel

Fifty milliliters of washed, Affigel-202 beads (22 μmoles functional groups/ml) was added to an equal volume of ethylene glycol (or ethanol) containing 1.3 mmoles PBA (see Fig. 1). The room temperature mixture was kept under nitrogen and suspended by a vibrating mixer. The pH was adjusted to 4.7 with 0.1 N HCl and coupling was initiated by the addition of 1.5 gm EDAC. The pH was maintained between 4.7 and 5.0 for 4 hr, after which the suspension was treated as in the PBA-

Sepharose preparation. Based on the PBA recovered, 95% coupling was achieved. The beads were stored under N_2 at $-20°C$ in 50% ethanol.

Aniline Affigel

To determine if the adsorption characteristics of PBA Affigel were ligand specific, a control medium was prepared by coupling, in an identical manner, a fivefold excess of freshly distilled aniline to the support material (see Fig. 1).

RESULTS

Glucose Uptake Into Liposomes/Nonsolubilized Membrane Fragments

Figure 2 shows the influx of D- and L-glucose into liposomes composed of phospholipids with or without incorporated erythrocyte membrane fragments at 22°C. Lipid-only vesicles were relatively impermeable to both sugars; longer than 70 hr was required for glucose to reach equilibrium. On the other hand, proteoliposomes, consisting of a 40:1 ratio of lipid to membrane proteins, displayed a rapid stereospecific intial uptake of D-glucose that was complete within 5 min; therafter, D-glucose uptake was essentially the same as its passively diffusing L-isomer. Ghost proteins must have fused and become embedded in the lipid bilayer vesicles since, when intact ghosts and liposomes were freeze-thawed and sonicated separately and then combined, the slow uptake similar to that of pure liposomes was observed (data not shown).

The facilitated influx process delivers D-glucose into an intravesicular space which is about 50% of the equilibrium (70 hr) volume (Fig. 2). One interpretation for this finding is that every second proteoliposome possesses an active, correctly oriented D-glucose transporter. This view is supported by the following theoretical argument: Incorporating erythrocyte membrane proteins into 700 Å diameter lipid bilayer vesicles (estimated from the intravesicular volume and lipid concentration) at

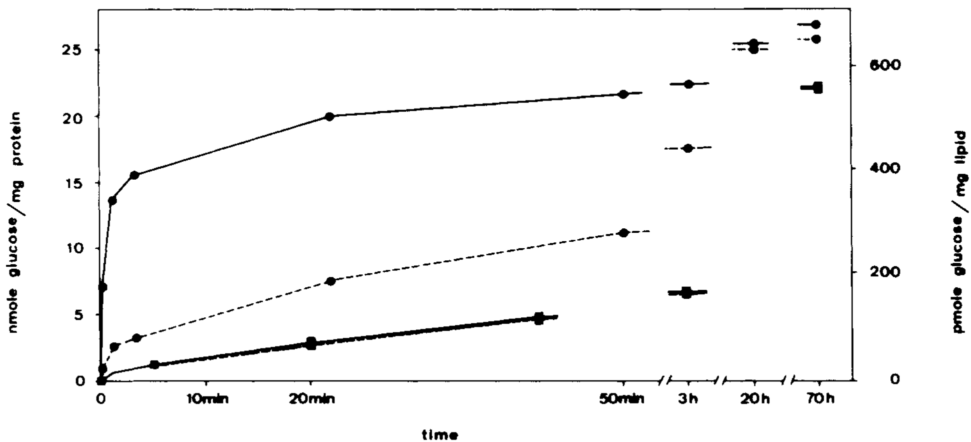

Fig. 2. Glucose uptake by liposomes and proteoliposomes at room temperature. Influx of 0.2 mM D-glucose (——) or L-glucose (-----) into pure liposomes (■) or proteoliposomes composed of ghost protein and a soybean lipid mixture at a ratio of 40:1 (w/w) (●). The protein concentration in the uptake medium was 0.069 mg per ml. Incorporation of ghost protein in lipid bilayers and influx measurement with radioactive labeled glucose was done as described under Materials and Methods.

a ratio of 1 to 40 (w/w) should result in 0.5 glucose carrier per liposome, assuming that 500 pmole transporters per milligram membrane protein exist in the red blood cell.

Influx of D- and L-glucose into proteoliposomes was also measured at 37°C and at this temperature L-glucose reached the D-glucose equilibrium value as early as 3 hr. These uptake data (not shown) fit an exponential curve for first-order kinetics from which a permeability coefficient, $P_{37°C} = 1.6 \times 10^{-10}$ cm/sec, could be determined [28].

