Reconstituted D-Glucose Transport from the Adipocyte Plasma Membrane. Chromatographic Resolution of Transport Activity from Membrane Glycoproteins Using Immobilized Concanavalin A†

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ABSTRACT: Adipocyte intrinsic membrane proteins were solubilized in sodium cholate containing buffer, added to cholate-dispersed phospholipids, and reconstituted on Sephadex G-50 columns as previously described [Shanahan, M. F., & Czech, M. P. (1977) *J. Biol. Chem. 252*, 8341–8343]. Reconstituted vesicles (0.02-μm diameter) which eluted from the columns were subjected to a freeze–thaw step which resulted in extensive vesicle fusion and enlargement. Brief sonication of the latter preparation reduced the size of the vesicles (0.1–0.5-μm diameter) which then exhibited a time-dependent uptake of D-[^{3}H]glucose that was inhibited by 50 μM cytochalasin B or 90 mM D-glucose and 3-*O*-methylglucose but not by L-glucose. Cytochalasin B sensitive D-glucose transport activity was obtained in reconstituted vesicles irrespective of the monovalent cation (KCl, NaCl, or choline chloride) used in their preparation and incubation. Transport activity was absolutely dependent upon the presence of divalent cations. Both Ca^{2+} and Mg^{2+} were active in this role and had to be added before the freeze–thaw and sonication steps to be effective. Cholate-solubilized intrinsic membrane proteins were fractionated prior to reconstitution by a number of chromatographic techniques in order to further characterize and purify the hexose transporter. Sepharose 6B chromatography in the presence of 0.5% sodium cholate indicated that the transport protein has a Stokes radius between 60 and 80 Å. Hydroxylapatite chromatography in cholate-containing phosphate buffer resolved the solubilized protein into a void peak and two broad peaks. The proteins in the void peak and in the peak eluting between 15 and 100 mM K_2HPO_4–KH_2PO_4 contained up to 80% of the total protein, yet exhibited no transport activity when reconstituted into artificial liposomes. The remaining proteins of the second peak which eluted between 150 and 350 mM K_2HPO_4–KH_2PO_4 contained all the D-glucose transport activity. No unique protein bands were visible following dodecyl sulfate gel electrophoresis of the transporting fraction compared to the nontransporting fractions, suggesting that the transport protein is a very minor component of the adipocyte membrane. In concert with this conclusion, passage of the intrinsic membrane proteins through a column of concanavalin A–agarose completely removed the two major adipocyte intrinsic membrane glycoproteins (94 000 and 78 000 daltons) as well as several minor glycoproteins without depleting the transport activity. Adsorption by five other immobilized lectins also failed to prevent subsequent reconstitution of D-glucose transport activity into phospholipid vesicles. All three major protein bands of the cholate-solubilized extracted membrane (94 000, 78 000, and 68 000 daltons) and the multiple minor protein bands could be resolved from the glucose transporter by using concanavalin A–agarose and Sepharose 6B chromatography sequentially. This resulted in substantial purification of the transport system. It is concluded that (1) the adipocyte D-glucose transporter is apparently devoid of oligosaccharide units that can bind to concanavalin A, a lectin known to activate hexose transport in intact fat cells, and (2) the D-glucose transporter is only a minor component of the adipocyte intrinsic membrane protein and exhibits a Stokes radius of 60–80 Å. This size exceeds the width of the plasma membrane phospholipid matrix, consistent with a transmembrane disposition.

Reconstitution of solubilized membrane components into artificial phospholipid bilayer membranes has proven to be a productive approach to the study of the molecular basis of biological transport phenomena. A well characterized system in which membrane proteins are inserted into phospholipid vesicles with recovery of transport activity is valuable as an assay tool for transport purification schemes as well as for investigating effects of specific bilayer components on transport activity. Only two facilitated D-glucose transport systems, the human erythrocyte (Kasahara & Hinkle, 1977; Kahlenberg & Zala, 1977; Goldin & Rhoden, 1978) and rat adipocyte (Shanahan & Czech, 1977b) systems, have been successfully reconstituted. The adipocyte D-glucose transporter is of particular interest because it is exquisitely sensitive to regulation by hormones and other agents (Czech, 1976).

We have previously demonstrated that elution of extrinsic proteins from the adipocyte plasma membrane by incubation with dimethylmaleic anhydride followed by selective extraction of the remaining proteins with sodium cholate results in a soluble preparation greatly enriched in a 94 000-dalton glycoprotein fraction but also containing smaller amounts of other proteins (Shanahan & Czech, 1977b). This extract was found to exhibit stereospecific D-glucose transport activity subsequent to its reconstitution into phospholipid vesicles composed of phosphatidylcholine and phosphatidylethanolamine (1:1) from egg yolk (Shanahan & Czech, 1977b). The aims of the present studies were twofold, to document the properties of this reconstituted adipocyte hexose transport system in more detail and to further purify and characterize the D-glucose transport protein or proteins.

Materials and Methods

Isolation of Fat Cells. Isolated white fat cells were obtained (Rodbell, 1966) by enzymatic digestion of the omental and parametrial adipose tissue of 250–700-g female Sprague-Dawley rats. Adipose tissue (100–300 g) from 10 or more rats was minced and digested for 60 min at 37 °C in Krebs-Ringer

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phosphate buffer, pH 7.4, containing 3% bovine serum albumin (Armour) and 1 mg/mL crude collagenase (*Clostridium histolyticum*, Worthington). Krebs-Ringer phosphate buffer contains 128 mM NaCl, 1.4 mM $CaCl_2$, 1.4 mM $MgSO_4$, 5.2 mM KCl, and 10 mM Na_2HPO_4. Following digestion, cells were filtered through one layer of nylon chiffon and washed twice with warm Krebs-Ringer phosphate buffer with 1% albumin and once with warm 0.25 M sucrose, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.4. Some cell preparations were washed twice with the warm sucrose buffer rather than with Krebs-Ringer phosphate buffer containing albumin to minimize possible contamination of the final membrane preparation with albumin.

Preparation of Plasma Membranes. A crude fat cell plasma membrane fraction was prepared as previously described with minor modifications (Shanahan & Czech, 1977a). Washed and diluted cells were disrupted by using either a Brinkman polytron for 30 s (large preparations) or a glass homogenizing tube fitted with a Teflon pestle (small preparations). Cell homogenates were centrifuged at 8500*g* for 10 min. The supernatant as well as a small amount of fluffy white material collected from the surface of the brown (mitochondrial) pellet was centrifuged at 40000*g* for 30 min. The resulting pellet was resuspended in ice-cold 1 mM EDTA and 10 mM Tris-HCl (pH 7.5). The cell "homogenate" was sometimes spun first at 32600*g* for 25 min to increase the membrane yield. The 32600*g* pellets were resuspended in ice-cold sucrose buffer and spun at 5000*g* for 10 min. The 5000*g* supernatants were saved, and the 5000*g* pellets were resuspended and centrifuged again at 5000*g* for 10 min. The combined 5000*g* supernatants were spun at 36000*g* for 10 min. The final pellet containing membranes was resuspended in 10 mM Tris-HCl and 1 mM EDTA, pH 7.5.

Membrane Extraction and Solubilization. Dimethylmaleic anhydride extracted membranes were prepared as previously described by Shanahan & Czech (1977a). Briefly, the membranes were diluted into water to give a final ratio of membranes from 1 g of fat per 1 mL of water of 1:15. Solid dimethylmaleic anhydride (1.6–2 mg/mL, Sigma) was added with constant stirring while maintaining the pH at 8.0 with 1 to 2 N NaOH. When the reaction was completed and the pH was stable, the suspension was centrifuged at 40000*g* for 45 min and the pellet was resuspended and frozen in 10 mM Tris-HCl and 1 mM EDTA, pH 6.8.

For sodium cholate extractions, frozen dimethylmaleic anhydride extracted membranes were thawed and resuspended in an appropriate buffer. Sodium cholate (Sigma) was added to a final concentration of 2% (w/v) to 1–12 mg of membrane protein in 1 to 2 mL of buffer. Following 30–60 min of incubation on ice, the solubilized membranes were centrifuged as above. The supernatant was stored on ice for column fractionation or reconstitution with exogenous phospholipids. Sixty to eighty percent of the membrane protein was routinely solubilized by using this procedure.

Hydroxylapatite Chromatography. For the experiments reported in Figures 2 and 3, solubilized protein was added to a column (28 × 1.0 cm) containing hydroxylapatite (HTP, Bio-Rad) equilibrated in 10 mM K_2HPO_4–KH_2PO_4, 0.5% sodium cholate, and 0.02% NaN_3, pH 7.4. The solubilized protein was eluted by using a linear K_2HPO_4–KH_2PO_4 gradient. Elution was complete by 350 mM K_2HPO_4–KH_2PO_4. Addition of 1 M K_2HPO_4–KH_2PO_4 did not elute more protein. All chromatography work was done at 4–6 °C.

Sepharose 6B Chromatography. For the experiments reported in Figures 4 and 5, 1 mL of solubilized protein was added to a column (89 × 1.5 cm and 43 × 1.5 cm, respectively) containing Sepharose 6B-100 (Sigma) equilibrated at 4 °C with 100 mM NaCl, 10 mM Na_2HPO_4–NaH_2PO_4, 0.5% sodium cholate, and 0.02% NaN_3, pH 7.4. The columns were calibrated by chromatographing standard proteins in the presence of the same cholate-containing buffers. The Stokes radii of the eluted fractions were calculated by using standards of known radius and the parameter $(-\log K_{av})^{1/2}$, where $K_{av} = (V_e - V_0)/(V_t - V_0)$ (Laurent & Killander, 1964). V_e is the elution volume corresponding to the peak concentration of the solute, V_0 is the void volume of the column as determined by the appearance of blue dextran (Sigma), and V_t is the total volume of the gel bed. A linear relationship exists between the Stokes radii of the standard proteins and the parameter $(-\log K_{av})^{1/2}$ even in the presence of 0.5% sodium cholate. Thyroglobulin (Type II), ferritin (Type 1), and aldolase (Grade III from rabbit muscle) were obtained from Sigma.

