Biochimica et Biophysica Acta 818 (1985) 171–182
Elsevier

BBA 72695

Reconstitution of the glucose transporter from bovine heart

Thomas J. Wheeler and Mary Anne Hauck

Department of Biochemistry, University of Louisville School of Medicine, Louisville, KY 40292 (U.S.A.)

(Received February 11th, 1985)

Key words: Glucose transport; Cytochalasin B; Membrane reconstitution; (Bovine heart)

Reconstitution of the glucose transporter from heart should be useful as an assay in its purification and in the study of its regulation. We have prepared plasma membranes from bovine heart which display D-glucose reversible binding of cytochalasin B (33 pmol sites / mg protein; $K_d = 0.2$ μM). The membrane proteins were reconstituted into liposomes by the freeze-thaw procedure. Reconstituted liposomes showed D-glucose transport activity which was stereospecific, saturable and inhibited by cytochalasin B, phloretin, and mercuric chloride. Compared to membrane proteins reconstituted directly, proteins obtained by dispersal of the membranes with low concentrations of cholate or by cholate solubilization showed 1.2- or 2.3-fold higher specific activities for reconstituted transport, respectively. SDS-polyacrylamide gel electrophoresis followed by electrophoretic protein transfer and labeling with antisera prepared against the human erythrocyte transporter identified a single band of about 45 kDa in membranes from both dog and bovine hearts, a size similar to that reported for a number of other glucose transporters in various animals and tissues.

Introduction

The plasma membrane of most animal cells contains an integral membrane protein, the glucose transporter, which catalyzes the facilitated diffusion of glucose. This protein has been purified from human erythrocytes and reconstituted into liposomes; a number of studies of its structure and kinetic features have been published. In recent years evidence from antibody cross-reactivity and photoaffinity labeling with cytochalasin B has indicated that the glucose transporters of several other tissues in various animals are antigenically related to the human erythrocyte transporter and have a similar size (about 45 kDa) (reviewed in Ref. 1).

Some progress has been made in the reconstitution and partial purification of transporters from cells other than the human erythrocyte, such as rat adipocytes [2–4], rat LM cells [5], bovine thymocytes [6], and Ehrlich ascites cells [7]. However, little has been reported in the case of heart and skeletal muscle.

Glucose transport in heart is regulated by a number of factors. Transport is increased by insulin, muscular contraction, anoxia, and hyperosmolarity, and is decreased by fatty acid oxidation [8–10]. Catecholamines and cardiac glycosides can either activate or inhibit depending on the conditions [11,12]. The regulation by insulin most likely involves translocation of transporters from an intracellular pool to the plasma membrane [13], as has been demonstrated for adipocytes [3,14] and diaphragm muscle [15], although an increase in the turnover number may also occur [16]. Much less is known about the mechanisms involved in the other types of regulation. As a step in the

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; Mops, 4-morpholinepropanesulfonic acid; SDS, sodium dodecyl sulfate; PC, phosphatidylcholine; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol.

characterization of the heart transporter and its regulation, we report here the reconstitution of glucose transport activity using plasma membranes from bovine heart.

Portions of this work were presented previously (Wheeler, T.J. and Hauck, M.A. (1984) Fed. Proc. 43, 1573).

Materials and Methods

Materials. Fresh bovine hearts were obtained from a local slaughterhouse and used immediately. In preliminary experiments hearts from mongrel dogs were also used. [4-^{3}H]Cytochalasin B, [^{14}C]urea, L-[1-^{3}H]glucose, and [*carboxyl*-^{14}C]cholic acid were obtained from New England Nuclear; D-[U-^{14}C]glucose from ICN; cytochalasin E from Aldrich; Na^{125}I from Amersham; soybean phosphatidylcholine, protein A, PMSF, and adenosine deaminase from Sigma; and scintillation cocktail (Liquiscint) from National Diagnostics. Cholic acid from Sigma was further purified by precipitation from water. Rabbit antisera prepared against the human erythrocyte glucose transporter were a gift of Dr. David Sogin.

Preparation of plasma membranes. Two plasma membrane preparations were tested. In each case all solutions were kept on ice. Preparations were generally done on two consecutive days, with intermediate stages frozen overnight. The final membranes were stored at −20°C.

Method I (modified from Kidwai [17] and Klip and Walker [18]): heart muscle (800–1000 g) was cut into small pieces and suspended in 3 ml/g tissue of a buffer containing 0.25 M sucrose, 5 mM Tris-HCl (pH 8), 0.5 mM dithiothreitol, and 0.2 mM PMSF. Portions of suspended muscle were homogenized three times for 15 s with a Polytron at half maximal speed and adjusted to pH 7.5 with Tris base. The homogenate was centrifuged at $4000 \times g$ for 6 min and the supernatant filtered through cheesecloth. The filtrate was centrifuged at $100\,000 \times g$ for 45 min. Pellets were resuspended to 80 ml with 0.25 M sucrose buffer as above and layered on 24% (w/v) sucrose buffer containing 5 mM Tris-HCl (pH 7.5), 0.5 mM dithiothreitol and 0.1 mM PMSF. Additional 0.25 M sucrose buffer was added on top of the resuspended pellets. The membranes were centrifuged at 24 000 rpm for 90 min in a Beckman SW27 rotor. The bands at the sample/24% sucrose interface were removed, diluted at least 4-fold with water, pelleted at $100\,000 \times g$ for 30 min, and resuspended in 10 mM Tris-HCl (pH 7.5).

Method II (modified from Slaughter et al. [19]): diced heart muscle was suspended in 2 ml/g tissue of a buffer containing 0.25 M mannitol, 70 mM Tris (adjusted to pH 7.4 with H_2SO_4), 0.5 mM dithiothreitol, and 0.1 PMSF. Portions were homogenized in a blender for two 5-s bursts, followed by 20 s with a Polytron at a setting of 6. The homogenate was centrifuged at $14\,000 \times g$ for 30 min. Pellets were resuspended to the original volume with mannitol buffer, homogenized three times for 30 s with the Polytron, and centrifuged as before. A second homogenization of the pellets from this step [19] was found not to increase the yield of cytochalasin B binding sites and was generally omitted. The supernatant was centrifuged at $48\,000 \times g$ for 30 min. Pellets were resuspended to 25 ml with mannitol buffer and layered on 0.64 M sucrose, 20 mM imidizole-HCl (pH 7.4), 0.5 mM dithiothreitol, and 0.1 mM PMSF. Additional mannitol buffer was added on top of the resuspended pellets. The membranes were centrifuged at 40 000 rpm for 90 min in a Beckman 60 Ti rotor. Bands were removed from the mannitol/sucrose interface, diluted at least 3-fold with 10 mM Tris-HCl (pH 7.5), pelleted at $100\,000 \times g$ for 30 min, and resuspended in 10 mM Tris buffer.