Osmotic Fragility of the Proteoliposomes

The proteoliposomes behaved like ideal osmometers when different amounts of glycerol were added to vary the external osmolarity between 240 and 852 mOsm (Fig. 3). The equilibrium value of D- and L-glucose, representing the internal liposome volume, was linearly related to the inverse of the total outside osmolarity. No adsorption (binding) of glucose to membranes but actual uptake into an aqueous intraliposomal compartment is indicated.

Initial Rate Determination From Equilibrium Exchange Experiments

The results of a short time equilibrium exchange experiment with proteoliposomes are shown in Figure 4. The 2-sec time point represents the initial exchange rate whereas the process is already 30% slower at 5 sec. Estimates of the D-glucose (10 mM) initial rate determinations varied between 20% and 80% of the corresponding physiological values (see below).

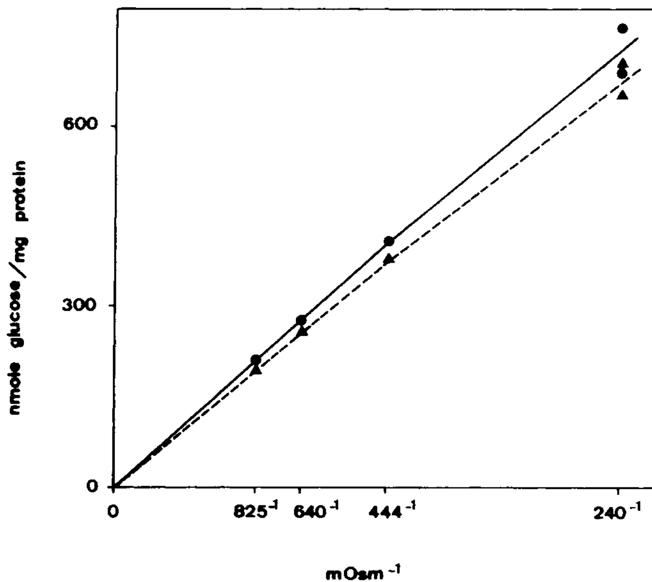

Fig. 3. Osmotic shrinking of proteoliposomes. Proteoliposomes (see Materials and Methods) in 100 mM KCl, 10 mM Tris-HCl, 0.02% KN_3, pH 7.5, were incubated with 10 mM D- and L-glucose together with increasing amounts of glycerol (0 to 612 mM). After 3 hr at room temperature the glucose uptake was determined and plotted as indicated; L-glucose, solid line, circles; D-glucose, broken line, triangles.

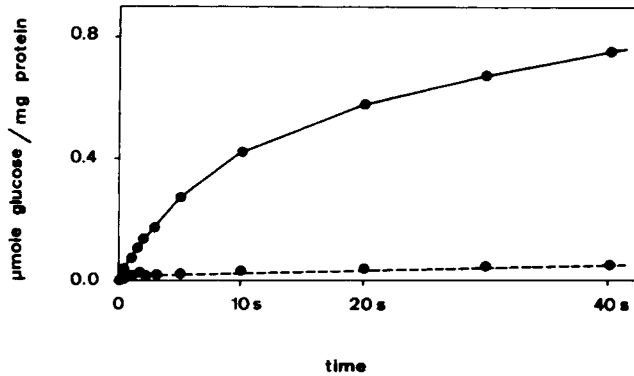

Fig. 4. Short time equilibrium exchange flux with glucose into proteoliposomes. Proteoliposomes as in Figure 2 were preequilibrated with 10 mM unlabeled D- and L-glucose and thereafter tested for tracer exchange rate capacity between 0.5 and 40 sec at room temperature. The protein concentration was 0.12 mg per ml. D-glucose, solid line; L-glucose, broken line.

Despite efforts to improve the reproducibiity of the system (eg, extensive washing of the ghosts, addition of proteolytic inhibitors, and so forth), loss of carrier activity from ghost to ghost preparation could not be controlled. On the other hand, within a reconstitution experiment from a single ghost preparation or extract, recovery of activity was highly reproducible. Similar exchange experiments as that shown in Figure 4 were conducted at room temperature over a concentration range of 5 to 200 mM D- and L-glucose from which K_m and V_{max} values of 25–30 mM and 4–6 μmole/min/mg membrane protein could be estimated. The native transporter under these conditions operates with a K_m of 20–40 mM and a V_{max} of 13 μmole/min/mg membrane protein [18,29].