Immobilized Lectin Chromatography. Solubilized membrane proteins were diluted 1:3 to obtain a final concentration of 0.5% sodium cholate in 100 mM NaCl and 10 mM Tris-HCl, pH 7.4 (buffer A). The diluted proteins were added to the appropriate immobilized lectin which had been extensively washed in the same detergent-containing buffer. The proteins were incubated with the immobilized lectins for 1 h at room temperature or on ice with occasional mixing. The nonadsorbed proteins were collected, and the immobilized lectins were washed with 1 to 2 bed volumes of the buffer. Adsorbed proteins were eluted following a 30-min incubation at room temperature (22 °C) with the same buffer containing 100–400 mM of the appropriate sugar. The temperature during adsorption or elution appeared to have little effect on the amount of protein bound or eluted or on the glucose transport activity observed in the reconstituted vesicles. Concanavalin A–agarose and wheat germ agglutinin–Sepharose were obtained from Sigma, peanut agglutinin–agarose, soybean agglutinin–agarose, and *Ulex europaeus* agglutinin 1–agarose were from Vector Laboratories, and *Ricinus communis* agglutinin 1–agarose was from P-L Biochemicals. Bio-Gel A1.5, 50–100 mesh, came from Bio-Rad Laboratories.

Sample Concentration. Chromatography column fractions (1 mL) were assayed for protein (see below) and pooled as indicated in the figure legends. The pooled samples were concentrated and/or dialyzed by using either vacuum dialysis (25 000 molecular weight cutoff collodion bags, Arthur Thomas) at 4 °C or ultrafiltration followed by diafiltration (PM 10 membranes, MMC Multiphor, Amicon Corp.) at room temperature (22 °C). A portion of the membrane protein (up to one-third) was lost during concentration using either method. Greater glucose transport activity was retained following concentration if 2.5 mg of egg phosphatidylethanolamine (Supelco) and 2.5 mg of egg phosphatidylcholine (Type III-E, Sigma) were first dispersed by sonication in 250 μL of the appropriate sample buffer containing 2% sodium cholate and then added to each pooled sample prior to concentration. Aliquots of the concentrated protein (300–600 μL) were used to assay for D-glucose transport activity by using the reconstitution assay and/or protein composition by using polyacrylamide gel electrophoresis.

Reconstitution of D-Glucose Transport Activity. Concentrated proteins (250 μL) were added to exogenous phospholipids (2.5 mg of egg phosphatidylethanolamine and 2.5 mg of egg phosphatidylcholine) dispersed by sonication in 250 μL of buffer A containing 2% sodium cholate. Liposomes were prepared from this mixture by using the method of Brunner et al. (1976) as modified by Shanahan & Czech (1977b).

Briefly, the samples were added to a Sephadex G-50-80 (Sigma) column (16 × 1 cm) equilibrated in and eluted with buffer A containing 2 mM $MgSO_4$ at 4 °C (flow rate 12 mL/h). The phospholipid-containing void volume (700–800 μL) was collected, rapidly frozen in dry ice–acetone, thawed under cold tap water, and sonicated in an ultrasonic (Branson) bath for 5 s unless noted otherwise. The data in Table I and Figure 1 outline the effects of the freeze–thaw and sonication steps on transport activity and morphology.

In several experiments, it was desirable to prepare vesicles in large amounts. In these experiments (Figure 1 and Tables I, II, and IV) 4 times the standard amount of cholate-solubilized membrane protein (1 mL) was combined with 4 times the amount of exogenous phospholipid (20 mg in 1 mL) before passage through a column (20 × 1.5 cm) containing Sephadex G-50. Vesicles (3 to 4 mL) were collected in the void volume and subjected to the freeze–thaw and sonication steps as described above.

Glucose Transport in Liposomes. Assay of D-glucose uptake was routinely performed by addition of 40–45 μL of the reconstituted liposomes to glass test tubes as described in detail previously (Shanahan & Czech, 1977a). Cytochalasin B in 0.5 μL of 95% EtOH (final concentration 20 μM unless noted otherwise, Aldrich) or an equal volume of 95% EtOH was added at least 5 min prior to addition of labeled glucose. Transport was initiated by the addition of 5 μL of D-[^{3}H]-glucose (2–4 μCi/tube, 250 μM final concentration, New England Nuclear) in 100 mM NaCl. The suspension was immediately decanted onto Amicon filters (0.2-μm pore size, 25-mm diameter) in a Gelman filter holder (50 mL) attached to a rotary Doerr pump and quickly washed twice with 1 mL of ice-cold buffer. Filters were air-dried and immersed in 4 mL of ACS (Amersham/Searle) liquid scintillation fluid for counting. Intravesicular space determinations were based on the assumption that 250 μM [^{14}C]urea (0.75–1.5 μCi/tube, New England Nuclear) equilibrates across the vesicles within 5 min of incubation or D-[^{3}H]glucose does within 60 min. Increased incubation times rarely altered the uptake in either case. The urea values may sometimes give slight underestimates of space because urea may leak out during the filtration process (not shown). Nevertheless, urea spaces should reliably indicate size differences between vesicles.

Uptake of label was expressed as the amount of radioactivity accumulated at a given time minus the radioactivity bound on filters containing membranes to which D-[^{3}H]glucose and 1 mL of cold buffer were added simultaneously. Under the conditions of our experiments, these control values represented between 8 and 25% of the total radioactivity obtained from 2-min incubations depending on the amount of membrane present. Most of the figures and tables give the results of one representative experiment in which the transport values were determined in triplicate or quadruplicate.

Electrophoresis. The membrane protein composition of the various column fractions was analyzed by using the NaDod-SO_4[1]–polyacrylamide gel electrophoresis procedure described by Laemmli (1970) with a 3.5% acrylamide stacking gel and 7.5% separating gel. Samples containing 50–100 μg of protein were boiled for 1 min in $NaDodSO_4$ (1%) and DTT (50 mM) containing buffer prior to gel application. The 1-mm thick slab gels were typically run at 25 mA for 4 to 5 h. The gels were stained overnight with 0.05% Coomassie Blue R (Sigma) in 25% 2-propanol and 7% acetic acid. The molecular weight standards routinely run were the following: β-galactosidase, 116 000 (gift of Dr. Boris Rotman, Brown University); phosphorylase *b*, 94 000 (Sigma); bovine serum albumin, 68 000 (Armour); ovalbumin, 45 000 (Sigma); carbonic anhydrase, 31 000 (Sigma). A sixth molecular weight is indicated in the figures in order to identify the previously described 78 000 molecular weight protein (Shanahan & Czech, 1977b; Czech & Lynn, 1973b).

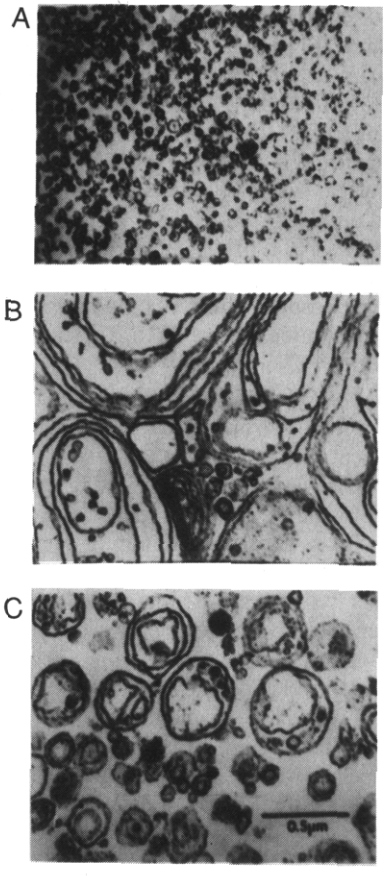

FIGURE 1: Electron microscopy of reconstituted vesicles at various stages during preparation. Reconstituted vesicles were prepared by the large-batch method and prepared for electron microscopy (see Materials and Methods) at the three stages of preparation as described in footnote *a* of Table I. (A) Vesicles eluted from Sephadex G-50; (B) vesicles following the freeze–thaw cycle; (C) vesicles subjected to freeze–thaw and sonication (20 s) steps.

Protein and Phosphate Assays. Membrane protein was estimated by a modification (Geiger & Bessman, 1972) of the method of Lowry et al. (1951) using a 5:3 mixture of human albumin and human globulin (Sigma protein standard) as a standard. $NaDodSO_4$ was added in sufficient quantities to clarify phospholipid-containing samples.

Phosphate concentrations of the fractions eluted from the hydroxylapatite column were determined by using the method of Ames (1966).

Electron Microscopy. The pelleted vesicles were fixed in Karnovsky's fixative, rinsed in sodium cacodylate buffer, and postfixed in 1% osmium in sodium cacodylate for 1 h. The samples were embedded in Epon 812, stained with uranyl acetate and lead citrate, and viewed with a Phillips 201 A electron microscope.

Results

Figure 1 presents the morphology of reconstituted phospholipid vesicles containing adipocyte membrane proteins and a 1:1 ratio of phosphatidylcholine and phosphatidylethanolamine at three stages of their preparation following elution

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol.