Cytochalasin B binding assays. The binding of cytochalasin B to membranes was measured using a modification of the procedure of Cushman and Wardzala [20]. Assays contained [^{3}H]cytochalasin B, [^{14}C]urea, 2 μM cytochalasin E, 500 mM D- or L-glucose, 0.14 mg membrane protein, and 10 mM Mops buffer (pH 7.5) in a total volume of 60 μl in 400 μl polyethylene micro centrifuge tubes (Bio-Rad). Tubes were centrifuged at $13\,000 \times g$ for 60 min in a Fisher microcentrifuge at 4°C. Supernatants were removed and 10 μl counted. The bottoms of the tubes containing the pellets were cut off and added directly to scintillation vials. All vials contained 4 ml scintillation fluid. No significant difference in quenching was observed between the vials containing aliquots of the supernatants and those containing the pellets and tips of the tubes. Assays were run in triplicate for both

D- and L-glucose-containing samples.

D-Glucose reversible cytochalasin B binding was calculated by subtracting the value of bound/free in the presence of D-glucose from that in the presence of L-glucose for the same total concentration of cytochalasin B. The most appropriate correction would be to use values obtained at the same free concentration. Our correction closely approximates this because the free values are similar for the two conditions and the ratio in the presence of D-glucose shows little change with concentration. Binding data were converted to double-reciprocal form (1/bound vs. 1/free) and a weighted least-squares fit performed, weighting points by the factor $(\text{bound})^4/(\text{S.E.})^2$ [21].

Reconstitution and assay of glucose transport activity. For most experiments reported here, membranes were dispersed with low concentrations of cholate by a modification of the procedure of Robinson et al. [22]. Membranes (2 mg/ml) were incubated with 0.3% sodium cholate in 10 mM Tris-HCl (pH 7.5) for 10 min at 0°C, sonicated 5 s in a bath-type sonicator (Laboratory Supplies Co.), and placed in a −20°C freezer for 15 min. For the experiment shown in Fig. 6, membranes at 5 mg/ml were incubated with 1% cholate. The frozen samples were allowed to thaw at room temperature and centrifuged at $15000 \times g$ for 10 min. Supernatants were passed through columns of Sephadex G-50M to remove the cholate. For cholate solubilization experiments, the incubation time was increased to 20 min. The samples were then sonicated, frozen, and thawed as above and centrifuged at $100000 \times g$ for 30 min, followed by gel filtration. In some experiments membranes were reconstituted directly without cholate treatment.

Samples were reconstituted into liposomes by the freeze-thaw procedure [23]. Liposomes were prepared by extensive sonication of soybean phosphatidylcholine (Sigma type II-S) at 50 or 150 mg/ml in 10 mM Tris-HCl (pH 7.5). A mixture of liposomes (25 mg lipid/ml) and membrane sample was frozen in liquid nitrogen, allowed to thaw at room temperature, and sonicated 5 s.

Transport was assayed using a filtration assay [24]. Assays contained 30 μl reconstituted liposomes, D-[^{14}C]glucose (0.2 mM or as noted), tracer L-[^{3}H]glucose, and 10 mM Mops buffer (pH 7.5) in a total volume of 250 μl. A cold stopping and washing solution contained 0.5 mM $HgCl_2$ and NaCl to give the same osmolarity as the assays. Liposomes were collected on 0.22 μm Millipore filters (type GSWP) and counted in 10 ml scintillation fluid. Assays were run in triplicate or quadruplicate *.

In our early studies we used 96% radiochemical purity D-[^{14}C]glucose from ICN (catalog number 11050). However, we noted that with one lot (1772113) there was a significant excess of apparent D-glucose over L-glucose uptake in liposomes containing no added protein, indicating an impurity more permeant than glucose. Subsequently we switched to 99.5% purity (catalog 11047), which showed little or no artifactual stereospecific uptake. The only experiments affected significantly by such uptake were those shown in Fig. 6. In these experiments the stereospecific uptake obtained with liposomes containing no protein was subtracted from that obtained using reconstituted protein.

Electrophoretic techniques. SDS-polyacrylamide slab gels (10% acrylamide) were run according to the procedure of Laemmli [25]. Proteins were transferred electrophoretically to nitrocellulose paper [26], labeled with antibody against the human erythrocyte glucose transporter and visualized with ^{125}I-protein A [27] as described [28]. Gels were calibrated using Bio-Rad low molecular weight protein standards.

Other assays. Protein was determined by the method of Lowry et al. [29] using bovine serum albumin as a standard. Assays contained 1 or 2% SDS. 5′-Nucleotidase activity was measured by monitoring the decrease in absorbance at 265 nm of mixtures containing 8 mM $MgCl_2$, 80 mM KCl, 8 mM sodium potassium tartrate, 0.8 mM AMP, 40 mM Tris-HCl (pH 7.5), and 0.08 units adenosine deaminase/ml.

* Since each assay contains its own control for nonspecific uptake in the form of L-[^{3}H]glucose, fluctuations in both D- and L-glucose between assays will tend to cancel out when the specific transport (D- minus L-glucose) is calculated for each assay. In our previous experiments on the reconstituted erythrocyte transporter [24], D- and L-glucose data were averaged separately before taking their differences, thus giving larger apparent standard errors than were actually present.

Results

Binding of cytochalasin B to heart membranes

Plasma membrane fractions were prepared from bovine hearts using modifications of two different procedures [17–19]. These procedures use homogenization in 250 mM sucrose or mannitol buffers rather than the high salt buffers used in some methods. Our modifications include the use of dithiothreitol, since the glucose transporters of some tissues are known to contain essential sulfhydryl groups, and the protease inhibitor PMSF.