Fractionation of Erythrocyte Membrane Proteins

Certain mild solubilization conditions can selectively remove intrinsic proteins leaving the membrane cytoskeleton as a residue [30]. Table I shows the course of a ghost extraction using the detergent Triton N-101. The extract contained portions of bands 3 and 6, region 4.5, and some minor proteins. Although recovery of transporter activity was excellent it was strictly dependent on the protein's time of exposure to the detergent (see below). Our results with other detergents tested for their ability to selectively solubilize an active transporter are shown in Table II. Neither cholate nor deoxycholate gave useful results in our hands whereas several nonionic detergents worked well, including Emulphogene.

Stability of the Carrier During Solubilization

Since sugar transporter activity was lost upon exposure to the Triton detergents (stability was not improved with proteolytic inhibitors like phenylmethylsulfonyl fluoride (PMSF), EDTA, p-chloromercuribenzoate (PCMB), tetrathionate, pepstatin; antioxidants like ascorbate, sulfite; pH changes of the buffer; or addition of D-glucose as a stabilizing ligand), we restricted our examinations on carrier stability to Emulphogene. This detergent was chosen for the affinity chromatography procedure because column eluates containing the detergent (which lacks aromatic groups) could

TABLE I. Triton N-101 Solubilization and Reconstitution of Ghosts

Sample	Protein recovery (%)		Phospholipid recovery (%)		Specific transport activity (μmol·min^{-1}·mg^{-1}; (10 mM glucose)	Transport recovery (%)	
Ghosts (not solubilized)	100		100		5.04	100 by def	
Solubilized ghosts							
Not cenrifuged	124		101				
Supernatant	48	130	26	100			
Pellet	82		74				
Ghosts solubilized and reconstituted							
Not centrifuged	84				3.30	55	
Supernatant	21	75			7.62	32	49
Pellet	54				1.55	17	

Triton N-101 (0.33%) was added to ghosts (1 mg protein per ml) in 100 mM KCl, 10 mM Tris-HCl, 0.02% KN_3, pH 7.5, and the mixture was allowed to stand for 10 min at 0°C (gentle shaking). After centrifugation of one part of the samples for 30 min at 200,000g, all fractions were treated immediately with Bio Beads SM-2 (1 gm wet beads per 20 mg Triton) overnight. After removing the beads, the fractions were concentrated (4 hr at 200,000g) and used for reconstitution studies (equilibrium exchange).

TABLE II. Solubilization of Ghosts by Various Detergents

Detergent	Solubilization: Efficient	Solubilization: Selective	Transport recovery compared to Triton X-100 (%)
0.5% Triton X-100	Yes	Yes	100 by def
0.5% Triton X-114	Yes	Yes	50
0.5% Triton N-101	Yes	Yes	90–120
0.5% Triton X-102	No		ND
0.5% Brij 76	No		ND
1.0% Brij 56	Little	Yes	30
0.5% Brij 35	No		ND
0.2% Aminoxid WS 35	Yes	Yes	60
0.5% Zwittergent 314	Yes	Yes	<7
0.1% SDS	100% Solubilized		<5
0.5% Tween 20	No		ND
0.5% Tween 60	No		ND
2.5% Na-cholate	No		ND
1.0% K-deoxycholate	Yes	Hardly	0
0.2% Emulphogene BC-720	Yes	Yes	>60

Intact ghosts were treated with the indicated detergents as described in Table I. Bio Beads incubation or dialysis (bile salts and Emulphogene) to remove the detergents was performed as explained in Materials and Methods. The solubilization was estimated qualitatively and was not optimized for each compound: efficient means more than only band 6 (SDS-PAGE) was in the supernatant, and selective means no remarkable amount of bands 1, 2, or 5 was in the supernatant.