Table I: Effect of a Freeze–Thaw Cycle and Sonication on the Transport Properties of Reconstituted Membranes[a]

	uptake [nmol/mg of protein (±SE)]			
vesicle treatment	1 min	1 min plus cytochalasin B	60 min	60 min plus cytochalasin B
none	0.08 ± 0.02	0.06 ± 0.01	0.07 ± 0.03	0.09 ± 0.04
freeze–thaw	1.1 ± 0.4	1.3 ± 0.04	1.9 ± 0	2.2 ± 0.1
freeze–thaw plus sonication	1.2 ± 0	0.65 ± 0.05	2.7 ± 0.1	2.1 ± 0.1

[a] Cholate-solubilized phospholipids (phosphatidylcholine–phosphatidylethanolamine, 1:1) were mixed with cholate-solubilized dimethylmaleic anhydride extracted membranes according to the large-batch procedure and chromatographed on Sephadex G-50. The reconstituted vesicles collected in the void volume (3.8 mL) were divided into three portions (1.2 mL). One received no further treatment, another was subjected to the standard freeze–thaw cycle, and the third was subjected to the freeze–thaw cycle followed by a 20-s sonication. The vesicles were assayed in triplicate at the indicated times. Cytochalasin B was present where indicated at a concentration of 50 μM.

Table II: Effects of Unlabeled L-Glucose, D-Glucose, and 3-*O*-Methylglucose on the Uptake of D-[^{3}H]Glucose by Reconstituted Vesicles[a]

	D-[^{3}H]glucose uptake (% of equilibration value)		
unlabeled hexose	control	cytochalasin B	net D-glucose transport[b]
L-glucose	54 ± 11	19 ± 2	35 ± 9
D-glucose	26 ± 4	18 ± 7	8 ± 2
3-*O*-methylglucose	17 ± 4	17 ± 1	0 ± 3

[a] Cholate-solubilized phospholipids were mixed with cholate-solubilized dimethylmaleic anhydride extracted membranes and applied to a Sephadex G-50 column using the large-batch reconstitution procedure. The reconstituted vesicles collected in the void volume (3 mL) were divided into three portions (0.7 mL). Then 70 μL of unlabeled L-glucose, D-glucose, or 3-*O*-methylglucose was added to the respective aliquots (90 mM final concentration) prior to the freeze–thaw sonication (20 s) cycle. The 1-min uptake values were normalized as percentages of a 60-min D-[^{3}H]glucose equilibration value (indicator of intravesicular water space) to account for small differences in the vesicle size among groups. The values represent the mean ± standard error of three experiments performed in triplicate on three separate days. Net D-glucose transport refers to the mean ± standard error of the cytochalasin B sensitive component [control minus cytochalasin B (50 μM) value] of total D-[^{3}H]glucose uptake measured at 1 min. [b] % of equilibration value.

from a Sephadex G-50 column. Panel A shows an electron micrograph of such reconstituted vesicles as they appear in the void volume of the column using the large-batch preparation procedure. The vesicles were relatively homogeneous and bounded by a single bilayer. The mean diameter was apparently in the 0.02-μm range. The filtration method employed in these studies proved inadequate to measure D-glucose transport into these small vesicles (Table I). They were not retained by the filters as evidenced by the recovery of protein in the filtrate (not shown). Attempts to measure uptake by using filters with smaller pore size have failed because of the very low flow rates obtained with such filters (not shown).

Subsequent to a rapid freeze–thaw cycle, a dramatic and extensive fusion of vesicles occurred. Large sheets of bilayers formed which appear to enclose a water space and other concentric bilayers (Figure 1B). A time-dependent uptake of D-glucose into these phospholipid–protein structures was observed. About twice as much D-glucose uptake was found after 60 min of incubation compared to that for 1 min (Table I). However, no sensitivity of this uptake to cytochalasin B was detected at either time point monitored.

Bath sonication of the freeze-thawed material for 20 s resulted in the formation of enclosed bilayers of ~0.1–0.5 μm in diameter, some of which enclosed smaller vesicles (Figure 1). In contrast to the nonsonicated vesicles, these vesicles were capable of D-glucose transport which was sensitive to inhibition by cytochalasin B (Table I). Cytochalasin B inhibited the uptake of D-glucose in 2 min by almost 50%. While most preparations exhibited a 40–60% inhibition of transport by cytochalasin B, some preparations were inhibited by as much as 80%. Transport was not decreased further by inclusion of cytochalasin B during the freeze–thaw step, suggesting that externally added cytochalasin B was effective in reaching both internal and external binding sites (data not shown). Cytochalasin B inhibited uptake at 60 min by only 22%, suggesting that D-glucose was equilibrated or nearly equilibrated across the membranes in the control but not in the cytochalasin B inhibited vesicles. In subsequent experiments we used the 60-min D-glucose values to estimate intravesicular water spaces.

The amount of measurable, time-dependent and cytochalasin B sensitive hexose transport activity was found to depend upon the actual length of sonication. Shorter times (5–10 s) resulted in vesicles having uptake values twice as high as those sonicated for 20 s, although the percent inhibition due to cytochalasin B remained constant (data not shown). Longer times (4 min) decreased the measurable uptake to a negligible amount. Electron microscopy showed an inverse correlation between vesicle size and the length of sonication period (not shown). The low rates of uptake in vesicles sonicated for long time periods probably reflect, at least in part, the escape of smaller vesicles through the Amicon filters.

Various unlabeled sugars were tested for their ability to inhibit the initial rates of D-glucose uptake in order to demonstrate that the reconstituted D-glucose transport system retains the sugar specificity of the adipocyte transport system. The unlabeled sugars were added to the vesicles prior to the freeze–thaw step to allow them to equilibrate across the membrane, thereby minimizing osmotic effects of high concentrations of hexoses on the reconstituted vesicle preparation. Subsequent to freeze–thaw and sonication, these vesicles were incubated with D-[^{3}H]glucose. Uptake of label was measured at 1 and 60 min in the presence and absence of cytochalasin B (Table II). The estimates of the initial rate of uptake measured at 1 min were expressed as a percent of the final equilibration value (60 min) of each preparation in order to normalize the slight differences in vesicle size characteristic of different preparations sonicated individually. Table II shows that vesicles equilibrated with 90 mM L-glucose before assay of D-[^{3}H]glucose uptake displayed the usual cytochalasin B sensitive transport rate at 1 min. Net transport (control minus cytochalasin B) in vesicles equilibrated with D-glucose, however, was markedly inhibited. Vesicles equilibrated with 90 mM 3-*O*-methylglucose exhibited no D-[^{3}H]glucose uptake over that observed in the presence of cytochalasin B.

Earlier studies on native adipocyte plasma membranes showed that similar hexose transport activity is observed in isoosmolar salt solutions or sucrose. However, cations tightly bound to the membrane matrix might not be released upon incubation of membranes in 0.25 M sucrose. The reconstituted preparation provides an advantage to studying the cation requirements of the transport system because bound cations should be released during the extraction with dimethylmaleic

Table III: Effectiveness of Potassium Chloride and Choline Chloride Substitution for Sodium Chloride in the Preparation of Reconstituted Vesicles Which Exhibit Hexose Transport Activity[a]

	D-[^{3}H]glucose (nmol/mg of protein)		
	3 min		
salt	control	plus cytochalasin B	60 min, control
NaCl	11.6	6.8	17.0
KCl	12.3	6.1	22.1
choline chloride	13.9	6.8	29.9

[a] Dimethylmaleic anhydride extracted membranes and phospholipids were solubilized in 2% Tris-cholate, pH 7.5, instead of sodium cholate. Solubilized membrane protein (0.23 mL) was added to 0.25 mL of solubilized phospholipids (2.5 mg each of phosphatidylcholine and phosphatidylethanolamine) in the standard buffer or in buffer where the NaCl was substituted with equimolar amounts of KCl or choline chloride as indicated. The membrane protein–phospholipid mixtures were passed through small Sephadex G-50 columns equilibrated in the appropriate buffer. The vesicles collected in the void volume were subjected to the freeze–thaw and sonication (5 s) steps and dispensed (45 μL) into test tubes with or without 50 μM cytochalasin B. Five minutes later, D-[^{3}H]glucose uptake was monitored for 3 and 60 min.

anhydride, the cholate solubilization in EDTA, and/or the passage through Sephadex G-50. Phospholipids were solubilized in the presence of 100 mM NaCl, KCl, or choline chloride before being mixed with solubilized protein in order to investigate possible effects of monovalent cations on transport activity. Vesicles were prepared on columns of Sephadex G-50 eluted with buffers containing 100 mM of the respective salt substituted for NaCl. Table III shows that vesicles reconstituted in the presence of KCl or choline chloride exhibited a time-dependent D-glucose uptake which was comparable to that observed in the presence of NaCl. A maximal dose of cytochalasin B inhibited hexose transport in all vesicle preparations to a similar extent.

A second series of experiments studied the effect of divalent cations on transport in reconstituted vesicles (Table IV). For protocol 1, two groups of vesicles were prepared on Sephadex columns eluted with buffer A (no $MgCl_2$). The vesicles were divided into two groups, one of which received an addition of $MgCl_2$ at a final concentration of 2.2 mM. Table IV shows that vesicles prepared in the absence of $MgCl_2$ throughout exhibited no cytochalasin B sensitive uptake of D-glucose. Furthermore, addition of 2.2 mM $MgCl_2$ to such vesicles after the freeze–thaw and sonication steps was ineffective in restoring cytochalasin B sensitivity to D-glucose uptake in these vesicles. In contrast, 2.2 mM $MgCl_2$ which was added to the vesicles before they were subjected to the freeze–thaw and sonication steps completely restored the capability of the vesicles to transport hexose by a cytochalasin B sensitive process. This transport activity was decreased but not abolished by the addition of 3 mM EDTA following sonication.