As a quantitative assay of the glucose transporter in these membranes, we used the D-glucose reversible binding of the transport inhibitor cytochalasin B. The binding of cytochalasin B at various concentrations is shown in Fig. 1. Binding in the presence of 500 mM D-glucose has a relatively constant value of bound/free, indicating that it is nonsaturable. When this is subtracted from the binding in the presence of L-glucose, the resulting data is consistent with a single class of binding

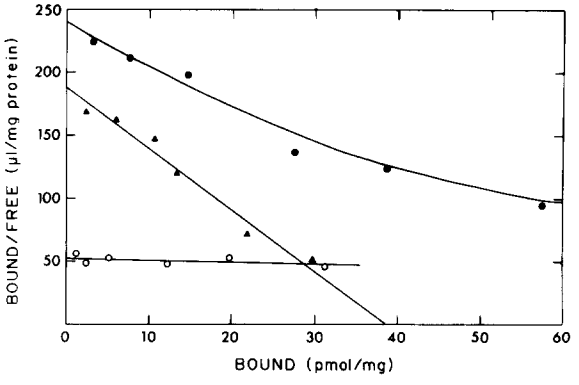

Fig. 1. Binding of cytochalasin B to bovine heart plasma membranes. Assays contained [^{3}H]cytochalasin B, [^{14}C]urea, 2 μM cytochalasin E, 500 mM D- (○) or L-glucose (●) and 0.14 mg membrane protein prepared by method II in 10 mM Mops buffer (pH 7.5). The total assay volume was 60 μl. the urea counts were used to correct for water trapped in the pellets. The D-glucose-reversible binding (▲) was calculated by subtracting the value of bound/free for D-glucose from that for L-glucose at each cytochalasin B concentration. The line through the triangles was drawn from a fit of the data (as described in Materials and Methods), which gave 39 pmol binding sites per mg protein and a K_d of 0.21 μM. The line through the open circles was drawn by eye. The curve through the solid circles is the sum of these two lines along radii representing equal free concentrations.

sites having a K_d of 0.21 μM and a total capacity of 39 pmol/mg. Similar binding curves gave K_d = 0.23 ± 0.05 μM and 0.22 ± 0.02 for membranes prepared by methods I and II, respectively (mean $\pm$ S.E. for three preparations of each type). This K_d is similar to that observed for the glucose transporter in a number of other cells, e.g. 0.18 μM for the purified human erythrocyte transporter [30], 0.08 to 0.14 μM for rat adipocyte membranes [14], and 0.28 μM for rat skeletal muscle plasma membranes [18].

We routinely measured the binding of cytochalasin B at a single concentration (about 0.1 μM), and then calculated the number of binding sites using a value of 0.2 μM for the K_d of cytochalasin B. Membranes prepared by method I had a specific activity of 14 ± 2 pmol/mg protein and yield of 2.0 ± 0.4 pmol/g muscle (mean $\pm$ S.E. for six preparations). Membranes prepared by method II gave a 2.4-fold higher specific activity of binding sites (33 ± 2 pmol/mg) but a 1.8-fold lower yield of total sites (1.1 ± 0.2 pmol/g muscle, $n = 12$). This yield, however, was more than adequate given the large amount of starting material available. Compared to the crude membranes (i.e., the pellets before centrifugation using 0.64 M sucrose), the plasma membranes of method II were enriched about 4-fold and 5-fold in the specific activities of 5′-nucleotidase and cytochalasin B binding, respectively. Because of the higher specific activity of plasma membranes prepared by this method, they were used for the remaining studies reported here. The specific activity of these membranes (33 pmol/mg protein) is similar to that of plasma membranes from insulin-stimulated rat adipocytes (32 pmol/mg [14]), insulin-stimulated rat diaphragm membranes (28 pmol/mg [15]) and rat skeletal muscle (about 10 pmol/mg [31]).

Time-course of reconstituted glucose transport

For most of our studies on the reconstitution of the heart glucose transporter, we have used a modification of the procedure of Robinson et al. [22] developed for the rat adipocyte transporter (see Materials and Methods). Membranes dispersed with low concentrations of cholate were reconstituted into liposomes of crude soybean phosphatidylcholine by the freeze-thaw procedure [23]. The time-course of glucose uptake into recon-

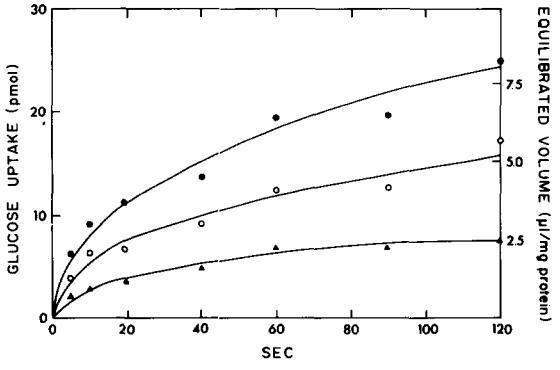

Fig. 2. Time-course of glucose uptake in liposomes reconstituted with heart membrane proteins. Assays contained 15 µg cholate-dispersed membrane protein, 750 µg soybean phospholipid, 0.2 mM D-[^{14}C]glucose, and tracer L-[^{3}H]glucose in a total volume of 0.25 ml. The equilibrated volume, expressed as µl/mg protein in the assay (right-hand scale), is shown for total (D-glucose) (●) and nonspecific (L-glucose) (○) uptake. The difference is the stereospecific uptake (▲). Multiplying the total and stereospecific volumes by 0.2 mM gives the uptake in pmol (left-hand scale).