TABLE III. Stability of D-Glucose Transporter Activity Solubilized in Emulphogene

Emulphogene concentration (%)	D-glucose transporter activity units/μg protein				
	Day 0	1	2	3	4
0.2	1.2 ± 0.2 (6)	0.34 ± 0.05 (10)	0.21 ± 0.08 (2)	0.06 ± 0.04 (3)	—
0.1	1.2 ± 0.4 (7)	1.1 ± 0.5 (4)	0.68 ± 0.35 (3)	0.58 ± 0.28 (4)	0.33 ± 0.05 (2)
0.05	0.85 ± 0.33 (4)	—	0.63 ± 0.33 (3)	0.34	—
0.025	1.7 ± 0.3 (2)	—	1.7 ± 0.1 (2)	—	—

To a 100,000g (1 hr) supernatant of a 25% ghost suspension in 0.1% Emulphogene (0°C, 20 min incubation), was added either buffer or Emulphogene to produce the indicated detergent concentrations. The solutions were kept at 4°C for periods up to 4 days and then assayed for transporter activity. Values represent the specific activity ± SD (N) of transporter; 1 unit = pmole net D-glucose transported (influx)/min. The initial 0.1% Emulphogene supernatant contained 60% of the original transporter activity (in the whole membrane/detergent suspension) and its specific activity was about 1.7 times that present in whole ghosts.

be scanned for protein by UV absorbance. A relatively slow decay of transporter activity occurred in the cold if detergent concentrations were kept low (Table III); in 0.1% detergent, the transporter was sufficiently stable to anticipate a respectable recovery from the affinity chromatography procedure if the operation could be completed within 2–3 days. We therefore routinely solubilized ghosts in 0.2% Emulphogene at 0°C and then diluted the mixture before its application to the affinity matrix.

Chromatography of Emulphogene-Solubilized Erythrocyte Membrane Proteins on PBA Affinity Columns

Extrinsic proteins were first removed from ghosts with 0.1 mM EDTA, pH 8. A standard extract (0.1% Emulphogene-5 mM EDTA, pH 7.4, 20 min at 0°C) of the resultant membranes (20 ml; 0.2 mg protein) was applied to a 3-ml Emulphogene preequilibrated column at 4°C (15 ml/hr). Pooled fractions were then concentrated and analyzed on SDS-PAGE. The initial unabsorbed protein peak (18% of that applied) contained diffuse staining bands, including residual 4.1, 4.2, and 6 (or 7). Elution with 1 M glucose in detergent (180 ml) delivered less than 3% of the added protein and the material was poorly defined on SDS-PAGE. (In the earliest experiments, the glucose eluate contained a single Coomassie blue staining doublet band in the 4.5 region but only after the large elution volume was extensively concentrated [31]. It was afterwards found to be a solvent contaminant.) Elution with 1 M KNO_3 yielded 10% protein and a final elution with lithium trichloroacetate (LiTCA) removed an additional 13%. These chaotropic agents eluted the intrinsic proteins, including bands 3, 4.5, and 7, but about half of the protein applied could not be recovered.

Interaction Between PBA Affigel and Glucose Transporter

The more hydrophilic matrix, Affigel-202 on which PBA is linked via a 10 atom succinamide leash, was also examined as an affinity chromatography medium. Varying amounts of PBA Affigel or the control matrix, aniline Affigel, were added to 0.1% detergent extracts of ghost proteins to determine the ability of the beads to selectively adsorb the sugar transporter. The results of a typical experiment are shown in Table IV. Loss of transporter is directly related to the amount of PBA Affigel added; more than 90% appears to be bound upon exposure to 100 mg of beads for 2 hr at 4°C. Except at the highest levels of beads used, the amount of adsorbed protein was low, variable, and did not correlate with the loss of transporter. Results using the aniline-derivatized matrix were markedly different; even though 100 mg of this material bound about ten times the amount of protein, no transporter activity was removed. Thus, in one step, the aniline matrix can increase the specific activity of the transporter about tenfold.

Other evidence that PBA Affigel selectively adsorbs the transporter was gained from preliminary tests with minicolumns to determine whether the transporter would survive percolation through the beads. Columns of PBA and aniline Affigel (150 μl settled volume) were deliberately overloaded with large volumes of Emulphogene extracts and the eluates were analyzed for carrier activity and protein. Figure 5 shows the elution profile obtained with the PBA matrix. Nearly 15 column void volumes of extract were applied before significant amounts of transporter activity was detected in the column outflow. Specific activity in the eluate reached that present in the applied sample only after 6 ml of extract had been added. In sharp contrast, high levels of protein appeared in the earliest fractions and all but about 2% was recovered in the combined eluate and wash. Results with the aniline derivative (not shown) were again quite different; specific activity of the transporter in the earliest eluate fractions was actually increased over that applied and nonspecifically adsorbed proteins were difficult to remove with buffered detergent.