For protocol 2, Ca^{2+} was substituted for Mg^{2+} in the standard column buffer to test the apparent dependency of the reconstituted adipocyte hexose transport system on Mg^{2+} (Table IV). Time-dependent and cytochalasin B sensitive D-glucose uptake was observed in both preparations. It is interesting to note that the total uptake of D-glucose at both 1 and 60 min was increased in the vesicles containing Ca^{2+} compared to Mg^{2+}. The data of Table IV suggest that divalent cations must be present before the freeze–thaw step for proper formation of assayable phospholipid vesicles and that vesicle size is somewhat increased when Ca^{2+} is substituted for Mg^{2+}. The question of what effect divalent cations have on the D-glucose transport mechanism itself remains unanswered.

Table IV: Effects of Divalent Cations on the Reconstitution of D-Glucose Transport Activity in Reconstituted Vesicles[a]

addn before freeze–thaw	addn after freeze–thaw–sonication	D-[^{3}H]glucose uptake [nmol/(mg of protein min)] control	plus cytochalasin B (50 μM)	net uptake
		Protocol 1		
none	none	0.29 ± 0.05	0.20 ± 0.09	0.08 ± 0.08
none	$MgCl_2$ (2.2 mM)	0.18 ± 0.09	0.19 ± 0.05	−0.01 ± 0.01
$MgCl_2$ (2.2 mM)	none	0.57 ± 0.03	0.27 ± 0.04	0.03 ± 0.07
$MgCl_2$ (2.2 mM)	EDTA (3 mM)	0.25 ± 0.06	0.15 ± 0.07	0.10 ± 0.05
		Protocol 2		
$MgCl_2$ (2.0 mM)	none	0.55 ± 0.15	0.37 ± 0.17	0.19 ± 0.02
$CaCl_2$ (2.0 mM)	none	1.3 ± 0.2	0.97 ± 0.16	0.34 ± 0.01

[a] Protein-containing phospholipid vesicles made in the absence (protocol 1) or presence (protocol 2) of divalent cations were collected in the void volume of the Sephadex G-50 column. In protocol 1, the vesicles were divided into a group (1.5 mL) receiving 10 μL of H_2O and a group (1.5 mL) receiving 10 μL of $MgCl_2$ (2.2 mM final concentration). Following the standard freeze–thaw and sonication steps, $MgCl_2$ (2.2 mM final concentration) was added to half of the first group and EDTA (3 mM) was added to half of the second group prior to the glucose transport assay. The values represent the mean ± standard error of data from three experiments performed on different days. Net uptake refers to cytochalasin B sensitive uptake. In protocol 2, the vesicles in the void volume were subjected to the standard freeze–thaw and sonication steps. Uptake was monitored at 1 and 60 min. The values are the means of two experiments ± the range of values obtained in those experiments. Equilibrium values (60 min) for D-[^{3}H]glucose uptake were 1.4 ± 0.4 and 2.3 ± 7 nmol/(mg of protein 60 min) for $MgCl_2$- and $CaCl_2$-treated vesicles, respectively.

Similar complications involving vesicle formation arose when we attempted to study the phospholipid requirement for transport. Of the phospholipid combinations assayed, a 1:1 mixture of phosphatidylcholine and phosphatidylethanolamine yielded optimal activity. Asolectin or phosphatidylethanolamine alone was ineffective. Phosphatidylinositol, phosphatidylserine, sphingomyelin, or brain gangliosides, either alone or in combination with phosphatidylethanolamine, resulted in little or no transport. Electron micrographs suggested that the poor transport activity found in these vesicles resulted from poor vesicle formation or formation of vesicles too small to assay by using our techniques (not shown).

Cholate-solubilized intrinsic adipocyte plasma membrane proteins were fractionated by using Sepharose 6B (Figures 2 and 3) in order to obtain a rough estimate of the molecular size of the transport protein or proteins. The concentration of sodium cholate in the column buffer was reduced to 0.5% from the solubilized concentration of 2% to avoid the loss of D-glucose transport activity observed upon prolonged exposure of the solubilized protein to the higher concentration of detergent. As seen in Figure 2, three distinct peaks were obtained: a void volume, a large broad peak containing 60–70% of the protein, and one small final peak. Although the void volume material contained some D-glucose transport activity when reconstituted into phospholipid vesicles, much greater activity was always found in the major peak than in the void volume peak. The majority of the transport activity was found associated with a band of proteins in the peak corresponding to a molecular size of between 50 and 90 Å, indicating a size in the 60–80-Å range if one accounts for zone broadening. The large spread of activity may be attributable to the presence

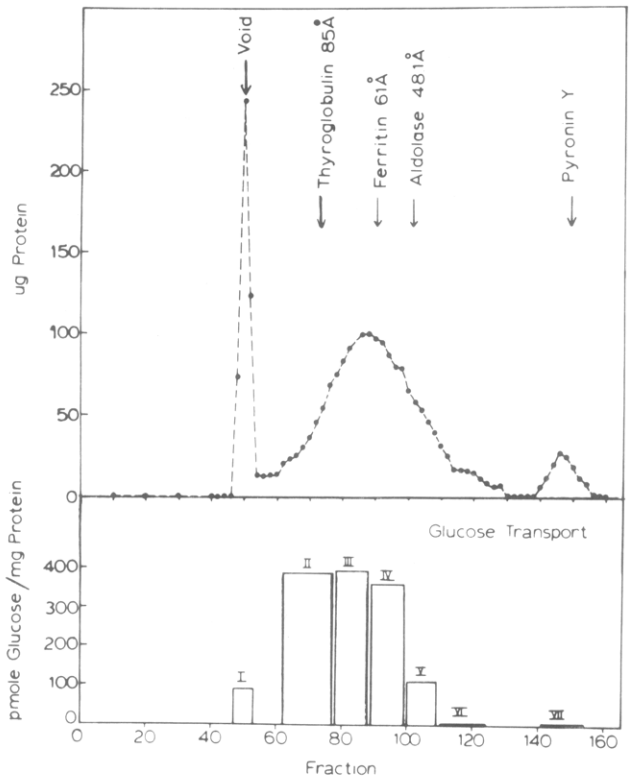

FIGURE 2: D-Glucose transport activity of cholate-solubilized intrinsic membrane proteins following fractionation by Sepharose 6B chromatography and reconstitution into artificial liposomes. Dimethylmaleic anhydride extracted membranes (5.1 mg of protein) were solubilized for 30 min on ice in 1 mL of 100 mM NaCl, 10 mM NaH_2PO_4–Na_2HPO_4, 2% sodium cholate, and 5 mM DTT, pH 7.4. A portion (100 μL) of the solubilized protein was diluted to 5 mL with the above buffer containing 0.5% sodium cholate (no DTT) and kept overnight at 4 °C as the control. The remainder (800 μL) of the protein was applied to a Sepharose 6B column (1.5 × 89 cm) and eluted with the same buffer (see Materials and Methods). The seven pooled fractions and the control were concentrated in the presence of exogenous phospholipids by using ultrafiltration. Protein-containing artificial liposomes were reconstituted following the standard procedures described under Materials and Methods. The upper panel shows the protein profile obtained following Sepharose 6B chromatography and the elution volumes and size of the molecular weight markers. The lower panel shows the corresponding net cytochalasin B sensitive D-glucose transport at 2 min for the seven pooled fractions. These results are from a representative experiment in which D-glucose transport was assessed by using quadruplicate sample points. Control uptake was 379 pmol of glucose per mg of protein. Protein concentrations in this and all further transport figures were determined directly from the reconstituted vesicles unless indicated otherwise.

of protein aggregates which contain the transport protein and are not dissociated by sodium cholate or the presence of protein–cholate–phospholipid complexes. It should be noted, however, that this Sepharose 6B profile is completely reproducible. If protein complexes do exist, similar complexes occur consistently.

Figure 3 shows the electrophoretic profile of solubilized proteins fractionated by Sepharose 6B gel filtration. The molecular size designations are given primarily as indicators of which gel fractions in Figure 3 correspond to the reconstituted fractions in Figure 2. Although no clear purification of transport protein was obtained, a number of interesting observations can be made from the electrophoretic gel patterns. The starting material (control) profile is dominated by two major bands (the 94 000- and 78 000-dalton bands), one secondary band (68 000 daltons), and multiple minor bands. The 68 000-dalton protein eluted primarily in the low molecular weight range (0–50 Å) fractions which did not exhibit D-

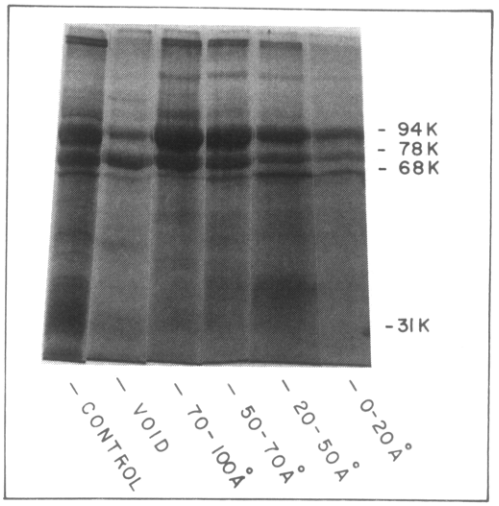

FIGURE 3: Polyacrylamide gel electrophoresis of cholate-solubilized intrinsic membrane proteins fractionated by using Sepharose 6B chromatography. Dimethylmaleic anhydride extracted proteins (4.0 mg of protein) were solubilized, separated by Sepharose 6B chromatography, pooled, and concentrated as described for Figure 2. The only variation in protocol was the column size (1.5 × 43 cm) and the cholate concentration of the column buffer (2% rather than 0.5%). The electrophoresis pattern is shown for 100 μg of all but the void and final fractions which are 80 and 45 μg, respectively. The indicated molecular sizes are rough approximations determined by using proteins of known size (see Figure 2). They serve primarily to permit comparison between the gel patterns of this figure and the transport profile of Figure 2.

glucose transport activity. In contrast, the 78 000-dalton protein was most concentrated in the poorly transporting void volume and decreased in concentration with increased elution volume. Of the three major proteins, only the 94 000-dalton protein was most concentrated in the fractions containing glucose transport.