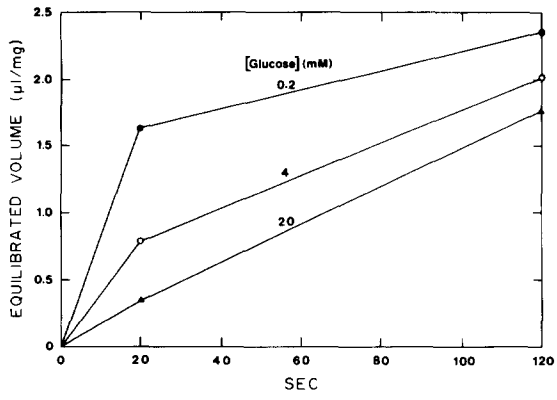

Fig. 3. Glucose uptake at various glucose concentrations. Liposomes were reconstituted with cholate-dispersed membranes and stereospecific uptake measured at 0.2 (●), 4 (○), or 20 mM (▲) glucose.

stituted liposomes is shown in Fig. 2. In this experiment the stereospecific (D- minus L-glucose) uptake was about one third of the total uptake and reached a maximum level at 1 to 2 min. In the remaining experiments reported here, only the stereospecific transport is given. Although the nonspecific uptake shown in Fig. 2 is about two-thirds of the total, it is lower in absolute amounts (0.08 µl/mg soybean lipid in 1 min) than that seen with the reconstituted erythrocyte transporter (0.13 µl/mg lipid, Fig. 8 of Ref. 23). In the latter case, where a purified protein has been reconstituted, the relative amount of uptake that is nonspecific is of course much lower.

Effects of glucose concentration

In order to demonstrate that we were in fact measuring the activity of the heart glucose transporter, we showed that the reconstituted activity was not only stereospecific (Fig. 2), but also saturable and sensitive to known glucose transport inhibitors. Fig. 3 shows the time-course of equilibration of reconstituted liposomes with 0.2, 4, and 20 mM glucose. Although the stereospecific equilibrated volume, or glucose space, achieved in 2 min is similar at each concentration, the initial rate of equilibration is reduced at higher levels of glucose. This indicates that the transport process is saturable. Using the 20 s uptake as a measure of initial rate, a K_m of 13 mM can be calculated from the data. However, since the uptake is clearly not linear for 20 s at low glucose concentrations (Fig. 2), the initial rates are underestimated, and the K_m must be somewhat lower than 13 mM. K_m values of 7 to 10 mM for methylglucose uptake in rat heart have been reported [32].

These results also confirm that we are measuring transport and not simply binding of glucose to the liposomes. The 2 min uptake at 20 mM glucose was 35 nmol/mg protein, or 2 mol/mol protein (assuming an average molecular mass of 50 kDa) for membranes that contain many other proteins in addition to the glucose transporter. Using the cytochalasin B binding capacity of the membranes as a measure of the content of glucose transporters, the uptake represents 1000 mol glucose/mol cytochalasin B sites.

Effects of glucose transport inhibitors

Fig. 4 shows the effects of cytochalasin B, $HgCl_2$, and phloretin on the uptake of glucose in 1 min. Each of these was able to inhibit the transport activity, with 50% inhibition occurring at total concentrations of about 0.4 µM, 0.15 mM, and 20 µM, respectively. The greatest inhibition produced by phloretin in the experiment shown in Fig. 4 was 58% at 60 µM; in two other experiments, 100 µM phloretin produced 77 and 90% inhibition. Since significant amounts of these inhibitors can bind to the lipids which are present at

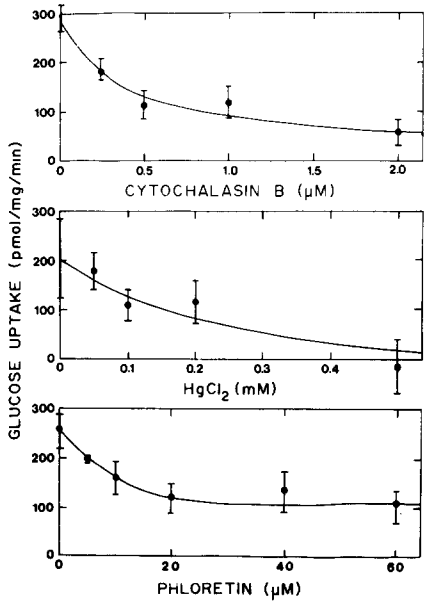

Fig. 4. Effects of glucose transport inhibitors. Inhibition of stereospecific uptake of 0.2 mM glucose in 1 min was measured. Assays contained 0.02%, 0, and 0.6% ethanol for the cytochalasin B, $HgCl_2$, and phloretin experiments, respectively.

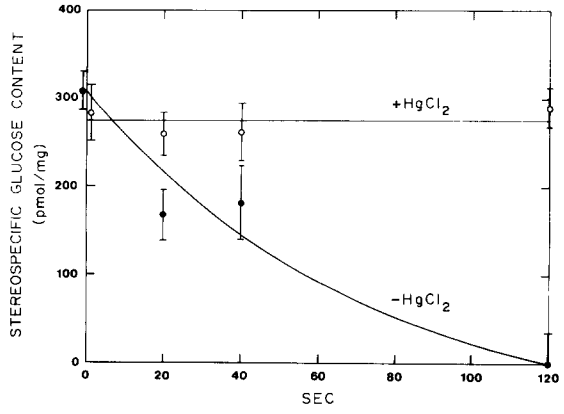

Fig. 5. Efflux of glucose from preloaded liposomes. Reconstituted liposomes were incubated with 0.2 mM D-[^{14}C]glucose plus tracer L-[^{3}H]glucose for 60 min. In each assay, liposomes (10 μl) were diluted with 3 ml glucose-free buffer with (○) or without (●) 0.5 mM $HgCl_2$, and the assay stopped at the indicated time. The stereospecific glucose content was determined by subtracting the equilibrated volume of the incubation mixture for L-glucose from that for D-glucose and multiplying by 0.2 mM.

high concentrations in the assay, the free concentrations, and thus the actual K_i values, are somewhat lower [24].

The results in Fig. 4 represent only the stereospecific transport; no significant effects on L-glucose uptake were produced by the inhibitors except at the highest concentration of $HgCl_2$ tested (0.5 mM), where about a 70% decrease was observed. No significant effect on the L-glucose permeability was produced by 0.2 mM $HgCl_2$; thus the effect is separate from the inhibition of stereospecific transport. We observed similar effects of $HgCl_2$ on L-glucose permeability in studies with the reconstituted human erythrocyte glucose transporter (Wheeler, T.J. and Hinkle, P.C., unpublished data). L-Glucose permeability produced by proteins incorporated into phosphatidylcholine liposomes has been suggested to be due to lipid-protein packing defects or channels produced by aggregated protein [33]. Since $HgCl_2$ both reacts with protein sulfhydryls and binds to the lipids of the liposomes (over 90% is bound at 0.5 mM [24]), it could possibly reduce either of these proposed permeability pathways.