TABLE IV. Glucose Transporter and Protein Binding Capacity of Affigel Derivatives

Beads (mg)	Derivative	Transporter activity ± SD (N) units/μg protein	Protein adsorbed (%)
0	Control	0.47 ± 0.16 (54)	—
5	PBA Affigel	0.44 ± 0.06 (8)	1.7
20	PBA Affigel	0.34 ± 0.06 (8)	4
50	PBA Affigel	0.08 ± 0.05 (28)	2.6
100	PBA Affigel	0.04 ± 0.03 (20)	2.9
350	PBA Affigel	0.02 ± 0.02 (8)	11
500	PBA Affigel	0.05 ± 0.02 (4)	16
20	Aniline Affigel	0.47 ± 0.01 (4)	2.6
100	Aniline Affigel	0.51 ± 0.11 (8)	29

Derivatized Affigel beads were first equilibrated in buffered 0.1% Emulphogene and recovered by suction such that 100 mg had a volume of 120 μl. Varying amounts were added to 1.4 ml of a standard 0.1% detergent extract in microtubes and then shaken for 2 hr at 4°C in an Eppendorf shaker. After 30 sec centrifugation, supernatants were assayed for protein and transporter activity. Protein concentration in the original extract was 350 μg/ml.

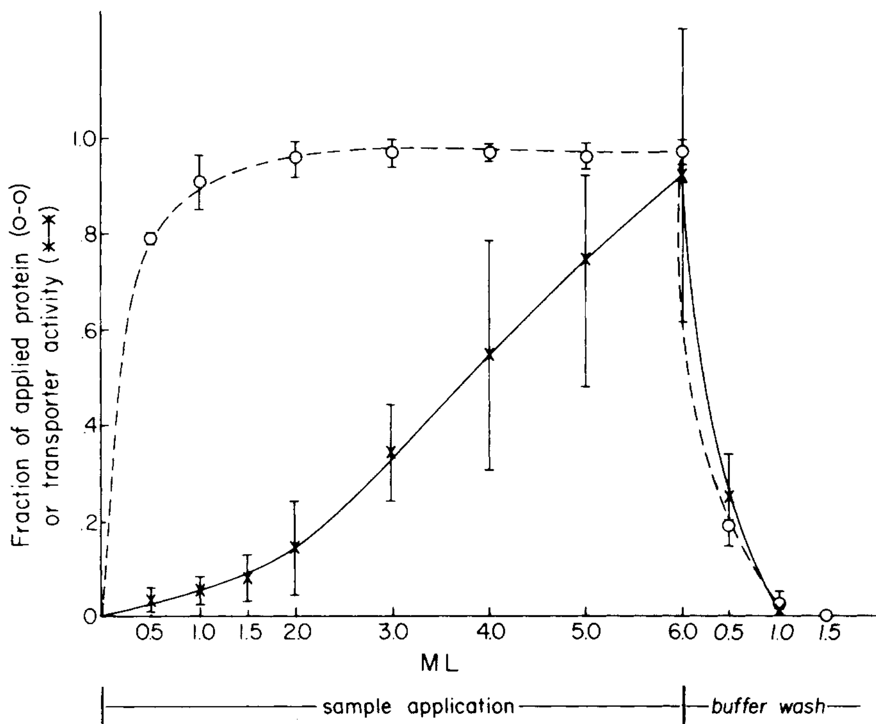

Fig. 5. Application of "saturating" quantities of Emulphogene extract to a PBA Affigel minicolumn. Six milliliters of an Emulphogene extract was applied to a 150-μl preequilibrated PBA Affigel column at 4°C over a 2-hr period; this was followed by a wash of 0.1% buffered detergent. Eluates were collected in the volumes indicated and assayed for protein and transporter activity. Results are expresed as the fraction of transporter activity and protein compared to the applied extract. Observations represent the mean ± SD of replicate assays of fractions from four columns. The mean transporter activity and protein concentration in the applied extracts for the four experiments were 164 ± 58 units/ml and 383 ± 47 μg/ml, respectively. Maximum binding capacity for 150 μl PBA Affigel was about 500 units of transporter.

Many experiments were performed with the PBA Affigel matrix in attempts to obtain a selective elution of the glucose transporter, but none were successful. Molar levels of D-glucose and high concentrations of phloretin failed to elute either transporter activity or a definable protein even when the Emulphogene extract applied to the column possessed high carrier specific activity and was composed primarily of bands 3, 4.5, and 7 (extrinsic proteins had been removed with a pH 12 extraction; see Table IV). Intrinsic proteins were eluted only when the columns were treated with 1 M KNO_3 or with a 1% SDS strip. As was the case for the PBA Sepharose, it seems evident that the transporter protein is bound too avidly also to the PBA Affigel matrix for it to be eluted in a functional condition.