More complete separation of transporting from nontransporting proteins was obtained by using a hydroxylapatite column equilibrated in 10 mM KH_2PO_4–K_2HPO_4, 0.5% sodium cholate, and 0.02% NaN_3, pH 7.4. When the proteins were eluted with a linear KH_2PO_4–K_2HPO_4 gradient, two major peaks and usually a void peak were obtained. The first peak (designated as fractions 1 and 2 in Figure 4) eluted roughly between 15 and 120 mM KH_2PO_4–K_2HPO_4 with the center at ~75 mM. Although the peak routinely contained ~50% of the total protein, the resident membrane components of this peak failed to exhibit cytochalasin B sensitive hexose transport activity when reconstituted into phospholipid vesicles. The small amount of apparent transport activity indicated in Figure 4 for fractions 1 and 2 was not statistically significant. In contrast, the proteins comprising the second major peak, which usually contained 25–30% of the total protein (designated as fractions 3 and 4), always exhibited significant transport activity, with a concentration of activity most often observed in the ascending side of the final peak (corresponding to the first part of fraction 4). This peak eluted roughly between 150 and 350 mM KH_2PO_4–K_2HPO_4, with the peak activity between 200 and 250 mM. The void volume protein peak was variable in size (compare Figures 4 and 5) and contained between 0 and 30% of the total protein. It appeared to be composed of relatively small molecular size proteins as evidenced by the large protein loss that occurred during concentration using vacuum dialysis (25 000-dalton pore size filters). The retained void volume proteins exhibited little or no D-glucose transport once they were reconstituted into phospholipid vesicles. Because of the variability in commercial hydroxylapatite preparations, each hydroxylapatite column

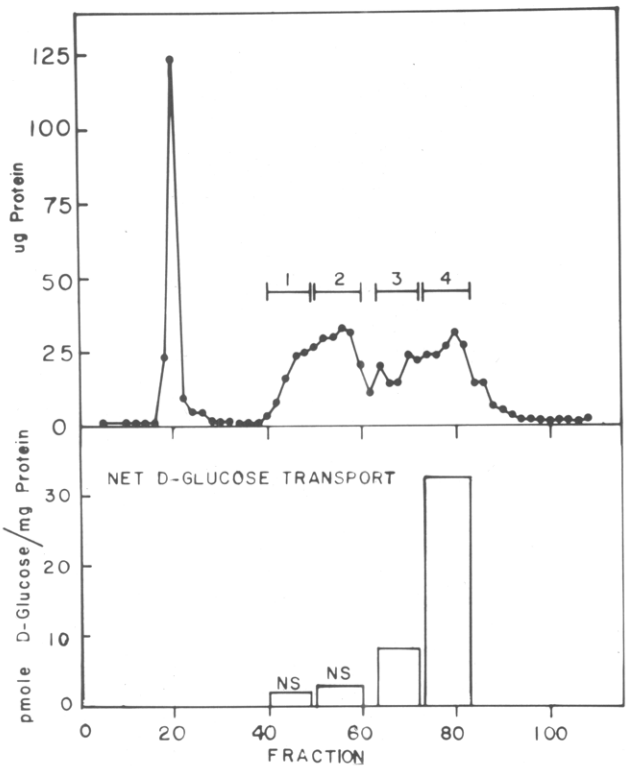

FIGURE 4: D-Glucose transport activity of cholate-solubilized dimethylmaleic anhydride extracted adipocyte plasma membranes following fractionation by hydroxylapatite chromatography and reconstitution into artificial liposomes. Dimethylmaleic anhydride extracted membranes from 200 g of fat were solubilized for 35 min on ice in 2 mL of 5 mM Tris-HCl, 0.5 mM EDTA, 5 mM DTT, and 2% sodium cholate, pH 6.8. A portion (200 μL) of the solubilized protein was diluted to 8 mL with 10 mM KH_2PO_4–K_2HPO_4, 0.5% sodium cholate, and 0.02% NaN_3 and set aside at 4 °C as a control. The remainder (1.7 mL) of the protein was applied to a hydroxylapatite column and eluted with a linear phosphate gradient as described under Materials and Methods. The four pooled fractions and the control were concentrated and dialyzed against 10 mM Tris-HCl, pH 7.5, in the presence of exogenous phospholipids by using vacuum dialysis. Protein-containing artificial liposomes were reconstituted as described under Materials and Methods. The upper panel shows the protein profile obtained following hydroxylapatite chromatography and the fractions pooled for the transport assay. Net D-glucose transport (lower panel) represents vesicular cytochalasin B sensitive D-glucose uptake at 2 min. The results are the average of quadruplicate sample points of vesicles from a representative column. The net transport shown for fractions 1 and 2 was found to be statistically insignificant at the 95% confidence level (Student's t test). Control uptake was 31 pmol of D-glucose per mg of protein. The protein values used in determining the transport numbers are the protein content of the pooled samples before concentration. Although these uptake values cannot be compared to those given in other figures, they permit comparison of the transport values obtained in the different fractions.

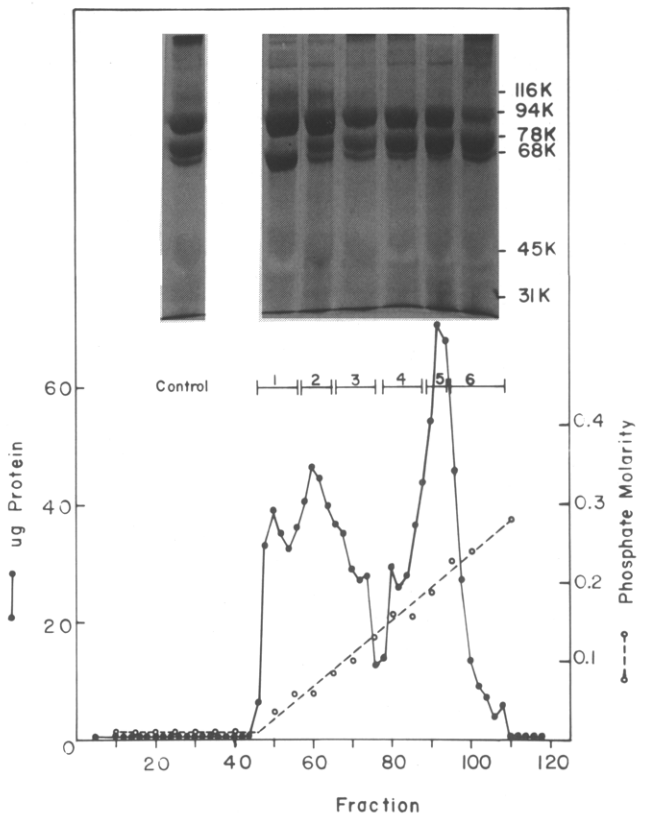

FIGURE 5: Polyacrylamide gel electrophoresis of cholate-solubilized intrinsic membrane proteins fractionated by using hydroxylapatite chromatography. Dimethylmaleic anhydride extracted membranes (2.8 mg in 0.8 mL) were prepared and fractionated as in Figure 4 with several minor differences. The protein was solubilized in 1.6 mL of 5 mM KH_2PO_4–K_2HPO_4, 5 mM Tris-HCl, 0.5 mM EDTA, and 2% sodium cholate, pH 7.0 (no DTT). The fractions were pooled as shown in the figure and then concentrated in the presence of phospholipids by using ultrafiltration and dialyzed against 10 mM Tris-HCl, pH 7.5, by using diafiltration. The electrophoretic pattern of 75 μg of each concentrated sample is shown above the protein profile of the fraction from which it was derived. The "control" was treated as described in Figure 4.

gave a slightly different profile (compare Figures 4 and 5). However, every column resolved the nonvoid protein into two well separated peaks, the first of which never exhibited transport and the second of which contained all the transport activity. This result was obtained whether or not the original solubilization was carried out in the presence (Figure 4) or absence (Figure 5) of DTT.

As observed with the Sepharose 6B column (Figure 3), all protein fractions from the hydroxylapatite column (Figure 5) contained at least a portion of all the major bands and most of the visible minor bands. However, a large concentration of the 68 000-dalton protein was obtained in fraction 1, a nontransporting fraction. The nontransporting fractions 1 and 2 also contained large amounts of the 94 000-dalton protein and decreased amounts of the 78 000-dalton protein compared to the control. In contrast, fraction 6, a transporting fraction, contained greatly reduced amounts of the 94 000-dalton protein but large amounts of the 78 000-dalton protein. In fact, all the transporting fractions (fractions 4–6) contained larger amounts of the 78 000-dalton protein and decreased relative amounts of the 94 000-dalton protein compared to the nontransporting fractions (fractions 1–3). The diffuse band of 116 000 daltons was completely removed. It seems unlikely from these data and the experiments with Sepharose 6B that the transport protein is a major component of the 116 000-, 94 000-, 78 000-, or 68 000-dalton bands.