Efflux of glucose from preloaded liposomes

In the experiment shown in Fig. 5, liposomes were preincubated with D- and L-glucose for 60 min and then diluted with a large excess of glucose-free buffer. The stereospecific efflux of D-glucose was complete by 2 min; however, 0.5 mM $HgCl_2$ prevented this efflux. Thus the reconstituted transporter is capable of inhibitor-sensitive, stereospecific transport in both directions across the liposomal membrane.

Requirements for reconstituted activity

We compared the stereospecific uptake of 0.2 mM glucose in 2 min per mg protein for different reconstitution conditions. Compared to cholate-treated membranes reconstituted at about 0.012 mg/mg lipid, samples with the liposomes omitted gave $29 \pm 1\%$ the activity (mean $\pm$ S.E., $n = 3$). This indicates that during the freeze-thaw and sonication steps the membranes themselves can form sealed vesicles which retain glucose transport activity. However, the equilibrated volume is much higher when soybean phospholipid vesicles are added, showing that the transporter has been incorporated into hybrid structures containing predominantly soybean lipids. Thus the trans-

porter has been reconstituted. Omitting the final sonication step resulted in a somewhat lower level of activity (found: 87 and 89%).

In our early experiments we obtained the most success with membranes dispersed with low concentrations of cholate [22]. This treatment does not result in a truly soluble fraction, as much of the protein 'extracted' will pellet when centrifuged at $100\,000 \times g$ for 30 min. However, it probably results in partial solubilization and dispersal of the membranes, with larger aggregates being removed in the $15\,000 \times g$ centrifugation. When untreated membranes were used directly in the reconstitution, the resulting activity was $77 \pm 11\%$ ($n = 3$) of that observed with the cholate dispersed membranes.

Effect of protein concentration

Because of the difficulty in measuring initial rates with the reconstituted liposomes, we have used as a measure of specific activity the stereospecific equilibrated volume, which reaches a maximum by 2 min (Fig. 2). This quantity will be a measure of the number of functional transporters that have been reconstituted, provided that (a) the size distribution of the liposomes is independent of the conditions being compared; and (b) most of the uptake is from liposomes containing single transporters.

The freeze-thaw reconstitution process generates liposomes having a range of sizes [23,34]. We analyzed the size distribution of reconstituted liposomes by chromatography on Bio-Gel A-150m (Bio-Rad Laboratories); no significant differences were observed for various types of samples and concentrations of protein. This is in agreement with the results of Sase et al. [34], where analysis by freeze-fracture electron microscopy revealed no effect of protein concentration on the size distribution of liposomes reconstituted with the purified human erythrocyte glucose transporter.

The effect of protein concentration on the equilibrated volume is shown in Fig.6 for membranes reconstituted directly, after cholate dispersal and after cholate solubilization. In the first two cases the volume was proportional to protein concentration in the range 0 to 0.01 mg protein/mg soybean phospholipid, giving a specific activity of 3 to 4 μl/mg protein. At higher levels of protein, the

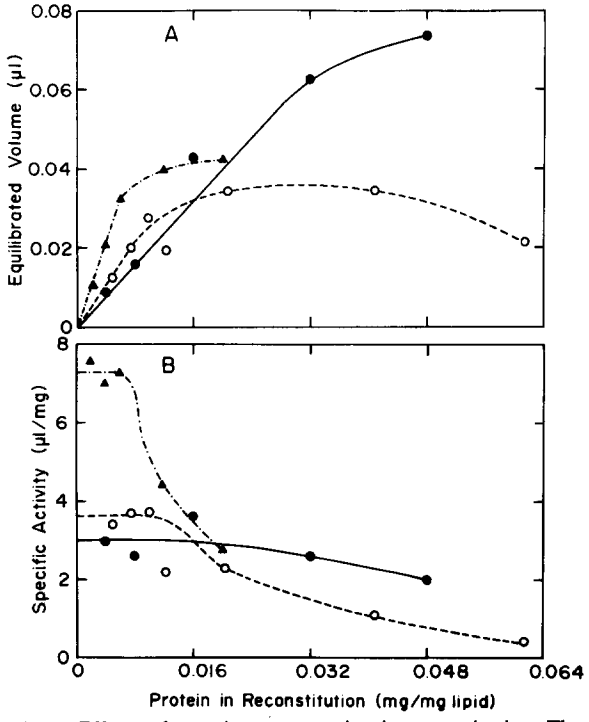

Fig. 6. Effects of protein concentration in reconstitution. The stereospecific uptake in 2 min was measured for liposomes reconstituted with plasma membranes directly (●), after cholate dispersal using 1% cholate and 5 mg protein/ml followed by $15\,000 \times g$ centrifugation (○), and after cholate solubilization using 1.5% cholate and 2 mg protein/ml followed by $100\,000 \times g$ centrifugation (▲). A, equilibrated volume; B, specific activity expressed as μl equilibrated per mg protein.

volume reaches a maximum and, in the case of the cholate-dispersed membranes, begins to decline. Two factors could explain these results: other proteins present in the membranes may interfere with the reconstitution and/or make the liposomes leaky; and higher protein concentrations could result in more liposomes with multiple transporters, giving no additional equilibrated volume [34]. Calculations based on results with the reconstituted erythrocyte transporter [34] and the relative numbers of cytochalasin B binding sites indicated that a ratio of one heart transporter per liposome would be achieved only above 1 mg protein/mg lipid (assuming a random distribution). Thus the latter explanation appears unlikely. In the experiments shown in Figs. 2–5 we used cholate-treated membranes reconstituted at 0.01 to 0.025 mg protein/mg lipid in order to give the

maximum uptake, although this results in a lower specific activity than the maximum shown in Fig. 6.

In the case of cholate solubilized proteins, the decrease in specific activity with increasing amounts of protein occurred at much lower protein concentrations. Experiments with [^{14}C]cholic acid showed that with a 1% cholate extract, 99% of the cholate was removed by the gel filtration. Interference with the reconstitution by residual cholate is therefore unlikely. At the lowest three concentrations tested (0.002 to 0.006 mg protein/mg lipid), the specific activity was constant, within experimental error, at about 7 μl/mg. We have therefore indicated a plateau of specific activity for this range of concentrations in Fig. 6. It should be noted, however, that at very low protein concentrations the uptake becomes more difficult to measure accurately and the curve could have a somewhat different shape than shown.