DISCUSSION

Liposomes have often been used as the system to demonstrate D-glucose transporter activity in detergent-solubilized erythrocyte membranes [1,2,23]. The two

common reconstitution methods—dialysis of a detergent solution of excess lipid and protein or sonication of a lipid-protein suspension—produce liposomes with different characteristics: 1) Sonicated liposomes are larger and less uniform (30–80 nm diameter) than, for example, vesicles formed by dialysis of a cholate solution of lipids and protein (30 nm diameter). 2) The orientation of the transporter (based on the availability of its cytochalasin B binding site to trypsin digestion) in sonicated liposomes is 50% inside out, while in the cholate liposomes, 75% are inside out [32]. Thus, liposomes formed by sonication possess the dual advantages of having a larger intramolecular space and of having twice the proportion of transporter molecules oriented in the influx mode.

In our hands, the reconstitution method of freezing and thawing preformed liposomes in the presence of added erythrocyte membrane proteins [1,24] was fairly successful. The behavior of proteoliposomes in media of different osmolarities (cf Fig. 4), the linearity of semilogarithmic influx curves over a wide range (data not shown), the similarity of equilibrium values among liposomes with different amounts of associated ghost proteins, and the distribution of sugar transporter in these liposomes (ca 50% carrier incorporation when the lipid to protein ratio was 40 to 1) suggest that a remarkably homogeneous population of reconstituted proteoliposomes was formed. Their formation is not directly influenced by the pretreatment of the ghosts (extractions, solubilization) if the lipid/protein ratio is kept high. The specific activity of the reconstituted transporter, determined by equilibrium exchange rates (which are not dependent on the orientation of the carrier on the membrane), was similar to or greater than that reported by Kasahara and Hinkle [1], Zoccoli et al [2], Zoccoli and Lienhard [20], or Fröman et al [33].

Transporter Stability

Kahlenberg and Zala [34] found that when the glucose transporter was solubilized in 0.1% Triton X-100, it had a half-life of 2 hr at 20°C and was completely inactivated after 10 min at 40°C. Phutrakul and Jones [35] found similar results using a black lipid membrane assay. The transporter is also unstable in 25 mM cholate; more than half activity is lost in 24 hr at 4°C [36]. Solubilization in Emulphogene has less of an adverse effect on transporter stability; its deterioration is dependent on detergent concentration. The carrier was most stable in 0.025% (Table III); even in 0.1% Emulphogene at 0°C ($t_{1/2}$ = 3 days) stability was greater than in 0.1% Triton X-100. Explanations for the instability include 1) the presence of denaturing contaminants in the detergent [22,37], 2) a detergent-concentration dependent, irreversible conformation change of the protein, 3) slow oxidation of essential amino acid groups, and 4) proteolysis, owing to detergent-activated endogenous enzymes. At least three proteases exist in ghost membranes [38–40] and their activity has been shown to be accelerated by nonionic detergents [39,41,42].

The Insolubilized Phloretin Derivatives

Both the batch treatment experiments and column chromatography provide evidence that the Emulphogene-solubilized glucose transporter was tightly bound to both PBA matrices; however, protein binding and carrier recovery differed. Even after treatment with chaotropic agents, protein recovery was incomplete although (some of) bands 3, 4.5, and 7 could be desorbed from both matrices. Elution with 1% SDS yielded all of the protein bound to PBA Affigel but recovery from the PBA

Sepharose was obtained only at elevated temperature with SDS. Strong hydrophobic binding plus poor solubility of the protein in the absence of natural lipid may account for the failure of glucose or phloretin to elute transporter, even from PBA Affigel; even if glucose or phloretin could compete for the ligand binding site, other portions of the protein apparently remain bound, either at the hydrophobic sites on the spacer arm or within the porous beads. Evidence for this latter consideration comes from some preliminary experiments showing that transporter adsorption to batches of PBA Affigel occurred at an unexpectedly slow rate and not by a simple first-order process. A very rapid loss occurred within 1 min and 50% of the activity was bound only after 30 min at 4°C. This suggests that a large proportion of the ligand groups are cryptic, situated in the interior of the beads, and constitute sites from which release of transporter is difficult. It is possible that conditions may be found under which these phloretin derivatives could display the expected selectivity as affinity adsorbents.

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