Because the red blood cell hexose transport protein has been identified as a glycoprotein (Kasahara & Hinkle, 1977; Kahlenberg & Zala, 1977; Goldin & Rhoden, 1978; Sogin & Hinkle, 1978), we attempted adsorption of the solubilized adipocyte membrane proteins to a series of immobilized lectins known to bind to specific glycoprotein oligosaccharide residues (Dulaney, 1979). Table V shows the reconstituted D-glucose transport activity of the cholate-solubilized proteins which did not bind to various immobilized lectins. Clearly, none of the five lectins appeared to bind the D-glucose transport system. D-Glucose transport in the absence and presence of 20 μM cytochalasin B after 2 min was remarkably similar within each experiment between those proteins treated with Sepharose 6B or Bio-Gel A1.5 and those treated with agarose-bound lectins. When bound proteins were eluted from the immobilized *U.*

Table V: Inability of Immobilized Lectins To Remove D-Glucose Transport Activity from Cholate-Solubilized Adipocyte Intrinsic Plasma Membrane Proteins[a]

column	sugar specificity of lectin	D-glucose uptake of vesicles containing nonadsorbed proteins [nmol/(mg of protein 2 min)]		net uptake [nmol/(mg of protein 2 min)]
		control	plus cytochalasin B	
expt 1				
Sepharose 6B		5.6 ± 0.1	4.0 ± 0.1	1.6
Con A-agarose	α-D-mannosyl- and α-D-glucosyl-	6.4 ± 0.4	4.9 ± 0.3	1.5
expt 2				
Sepharose 6B		2.6 ± 0.1	1.5 ± 0.1	1.1
SBA-agarose	*N*-acetylgalactosaminyl-	1.8 ± 0.2	1.1 ± 0.3	0.7
PNA-agarose	α-D-Gal-β(1→3)-GalNAc and α-D-galactosyl-	4.0 ± 0.3	2.6 ± 0.2	1.4
expt 3				
Bio-Gel A1.5		2.5 ± 0.1	1.8 ± 0.0	0.7
UEA 1-agarose	L-fucosyl-	3.0 ± 0.1	2.0 ± 0.0	1.0
RCA 1-agarose	β-D-galactosyl-	3.8 ± 0.2	2.9 ± 0.0	0.9

[a] In experiment 1, intrinsic membrane proteins (1.6 mg of protein in 800 μL) were diluted 1:1 with buffer A + 4% sodium cholate in 0.02% NaN_3. The solubilized protein was diluted 1:3 with buffer A and divided into two portions. The first portion was incubated for 1 h on ice with 2 mL of Sepharose 6B, and the second portion was incubated with 2 mL of concanavalin A (Con A)-agarose. The nonadsorbed proteins were collected and concentrated to 485–490 μL by using ultrafiltration. Portions (235–240 μL) of each fraction were reconstituted into liposomes by using the standard procedure. Experiments 2 and 3 were carried out similarly to experiment 1 with minor variations. In experiment 2, intrinsic membrane proteins (2.1 mg of protein in 500 μL) were solubilized as above and split into three portions for treatment with Sepharose 6B, peanut agglutinin (PNA)-agarose, or soybean agglutinin (SBA)-agarose. Incubation with 2 mL of gel was for 1 h at room temperature (22 °C). The nonadsorbed proteins were concentrated to 350–380 μL with all but 35 μL used for reconstitution. Experiment 3 proceeded as in experiment 2, using 1 mL of either Bio-Gel A1.5 (control), *U. europaeus* agglutinin 1 (UEA 1)-agarose, or *R. communis* agglutinin 1 (RCA 1)-agarose. The nonadsorbed proteins were concentrated to 290–310 μL with all but 35 μL used for reconstitution. Transport values represent the mean ± standard error of quadruplicate sample points. [^{14}C]Urea incorporation did not differ significantly between the vesicles within any experiment.

europaeus agglutinin, *R. communis* agglutinin 1, and concanavalin A with the appropriate sugar, little or no transport was observed in the reconstituted vesicles (see Figure 7). These data suggest that the glucose transport protein does not have any commonly found membrane glycoprotein sugar residues that are accessible for binding to these lectins. In experiments not depicted, wheat germ agglutinin–Sepharose was also tested for its ability to remove D-glucose transport activity from the cholate-solubilized intrinsic membrane proteins. A variable amount of hexose transport activity appeared to be removed from the soluble proteins by the wheat germ agglutinin–Sepharose. Only a portion of this activity could usually be recovered when the column was eluted with 100 mM *N*-acetylglucosamine. Unfortunately, the variability in the results was so great that it was impossible to draw any conclusions concerning the ability of wheat germ agglutinin to bind the transport protein or to use this immobilized lectin in our purification studies.

The electrophoretic gel pattern of the lectin-treated membrane proteins illustrates that despite the inability of concanavalin A to remove transport activity from the preparation, this lectin bound all the 94 000- and 78 000-dalton glycoprotein components (Figure 6). Wheat germ agglutinin was also observed to adsorb these glycoproteins. Gels containing more membrane protein per lane showed a number of minor bands being removed as well (not shown). In contrast, soybean agglutinin–agarose, *U. europaeus* agglutinin 1–agarose, and peanut agglutinin–agarose appeared not to remove any protein in significant quantities. The prominent low molecular weight bands found on the concanavalin A–agarose and peanut agglutinin–agarose gels represent contamination of the nonadsorbed protein by lectin subunits liberated from the columns.

When the concanavalin A–agarose and wheat germ agglutinin–Sepharose columns were eluted with 400 mM α-methyl mannoside (see Figure 7) and 100 mM *N*-acetylglucosamine (not shown), respectively, the 94 000- and/or 78 000-dalton proteins were recovered along with several other glycoproteins. The recovery was nearly complete from the wheat germ ag-

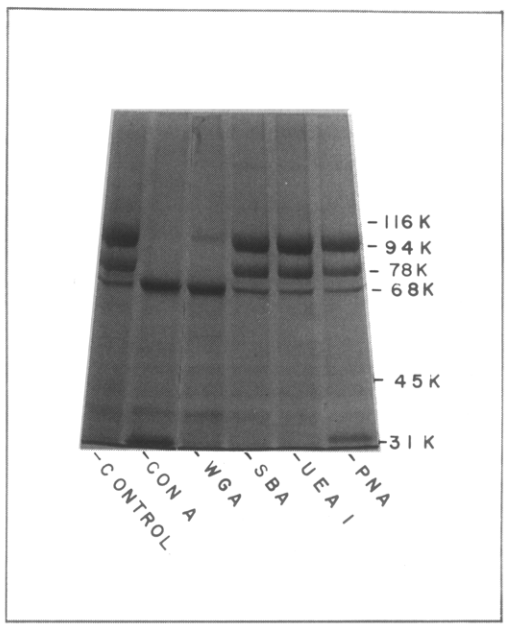

FIGURE 6: Ability of immobilized lectins to remove specific proteins from cholate-solubilized adipocyte plasma membranes. Dimethylmaleic anhydride extracted membranes from 200 g of fat were solubilized for 40 min on ice in 2 mL of 10 mM Tris-HCl, 0.5 mM EDTA, 50 mM NaCl, and 2% sodium cholate, pH 7.4. The solubilized protein was separated into six groups of 225 μL, each of which was brought up to 1 mL by using buffer A. One group was incubated for 1 h at room temperature with 1 mL of one of the following immobilized lectins: concanavalin A–agarose (Con A), wheat germ agglutinin–Sepharose (WGA), soybean agglutinin–agarose (SBA), *U. europaeus* agglutinin 1–agarose (UEA 1), or peanut agglutinin–agarose (PNA). The nonadsorbed protein was concentrated for gel electrophoresis by using ultrafiltration in the absence of added phospholipids. Each electrophoresis lane contains 50 μg of protein.

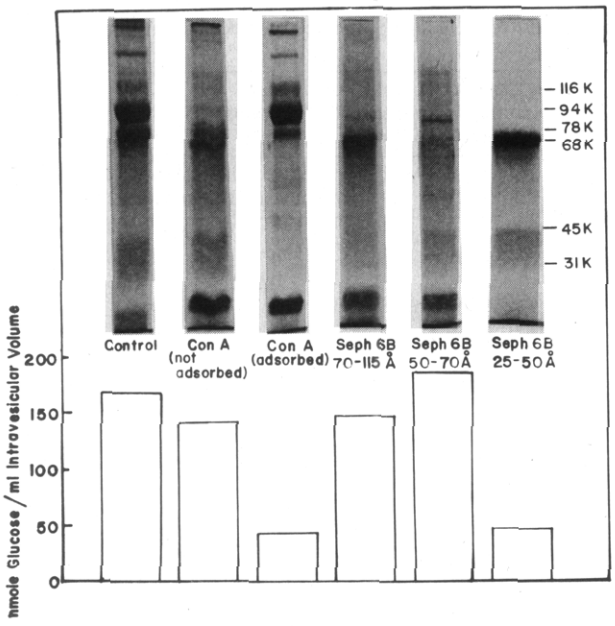

FIGURE 7: Retention of D-glucose transport activity in cholate-solubilized membranes depleted of the majority of protein bands by passage through a concanavalin A–agarose column followed by fractionation by Sepharose 6B. Dimethylmaleic anhydride membranes (11 mg of protein in 2 mL) were diluted 1:1 with 100 mM NaCl, 10 mM NaH_2PO_4–Na_2HPO_4, and 4% sodium cholate, pH 7.4. The solubilized proteins were diluted 1:3 with the above buffer (no cholate). A portion (2 mL) was incubated on ice as the control. The remaining 12.4 mL was incubated with 9 mL of concanavalin A (Con A)–agarose for 1 h at 22 °C. The nonadsorbed proteins were collected and concentrated to 1.5 mL by ultrafiltration in the presence of 5 mg of phosphatidylcholine and 5 mg of phosphatidylethanolamine. These proteins were resolubilized in 2% sodium cholate for 40 min on ice and added directly (no centrifugation) to a Sepharose 6B column (1 × 89 cm) equilibrated with 100 mM NaCl, 10 mM NaH_2PO_4–Na_2HPO_4, 0.5% sodium cholate, and 0.02% NaN_3, pH 7.4. A portion (250 μL) was retained prior to Sepharose 6B chromatography and diluted to 5 mL with the above buffer (Con A, not adsorbed). The Sepharose 6B fractions were pooled and designated by the molecular size range as determined in Figure 2. Following collection of the nonadsorbed proteins, 6 mL of column buffer plus 400 mM α-methyl mannoside was added to the concanavalin A–agarose. After 30 min of incubation at room temperature, the eluted proteins were collected (Con A adsorbed). All fractions were concentrated to 500 μL in the presence of phospholipids by using ultrafiltration with 250 μL being used for reconstitution studies. The transport data of the lower panel represent the cytochalasin B sensitive transport as determined from quadruplicate 2-min time points and as normalized per microliter of intravesicular volume and per amount of protein run on the gel lanes. Lack of sufficient protein per Sepharose 6B fraction as well as the presence of eluted concanavalin A prevented accurate measurements of the membrane protein content. The transport data were normalized, therefore, to the amount of protein used to make the corresponding gel electrophoresis pattern in the upper panel. Significant differences in intravesicular volume as determined by [^{14}C]urea equilibrium values of the reconstituted vesicles containing various protein fractions necessitated including this parameter in the transport values. Each transport value has thus been normalized by (picomoles of D-glucose uptake)/[(microliters of concentrated protein used for electrophoresis)(microliters of intravesicular volume)].