Thus for each of these three types of samples it is possible to perform the reconstitution under conditions (low ratios of protein to lipid) where equilibrated volume is proportional to the amount of protein. The results of Sase et al. [34] revealed that the reconstituted erythrocyte transporter was distributed randomly, independent of the size of the liposomes and the amount of protein used. If the reconstituted heart transporter is also distributed randomly, then under the conditions of our experiments most uptake is from liposomes with single functional transporters. The volume is, therefore, a measure of the number of transporters and an appropriate measure of specific activity. This type of measurement, termed an 'all-or-none assay', has been reported by Baldwin et al. [35]. We also tested crude membranes reconstituted at 0.01 to 0.06 mg protein/mg lipid, but no significant transport activity was observed.

Effects of different lipids

Reconstituted transport activity was tested using liposomes prepared from various types of phosphatidylcholine (Table I). The activity was measured for both cholate-treated heart membranes and untreated human erythrocyte ghosts. Compared to type II-S from soybeans (commercial grade), which we used routinely, acetone-washed [36] PC and more purified preparations showed progressively lower activity with heart membranes. This indicates that other components present in the crude PC preparations are important for either the reconstitution process or the activity of the transporter in the reconstituted liposomes. Previous studies of the purified human erythrocyte transporter showed that a mixture of PC and phosphatidylethanolamine, but not either lipid alone, resulted in successful reconstitution (Hinkle, P.C., personal communication). Thus the crude PC preparations are probably providing the proper lipid mixture in this case also. Egg PC (Type IX-E) gave an activity within the range of activities given by two lots of type II-S.

The reconstituted heart transporter showed a

TABLE I

EFFECTS OF LIPIDS IN RECONSTITUTION PROCEDURE

Liposomes were prepared by sonication of various types of phosphatidylcholine from Sigma and reconstituted with cholate-treated heart membranes or sonicated human erythrocyte ghosts. Uptake of 0.2 mM glucose in 2 min was measured. Data represent experimental figures or means ± S.E. ($n = 3$) Negative numbers refer to an excess of L- over D-glucose uptake; this was not statistically significant.

Type	Lot	Relative uptake (%)	
		Heart	Ghosts
Soybean II-S (10–20%)	640-03132	100	100
	14F-0159	178 ± 13	106, 163
	45C-0060	−10, −16	0, 0
Acetone-washed II-S	640-03132	59 ± 8	70 ± 32
Soybean IV-S (40%)	101F-7200	12, 29	1, 6
Soybean III-S (99%)	083F-8390	2, 12	−9, 7
Egg IX-E (60%)	61F-7005	124, 180	83, 131

lipid sensitivity similar to that of the erythrocyte transporter when ghost proteins were reconstituted at a low concentration (0.004 mg protein/mg lipid) (Table I). When much higher amounts of ghost proteins were used (about 0.08 mg protein/mg lipid), types II-S, acetone-washed II-S, and IV-S all gave the same activity (not shown). Under these conditions some liposomes probably have multiple transporters, and only the total internal volume is being measured; thus the uptake is less sensitive to the efficiency with which transporters are reconstituted.

Studies with the reconstituted rat adipocyte transporter [22] also showed lower activity with soybean type IV-S than with type II-S. However, in the experiments with the adipocyte transporter a 2-fold greater activity was seen for type IX-E compared to type II-S.

We obtained similar activity with two lots of type II-S PC. However, another lot (45C-0060) gave no activity with either reconstituted heart or ghost proteins. Assays using this lot not only had no stereospecific transport activity, but also showed very little nonspecific uptake, indicating inability of the lipids to form sealed liposomes in the reconstitution process.

Extraction of transport activity with cholate

Fig. 7 shows the results of solubilization of heart membranes with various concentrations of sodium cholate, followed by centrifugation at $100\,000 \times g$ for 30 min and reconstitution of the supernatants. The specific activity, measured by the equilibrated volume using low protein levels in the reconstitution (0.008 mg protein/mg lipid), increased with increasing cholate, reaching a maximum of 6.6 μl/mg at 1.5% cholate. Since each point represents the activity of all proteins solubilized by the indicated cholate concentration, the specific activity of the additional proteins solubilized by progressively higher concentrations must have been somewhat higher. By comparing the increase in the yield of total activity between 0.6 and 1.5% cholate to the increase in protein solubilized, it can be estimated that the additional protein solubilized in this interval had a specific activity of about 10 μl/mg. This is more than three times the specific activity of the unextracted membranes (about 3 μl/mg, Fig. 6B).

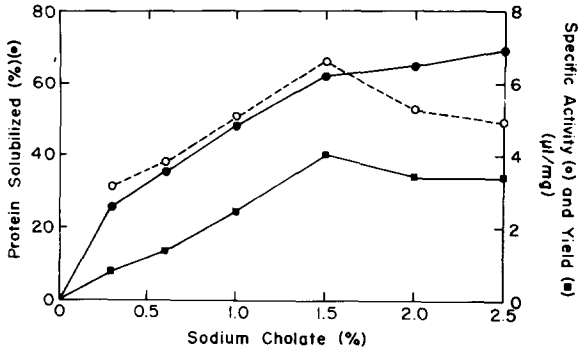

Fig. 7. Extraction of transport activity with sodium cholate. Heart membranes were solubilized with the indicated concentrations of cholate and centrifuged at $100000 \times g$ for 30 min. After removal of cholate by gel filtration, the extracts were reconstituted at 0.008 mg protein/mg lipid and uptake of 0.2 mM glucose in 2 min measured. Protein solubilized (●); specific activity (μl equilibrated/mg protein in assay) (○); yield of transport activity (μl/mg membrane protein) (■), obtained by multiplying the specific activity by the fraction of protein extracted. Results are means of two experiments.

Measurement of cytochalasin B binding to the supernatant and pellet after solubilization with 1.5% cholate, gel filtration, and lyophilization indicated that 80% of the binding sites were extracted. At cholate concentrations higher than 1.5%, it appeared that no additional transport activity was solubilized (Fig. 7), such that the total yield of activity remained constant while the specific activity decreased.