glutinin–Sepharose column but was incomplete from the concanavalin A–agarose column. These data in conjunction with the transport activity measurements provide clear evidence that the 94 000- and 78 000-dalton glycoproteins as well as multiple glycoproteins of higher molecular weight do not represent the adipocyte D-glucose transporter.

Cholate-solubilized intrinsic membrane proteins were chromatographed sequentially by using concanavalin A–agarose prior to Sepharose 6B in order to remove all three of the major protein bands (94 000-, 78 000-, and 68 000-dalton bands) and as many minor bands as possible. Figure 7 shows that when the solubilized proteins were treated with concanavalin A–agarose, virtually all of the 94 000-dalton band and most of the 78 000-dalton band were removed by the lectin as seen in Figure 6. In addition, three large molecular weight proteins were removed, including the diffuse 116 000-dalton band, the lower of the two bands at the top of the gel, and a sharp band at 140 000 daltons. Several other bands were not removed, including the 68 000-dalton band, the upper of the two bands at the top of the gel, and a light band at 120 000 daltons. Two new bands appeared, one at 26 000 daltons (concanavalin A) and a faint band at 84 000 daltons. This fraction has slightly less transport activity per amount of protein run on the gel than the control. If the vast difference in the amounts of solubilized membrane protein used in each gel lane was taken into account, however, the nonadsorbed proteins would have a much higher specific activity of transport than the control. The gel electrophoresis protein profile obtained when the bound proteins were eluted from the immobilized concanavalin A with 400 mM α-methyl mannoside contained precisely those bands that were missing from the previous fraction: the 94 000-, 78 000-, 116 000-, and 140 000-dalton bands as well as the lower of the two top bands. Dissociated concanavalin A appeared at 26 000 daltons. These proteins had very little transport capability, particularly when the large protein content is taken into account. Those proteins that did not bind the concanavalin A were subsequently chromatographed on Sepharose 6B, and the transport-containing peak was divided into three fractions. These corresponded approximately in elution volume to the first three nonvoid fractions of Figure 3. The vast majority of the 68 000-dalton protein eluted in the poorly transporting 25–50-Å fraction, as predicted from Figure 3. The primary transporting fraction, the 50–70-Å fraction, had virtually no 68 000-, 94 000-, 78 000-, 116 000-, or 140 000-dalton protein bands. Several minor bands were present, including the 130 000-dalton band, which is also present in the 70–115-Å fraction, and the 84 000-dalton band. The 50–70-Å fraction thus exhibited high levels of cytochalasin B sensitive hexose transport, yet contained only a small number of minor proteins. This indicates that a significant purification of the transport protein was achieved.

Discussion

The data presented in this manuscript underscore both the utility and the limitations of the reconstituted adipocyte hexose transport system as a tool for future efforts at final purification and characterization of the transport system. As observed in intact cells, this reconstituted transport system is inhibited by cytochalasin B, phloretin, phlorizin, and dipyridamole (Shanahan & Czech, 1977b). 3-*O*-Methylglucose and D-glucose but not L-glucose also inhibited D-[^{3}H]glucose uptake (Table II). Minimum requirements for obtaining net D-glucose transport activity in the reconstituted system included a brief sonication step and the presence of divalent cations. The sonication step may serve to decrease general membrane permeability, thus lowering the nonspecific D-glucose transport component of the total membrane permeability to D-glucose. Similar transport rates were observed for both sonicated and nonsonicated vesicles in the absence of cytochalasin B (Table I). Transport rates were decreased in the presence of cytochalasin B in the sonicated vesicles only. Sonication may instead ensure proper insertion or orientation of the transport system into the phospholipid bilayer. It remains unclear whether vesicles which are sonicated but not subjected to the freeze–thaw cycle would exhibit transport activity because they are too small to be assayed by our filtration assay. It is thus

possible that the freeze–thaw step also plays an important role in the insertion process.

The requirement of exogenous divalent cations for transport system activity in reconstituted vesicles appears to be absolute (Table IV). These ions must be present prior to the freeze–thaw and sonication steps, probably reflecting a divalent cation requirement for proper vesicle formation. Electron micrographs support this hypothesis (not shown). With the possible exception of cations that are tightly bound, transport activity does not appear to have any absolute dependence upon extravesicular divalent cations. When EDTA is added to the vesicles after the sonication step, D-glucose transport activity is reduced but not abolished. Interestingly, the adipocyte hexose transport system in reconstituted phospholipid vesicles exhibits no specificity in its monovalent ion requirement. This suggests that the increase in glucose uptake which occurs when K^+ is omitted from the Krebs-Ringer phosphate medium bathing of intact fat cell (LeTarte et al., 1969; Fain, 1968) or muscle (Bihler, 1968) preparations is not a direct ion effect on transport.

The reconstituted adipocyte hexose transport system does not appear to be amenable to precise determination of specific activity. Figure 1 shows that the vesicle size following the freeze–thaw and sonication steps encompassed a wide range of diameters. Many of the larger vesicles enclosed small vesicles. Any estimates of transport capability would not include the transport contributed by proteins incorporated into either the enclosed vesicles or the vesicles small enough to go through the Amicon filters used to assay uptake. It is possible, though, to compare transport activity of single vesicle preparations which are divided into groups and then exposed to various agents or conditions. Reasonably consistent data (e.g., Tables III and IV) are obtained when vesicles are prepared on the same day by using the same solubilized protein sample and separate Sephadex G-50 columns. The system thus allows one to obtain a reasonable estimate of the effects of various conditions on transport activity.

Specific activity comparisons are more difficult to make between preparations made on different days or between proteins fractionated by chromatography. A rather wide variability in rates of D-glucose uptake into reconstituted vesicles is observed among vesicles prepared on different days. This may reflect variations in the sonication step which would affect the degree of vesicle entrapment, vesicle size distribution, membrane permeability, and/or proper insertion of transport proteins into the bilayer. Alternatively, it may reflect differences in the efficiency of either the dimethylmaleic anhydride to extract extrinsic membrane proteins without removing the transport proteins or the cholate to solubilize a constant percentage of the transport protein. Other investigators attempting to incorporate transport proteins into artificial liposomes have reported the dependence of measurable transport capability but not intravesicular space on the protein/phospholipid ratio. Fairclough et al. (1979) claimed that the transport dependence on protein was based on the amount of transport protein and not total protein present. Thus, transport protein purification could be monitored if transport rates were corrected for the amount of protein present. We have also observed this transport dependence on the protein/phospholipid ratio, although our data (not shown) suggest that the presence of nontransport proteins may also affect the efficiency of vesicle formation. This is less easily corrected for and makes precise specific activity comparisons between control and purified fractions having different amounts of transport and nontransport proteins essentially impossible. We have attempted to minimize this problem by several means. Either the amount of total protein or the amount of transport protein was kept approximately constant for each fraction in each experiment, and uptake values were subsequently corrected for protein incorporated into each vesicle preparation. When proteins were fractionated by using Sepharose 6B or hydroxylapatite chromatography, each reconstituted fraction and the control contained approximately equal amounts of protein (Figures 2 and 4). For the experiments described in Figure 5, the starting amount of protein was the same for the control and lectin-treated samples. On the assumption that the immobilized lectins bound either all or none of the hexose transporters, the fraction containing the D-glucose transporter should have as much of the transporter as the control fraction. For the experiment described in Figure 7, the amount of protein removed as the initial control was an underestimate (see the electrophoretic gel patterns in Figure 7) of the amount of protein expected to be recovered as Sepharose 6B 50–70 Å. The amount of protein removed immediately prior to Sepharose 6B chromatography more closely approximates the amount in the Sepharose 6B 50–70-Å fraction. The uptake values in Figure 7 were also corrected for differences in intravesicular volume resulting from differences in efficiency of vesicle formation of the different fractions. Intravesicular volumes did not sufficiently differ to warrant such corrections in the other experiments with the exception of the experiment described in Table II.