Immunological identification of the heart glucose transporter

Fig. 8 shows the results of electrophoretic protein transfer of heart membrane proteins from SDS-polyacrylamide gels to nitrocellulose paper [26]. The transferred proteins were labeled with rabbit antisera prepared against the human erythrocyte glucose transporter and ^{125}I-protein A [27]. With dog heart membranes prepared by method I the antibody labeled a broad band centered at an apparent molecular mass of 46 kDa. With bovine heart membranes prepared by method II, a much sharper band at about 43 kDa was labeled.

The identity of the human erythrocyte glucose transporter is controversial. The purified preparations of a component of band 4.5 (nomenclature of Fairbanks et al. [37]), which display cytochala-

sin B binding and reconstituted transport activity, have an apparent mass of 46 kDa after removal of most of the carbohydrate [38]. Similar size proteins (40 to 60 kDa) have also been observed in HeLa cells [39], human placenta [40,41], rat adipocytes [28,42,43] and skeletal muscle [31], and chick embryo fibroblasts [43–45] by photolabeling with cytochalasin B and/or labeling with antibodies against the human erythrocyte band 4.5 protein. The labeling in Fig. 8 indicates that such antibodies also recognize polypeptides of similar size in dog and bovine heart membranes.

It has been suggested that glucose transport is catalyzed by a 100 kDa component of band 3 (see for example, Ref. 46), and that either the band 4.5 protein is a proteolytic fragment or both the band 3 and band 4.5 proteins are glucose transporters (reviewed in Refs. 47 and 48). We feel, however, that the antibody results with fresh membranes and the cytochalasin B photolabeling experiments argue strongly against the identification of band 3 as the transporter [1]. If the human erythrocyte transporter is indeed a 46 kDa protein, the results in Fig. 8 indicate a similar size for the dog and bovine heart transporters.

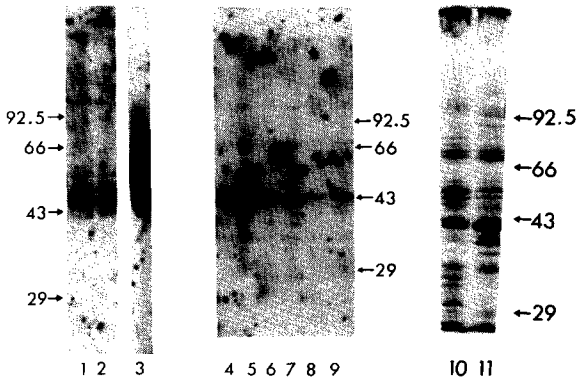

Fig. 8. Immunological identification of the heart glucose transporter. Lanes 1–9: Membrane fractions were separated by SDS-polyacrylamide gel electrophoresis, transferred electrophoretically to nitrocellulose paper, and identified using antisera prepared against the purified human erythrocyte glucose transporter and ^{125}I-protein A. Lanes 1 and 2: Dog heart membranes prepared by method I, 100 and 50 μg protein, respectively. Lane 3: Alkaline-washed human erythrocyte membranes, 0.25 μg. Lanes 4–9: Bovine heart membranes prepared by method II. Lanes 4 and 5, 50 and 100 μg of one preparation; lanes 6 and 7, 50 and 100 μg of another preparation; lanes 8 and 9, 50 and 100 μg of cholate-dispersed membrane proteins similar to those used in the reconstitution studies. Lanes 1–3 and 4–9 are from two different gels, and the relative intensities of labeling are not comparable. Lanes 10 and 11: Proteins (50 μg) from bovine heart membranes prepared by method I (lane 10) and method II (lane 11) were separated by electrophoresis and stained with Coomassie blue. The arrows designate the M_r ($\times 10^{-3}$) of protein standards: carbonic anhydrase (29000), ovalbumin (43000), bovine serum albumin (66000), and phosphorylase *b* (92500).

Fig. 8 also shows a Coomassie-stained SDS-polyacrylamide gel with bovine heart membranes prepared by methods I and II. The overall protein compositions appear similar. In the case of cholate dispensed or cholate-extracted proteins, the electrophoretic patterns are quite similar to the starting membranes (not shown). Since the cytochalasin B binding data indicates that less than 0.2% of the membrane protein is the glucose transporter, identification based on changes in staining patterns would require a much higher degree of purification than is achieved by the cholate extraction.

Discussion

The experiments reported here show that the glucose transporter from bovine heart plasma membranes can be reconstituted into liposomes. The transporter is functional after reconstitution by the criteria of stereospecificity, saturability, and sensitivity to inhibitors.

The bovine heart appears to be an excellent tissue source for the purification of the heart transporter. Plasma membranes prepared by method II showed a cytochalasin B binding capacity (33 pmol/mg protein) comparable to plasma membranes prepared from insulin-stimulated rat adipocytes [14] and diaphragm [15]. Since bovine hearts can readily be obtained in kg quantities at low cost, the preparation of large amounts of starting material for purification of the transporter is feasible.

The experiments shown in Fig. 7 demonstrate that the transport activity can be solubilized by sodium cholate. Thus the use of this detergent in further fractionation of the transporter appears promising. Preliminary experiments using Triton X-100 proved unsuccessful, but the use of this and other detergents remains to be tested in detail.

The bovine heart transporter also appears to be a suitable system for the study of the regulation of glucose transport in heart. Transport activity can

be demonstrated at various stages of purification: using intact membranes both before (not shown) and after a freeze-thaw step; with membranes reconstituted directly (Fig. 6); and with detergent extracts (Figs. 6 and 7). Proposed regulatory mechanisms, which might require the presence of other membrane components, can be tested at each of these stages. In addition, the effects of the phospholipid environment on the transport activity can be tested by modifying the lipid composition of the liposomes [5].

Kinetic studies of transporters in different tissues have shown different degrees of asymmetry and exchange acceleration [1], and it is of interest to compare the properties of the heart transporter. However, such studies are difficult in the intact heart. The reconstituted heart transporter should be a suitable system for kinetic studies. We have not yet attempted such studies in detail because the fairly high proportion of uptake that is non-specific (Fig. 2) makes initial rate measurements difficult. With a partially purified preparation studies such as those reported for the purified human erythrocyte transporter [24] should be feasible.