Our results do indicate that the reconstitution procedure can be used as a simple assay for the presence of transport activity in soluble protein fractions obtained by column chromatography in cholate. Although it is difficult to quantitate recovery of transport protein in each fraction, it is possible to identify fractions which contain the transport system and those that do not. This information alone proved sufficient to identify certain properties of the D-glucose transporter. Transport activity was found associated with those cholate-solubilized membrane proteins having a Stokes radius of 60–80 Å with a center of activity at 65–70 Å as determined by using calibrated molecular weight markers run on a Sepharose 6B column. This size measurement may be misleading if the protein is bound to numerous phospholipids and to cholate in a manner different than that of the standards or if the transport protein is a part of a protein aggregate. Evidence against the presence of nonspecific protein aggregates due to incomplete solubilization includes the inability of increased cholate concentrations (4%), increased cholate/protein ratios (up to 2.9 mg of cholate to 1 mg of protein), the use of dithiothreitol during the solubilization, and the use of 2% rather than 0.5% sodium cholate in the column buffer to bring about significantly better solubilization (not shown). In addition, the immobilized lectins concanavalin A–agarose and wheat germ agglutinin–Sepharose succeeded in removing very specific proteins. If the transport protein was part of complexes containing either the 94 000- or 78 000-dalton proteins or both, one would expect it to be removed by the lectins. The data in Table V show that concanavalin A–agarose did not remove the transport protein. There is also evidence to suggest that the 94 000- and 78 000-dalton proteins are not complexed to each other. While both are eluted from wheat germ agglutinin–Sepharose, low concentrations of α-methyl mannoside eluted much of the 94 000-dalton protein from the concanavalin A and almost none of the 78 000-dalton protein. Figure 7 illustrates that even 400 mM α-methyl mannoside preferentially elutes the 94 000-dalton protein. Despite the evidence against the existence of specific protein aggregates, a small

proportion of the transport protein may elute as part of a complex. This would explain why a small amount of D-glucose transport activity is sometimes found in reconstituted proteins that bind to concanavalin A–agarose (Figure 7). Alternatively, the transport protein may have a very low affinity for concanavalin A or it may not have been completely washed from the column before the nonadsorbed proteins were eluted with sugar.

It is also not unreasonable to expect the D-glucose transporter itself to exhibit such a large Stokes radius. The 65–70-Å radius compares favorably to the 65–68-Å radius reported by Zoccoli et al. (1978) for the D-glucose-sensitive cytochalasin B binding complex of solubilized red blood cell membranes. This value was determined by using proteins solubilized in 0.025–0.10% Triton X-100 and run on a Sepharose 4B column equilibrated in Triton X-100. This cytochalasin B binding complex has been identified as band 4.5 (Sogin & Hinkle, 1978) [nomenclature of Steck (1974)], which is known to exhibit specific D-glucose transport when incorporated into phospholipid bilayers (Kasahara & Hinkle, 1977; Kahlenberg & Zala, 1977; Goldin & Rhoden, 1978; Sogin & Hinkle, 1978). Sogin & Telford (1978) also reported the presence of 62-Å particles in reconstituted vesicles containing band 4.5 as judged by freeze-fracture microscopy. This particle size is in the range one would predict for a protein that spanned the membrane. Cholesterol-rich biological membranes like the rat adipocyte membrane (Czech & Lynn, 1973b) have been shown to have widths of ~50 Å (Caspar & Kirschner, 1971). Other well characterized eucaryotic transport systems, such as the erythrocyte anion carrier, band 3 (Bretscher, 1971), and Na^+-K^+-ATPase (Hart & Titus, 1973), have been shown to be transmembrane proteins.

The fact that D-glucose transport activity is not removed by any of the five lectins tested suggests that the D-glucose transporter is not a glycoprotein or that its oligosaccharides are not readily accessible to binding by the immobilized lectins. This is particularly interesting in light of the fact that concanavalin A has been reported to stimulate glucose oxidation (Czech & Lynn, 1973a; Cuatrecasas & Tell, 1973) and 3-*O*-methylglucose transport (Czech et al., 1974) in isolated fat cells. Czech et al. (1974) concluded that the physiological activity of concanavalin A in fat cells was mediated by receptor moieties distinct from those involved in insulin action. These data suggest that those moieties are also distinct from the D-glucose transporter itself. Treatment with concanavalin A–agarose also proved to be an important step in the purification of the transport system. Over 70% of the total protein and all of the 94 000- and 78 000-dalton proteins were removed without removing the transport protein. This provides strong evidence that the 94 000-dalton protein is not the transport protein, a conclusion contrary to that suggested previously by this laboratory (Shanahan & Czech, 1977b). It is also clear that the transporter cannot be any of the other glycoproteins removed by concanavalin A. This includes the 78 000-, 116 000-, and 140 000-dalton proteins described in the text. Hydroxylapatite chromatography also successfully separated the cholate-solubilized intrinsic membrane proteins into transporting and nontransporting fractions. The transporting fraction bound more tightly than did the nontransporting fraction to the $CaPO_4$ matrix. The lack of any specific protein visible in the electrophoretic gels of the transporting fractions compared to the nontransporting fractions suggests that the transport protein either is obscured by one of the larger protein bands or is present in such small quantities that it would not show up as a band unless the gels were overloaded. The latter possibility seems most probable. The amount of glucose transport protein present in an adipocyte plasma membrane as a percentage of total membrane protein can be estimated on the basis of the number of D-glucose-specific cytochalasin B binding sites of the adipocyte plasma membrane (4 pmol/mg of protein; Wardzala et al., 1978) and an approximate molecular weight of 100 000. The calculated value of 0.04% compares to a value of 3.5% for the red blood cell (Zoccoli et al., 1978), suggesting that the red blood cell transport protein is present at a 100 times greater concentration than the adipocyte transport protein.

The most purified D-glucose transport system was obtained by treating cholate-solubilized intrinsic adipocyte membrane proteins first with concanavalin A–agarose and then with Sepharose 6B (Figure 7). Although estimates of purification are clouded by the difficulty in obtaining precise specific activity values as previously discussed, cytochalasin B sensitive D-glucose transport was found to reside in a fraction (Sepharose 6B 50–70 Å) from which the 140 000-, 116 000-, 94 000-, 78 000-, and 68 000-dalton proteins were removed. These bands included over 90% of the soluble starting material. In fact, only a few minor bands were visible on $NaDodSO_4$ electrophoresis gels, including the 84 000- and 130 000-dalton bands. Studies in this laboratory and in other laboratories indicate several similarities between the reconstituted D-glucose transport systems of the red blood cell and the adipocyte, including the sensitivity to cytochalasin B and phloretin (Goldin & Rhoden, 1978; Kasahara & Hinkle, 1976; Shanahan & Czech, 1977b), the substrate specificity (Kasahara & Hinkle, 1976; Table II), and the Stokes radius (Zoccoli et al., 1978; Figure 2). However, differences in the properties of these systems seem to be apparent as well (Czech et al., 1978). Further purification of the adipocyte hexose transport system will be required to resolve the question of whether the insulin-sensitive adipocyte and insulin-insensitive red blood cell glucose transporters are similar or distinct proteins.

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Carbon-13 Nuclear Magnetic Resonance Studies on the Interaction of Glycophorin with Lecithin in Reconstituted Vesicles†

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ABSTRACT: Glycophorin, the MN blood group substance, is a major intrinsic glycoprotein in erythrocyte membranes. The interaction of glycophorin with phosphatidylcholine, ^{13}C-labeled in specific positions in reconstituted unilamellar vesicles, was investigated by using the ^{13}C NMR technique. 1-Palmitoyl-2-([14-^{13}C]linoleoyl)-*sn*-glycero-3-phosphocholine was synthesized and used as a probe. At 37 °C the spin–lattice relaxation time (T_1) of vesicle bilayers consisting of this phospholipid was 0.74 s in the absence of glycophorin. The incorporation of glycophorin decreased the T_1 to 0.63 s, indicating that the bulk lipid molecules are somewhat immobilized by glycophorin. In addition to the reduction in time, a broad component ($\Delta H_{1/2} = \sim 40$ Hz) superimposing the sharp resonance was observed in the ^{13}C NMR spectrum of reconstituted vesicles. The T_1 of the broad component was 0.32 s, suggesting that the lipid molecules contributing to the broad component may be more restricted than that of the sharp component. In order to quantify the broad component, a computer simulation was performed. The intensity of the broad component estimated from the simulation depended linearly on the concentration of glycophorin. Therefore, the broad component is considered to be contributed by a phospholipid domain surrounding the glycophorin molecules, a so-called "boundary lipid". The relationship between the broad component and the stoichiometry of the reconstituted vesicles allows the conclusion that about 30 lipid molecules are immobilized by one glycophorin monomer.

Recently, many investigations on the molecular motions of membrane lipids have provided evidence that lipid molecules show motions within the membrane, e.g., trans–gauche isomerization in the acyl chains, rotational motion, and lateral and transverse diffusion. These motions are affected by the incorporation of membrane proteins. In 1973, Jost et al. concluded from their results of spin-labeling studies on a cytochrome *c* oxidase–lecithin complex that a "boundary lipid" exists around membrane proteins (Jost et al., 1973). They are less mobile than the bulk lipids. Dahlquist et al. (1977) have also provided evidence for a boundary lipid using ^2H NMR.[1] Other groups, however, deny the existence of boundary lipids (Oldfield et al., 1978; Seelig & Seelig, 1978). The discrepancy might arise from differences in experimental techniques or experimental systems. ^{13}C NMR has some advantages over spin-labels in studying protein–lipid interactions, since there is no perturbation of the probe.

Glycophorin is a major intrinsic glycoprotein of the erythrocyte membrane. It may play a role as a membrane receptor, since glycophorin is well-known to have MN blood group characteristics. The extensive work of Marchesi's group has characterized glycophorin thoroughly. It seems to exist as a dimer in the membrane, with its C-terminal end at the cytoplasmic side of the membrane, and its N-terminal end, containing the sugar residues, exposed to the exterior of the cell (Marchesi et al., 1976). The interaction of glycophorin with lipids in the membrane is, however, not well understood from a dynamic standpoint, although several investigations have

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[1] Abbreviations used: NMR, nuclear magnetic resonance; CD, circular dichroism; HDL, high-density lipoprotein; $NaDodSO_4$, sodium dodecyl sulfate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid.

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