Acknowledgements

We thank Drs. Peter Hinkle and William Dean for helpful discussion, and Dr. Hinkle for providing the samples of soybean PC; and Dr. Ian Simpson for advice concerning the cytochalasin B binding assays. This work was supported by a grant from the Kentucky Affiliate of the American Heart Association and by National Institutes of Health grant AM31332.

References

1 Wheeler, T.J. and Hinkle, P.C. (1985) Annu. Rev. Physiol. 47, 503–517
2 Shanahan, M.F. and Czech, M.P. (1977) J. Biol. Chem. 252, 8341–8343
3 Suzuki, K. and Kono, T. (1980) Proc. Natl. Acad. Sci. USA 77, 2542–2545
4 Carter-Su, C., Pillion, D.J. and Czech, M.P. (1980) Biochemistry 19, 2374–2385
5 Baldassare, J.J. (1983) J. Biol. Chem. 258, 10223–10226
6 Schraw, W.P. and Regen, D.M. (1983) Arch. Biochem. Biophys. 220, 214–224
7 McCormick, J.I., Tsang, D. and Johnstone, R.M. (1984) Arch. Biochem. Biophys. 231, 355–365
8 Morgan, H.E. and Whitfield, C.F. (1973) Curr. Top. Membranes Transp. 4, 255–303
9 Elbrink, J. and Bihler, I. (1975) Science 188, 1177–1184
10 Bihler, I., Hoeschen, L.E. and Sawh, P.C. (1980) Can. J. Physiol. Pharmacol. 58, 1184–1188
11 Bihler, I. and Sawh, P.C. (1976) Can. J. Physiol. Pharmacol. 54, 714–718
12 Bihler, I. and Sawh, P.C. (1979) J. Mol. Cell. Cardiol. 11, 407–414
13 Watanabe, T., Smith, M.M., Robinson, F.W. and Kono, T. (1984) J. Biol. Chem. 259, 13117–13122
14 Cushman, S.W., Wardzala, L.J., Simpson, I.A., Karnieli, E., Hissin, P.J., Wheeler, T.J., Hinkle, P.C. and Salans, L.B. (1984) Fed. Proc. 43, 2251–2255
15 Wardzala, L.J. and Jeanrenaud, B. (1981) J. Biol. Chem. 256, 7090–7093
16 Simpson, I.A., Yver, D.R., Hissin, P.J., Wardzala, L.J., Karnieli, E., Salans, L.B. and Cushman, S.W. (1983) Biochim. Biophys. Acta 763, 393–407
17 Kidwai, A.M. (1974) Methods Enzymol. 31, 134–144
18 Klip, A. and Walker, D. (1983) Arch. Biochem. Biophys. 221, 175–187
19 Slaughter, R.S., Sutko, J.L. and Reeves, J.P. (1983) J. Biol. Chem. 258, 3183–3190
20 Cushman, S.W. and Wardzala, L.J. (1980) J. Biol. Chem. 255, 4758–4762
21 Cleland, W.W. (1967) Adv. Enzymol. 29, 1–32
22 Robinson, F.W., Blevins, T.L., Suzuki, K. and Kono, T. (1982) Anal. Biochem. 122, 10–19
23 Kasahara, M. and Hinkle, P.C. (1977) J. Biol. Chem. 252, 7384–7390
24 Wheeler, T.J. and Hinkle, P.C. (1981) J. Biol. Chem. 256, 8907–8914
25 Laemmli, U.K. (1970) Nature 227, 680–685
26 Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354
27 Renart, J., Reiser, J. and Stark, G.R. (1979) Proc. Natl. Acad. Sci. USA 76, 3116–3120
28 Wheeler, T.J., Simpson, I.A., Sogin, D.C., Hinkle, P.C. and Cushman, S.W. (1982) Biochem. Biophys. Res. Commun. 105, 89–95
29 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
30 Sogin, D.C. and Hinkle, P.C. (1980) Biochemistry 19, 5417–5420
31 Klip, A., Walker, D., Ransome, K.J., Schroer, D.W. and Lienhard, G.E. (1983) Arch. Biochem. Biophys. 226, 198–205
32 Cheung, J.Y., Conover, C., Regen, D.M., Whitfield, C.F. and Morgan, H.E. (1978) Am. J. Physiol. 234, E70–E78
33 Van Hoogevest, P., Du Maine, A.P.M. and De Kruijff, B. (1983) FEBS Lett. 157, 41–45
34 Sase, A., Anraku, Y., Nagano, M., Osumi, M. and Kasahara, M. (1982) J. Biol. Chem. 257, 11100–11105
35 Baldwin, J.M. Gorga, J.C. and Lienhard, G.E. (1981) J. Biol. Chem. 256, 3685–3689

36 Kagawa, Y. and Racker, E. (1971) J. Biol. Chem. 246, 5477–5487
37 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617
38 Gorga, F.R., Baldwin, S.A. and Lienhard, G.E. (1979) Biochem. Biophys. Res. Commun. 91, 955–961
39 Sogin, D.C. and Hinkle, P.C. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 5725–5729
40 Johnson, L.W. and Smith, C.H. (1982) Biochem. Biophys. Res. Commun. 109, 408–413
41 Ingermann, R.L., Bissonnette, J.M. and Koch, P.L. (1983) Biochim. Biophys. Acta 730, 57–63
42 Lienhard, G.E., Kim, H.H., Ransome, K.J. and Gorga, J.C. (1982) Biochem. Biophys. Res. Commun. 105, 1150–1156
43 Shanahan, M.F., Olson, S.A., Weber, M.J., Lienhard, G.E. and Gorga, J.C. (1982) Biochem. Biophys. Res. Commun. 107, 38–43
44 Salter, D.W., Baldwin, S.A., Lienhard, G.E. and Weber, M.J. (1982) Proc. Natl. Acad. Sci. USA 79, 1540–1544
45 Pessin, J.E., Tillotson, L.G., Yamada, K., Gitomer, W., Carter-Su, C., Mora, R., Isselbacher, K.J. and Czech, M.P. (1982) Proc. Natl. Acad. Sci. USA 79, 2286–2290
46 Mullins, R.E. and Langdon, R.G. (1980) Biochemistry 19, 1205–1212
47 Jones, M.N. and Nickson, J.K. (1981) Biochim. Biophys. Acta 650, 1–20
48 Carruthers, A. (1984) Prog. Biophys. Mol. Biol. 43, 33–69