Partial Purification of the d-Glucose Transport System in Rat Adipocyte Plasma Membranes*

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Partially purified rat adipocyte plasma membranes were subjected to selective protein extractions using sodium hydroxide, sodium iodide, and dimethylmaleic anhydride in an effort to identify membrane components associated with glucose transport. Up to 80% of the membrane proteins were extracted in these procedures with the remaining proteins consisting almost entirely of two glycoprotein fractions, 78,000 and 94,000 daltons, as determined by dodecyl sulfate polyacrylamide gel electrophoresis. Sonic disruption of these extracted membrane residues resulted in the formation of vesicular structures as revealed by electron microscopy. These vesicles demonstrated high affinity binding of \([\text{3H}]\text{cytochalasin B}\) in all three preparations. Vesicles from the dimethylmaleic anhydride-extracted membranes also exhibited a marked stereospecific uptake of \(\text{d}-\text{glucose}\) compared to \(\text{l}-\text{glucose}\), as measured by a rapid filtration method. This uptake was markedly inhibited by cytochalasin B and this inhibition closely paralleled the high affinity binding of \([\text{3H}]\text{cytochalasin B}\) to these vesicles. In addition, uptake of \(\text{l}-[\text{3H}]\text{glucose}\) was inhibited by phoretin, phlorizin, and dipyriramole, all potent inhibitors of \(\text{d}-\text{glucose}\) transport in the intact adipocyte. Competitive inhibitors of glucose transport such as 3-O-methylglucose and unlabeled \(\text{d}-\text{glucose}\) itself, also inhibited uptake, while \(\text{l}-\text{glucose}\) and sucrose exhibited almost no effect. These results are consistent with the hypothesis that fat cell hexose transport system activity is associated with membrane components in one or both of the 78,000- and 94,000-dalton glycoprotein fractions.

Hexose transport has been characterized in a variety of mammalian cell types as operating via a system of facilitated diffusion. Although the human erythrocyte has been the model of major interest in studying this mode of transport, other systems have come under closer scrutiny in recent years (1-4). This has been due, in part, to improved techniques in attempts to identify the membrane components associated with hexose transport activity. Methods using affinity and differential labeling of membranes have begun to prove successful in localizing transport-associated components to specific membrane protein bands on polyacrylamide gels (15-18). Other approaches have utilized various extraction procedures in attempts to purify transport-associated and high affinity cytochalasin B-binding proteins (18, 21, 22). Very recently, successful reconstitution of hexose transport activity has been achieved subsequent to incorporation of partially purified erythrocyte membrane proteins into phospholipid vesicles (20, 23-25). We report here that extraction of partially purified fat cell plasma membranes with 2,3-dimethylmaleic anhydride yields a membrane preparation which consists almost entirely of two glycoprotein bands on SDS-polyacrylamide gel electrophoresis. These extracted membranes retain stereospecific \(\text{d}-\text{glucose}\) transport activity that is sensitive to a variety of known inhibitors of glucose transport. Furthermore, the extracted membranes contain high affinity cytochalasin B binding sites which appear to be involved in the inhibition of glucose transport by cytochalasin B.

MATERIALS AND METHODS

Isolation of Fat Cells - White fat cells were obtained (6) by enzymatic digestion of the parametrial adipose tissue of 200- to 700-g

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The abbreviations used are: SDS, sodium dodecyl sulfate; KRP buffer, Krebs-Ringer phosphate buffer; PAS, periodic acid-Schiff.
female rats (Charles River CD strain) fed laboratory chow ad libitum. For each experiment involving isolated cells and plasma membranes the parametrical adipose tissue (40 to 80 g) from 10 or more rats was pooled and cut into small pieces with scissors, blotted, and added to small plastic bottles. Each bottle normally contained from 5 to 10 g of tissue and 10 ml of Krebs-Ringer phosphate buffer (pH 7.4) containing 128 mM NaCl, 1.4 mM CaCl₂, 1.4 mM MgSO₄, 5.3 mM KCl, and 10 mM NaHCO₃. At the end of the digestion period cells were filtered through one layer of nylon chiffon, washed twice with albumin buffer, and once with warm 0.25 M sucrose, 1 mM EDTA, 5 mM Tris at pH 7.5 (Buffer A).

Preparation of Plasma Membrane Fraction – The experiments described herein were performed with a crude fat cell plasma membrane preparation as well as a purified plasma membrane preparation (26). We found that the crude membrane preparation was often more sensitive to selective extraction by dimethylmaleic anhydride than the more purified preparation obtained by high speed centrifugation on sucrose density gradients. The crude preparations of plasma membranes were prepared by homogenization of cells in cold Buffer A in a loose fitting glass homogenizing tube with seven up and down strokes using a Teflon pestle. The homogenate was centrifuged at 8500 × g for 10 min and the supernatant as well as a small amount of white matter collected from the surface of the brown (mitochondrial) pellet were then vigorously sonicated for 2 min and the resulting pellet resuspended in ice-cold 1 mM EDTA, 5 mM Tris at pH 7.5 (Buffer B).

Membrane Extraction – Extraction of plasma membranes with NaOH was carried out in a similar manner to that of Steck and Yu (27). For NaOH extraction 1 volume of membrane suspension (4 mg/ml) was suspended in 10 volumes of ice cold water previously brought to pH 12 with 1N NaOH. The extraction proceeded for 5 min after which the solution was neutralized to pH 7.0 with 0.5N HCl and centrifuged at 1.5 × 10⁶ g-min on a Sorvall RC-2 centrifuge. For dimethylmaleic anhydride extractions, 1 volume of membranes was added to 15 volumes of water and 2 mg/ml of solid dimethylmaleic anhydride was added with constant stirring while maintaining the pH at 8.0 with continuous aliquots of 0.1N NaOH solution. After acid ceased to evolve, the suspension was centrifuged as above, and the pellet resuspended in 5 mM Tris buffer, 1 mM EDTA, pH 6.8. This suspension was either frozen overnight and the next day centrifuged and the pellet resuspended in Krebs-Ringer phosphate buffer, pH 7.4, for uptake and binding studies, or centrifuged and resuspended immediately after preparation.

NaOH extraction, based on the procedure of Kahlenberg (19). One volume of membrane suspension was suspended in 7 volumes of 1M NaOH in water, adjusted to pH 7.5, and incubated for 30 min after which the suspension was centrifuged as above. The pellet was washed three times in 5 mM sodium phosphate buffer, pH 7.4, then resuspended in Krebs-Ringer phosphate buffer. After final resuspension, all pellets were dispersed for 2 s using a Brinkman Polytron apparatus which is similar to the sonication step recommended by Carter et al. (12) and uptake or binding studies were then performed.

Glucose Transport in Membrane Vesicles – Assay of glucose uptake by membranes was routinely performed by addition of 20 to 50 μl of a membrane suspension in Krebs-Ringer phosphate buffer to glass test tubes (12 × 100 mm). At this point, 0.5 μl of any inhibitors used was added to the appropriate final concentration. Five minutes later transport was initiated by the addition of 1 μl of a solution containing 0.1% BSA, 0.1% glucose, or 1 μCi/ml of [3H]glucose. The labeled hexoses were dissolved in acidic NaCl before addition. The tubes were immediately vigorously shaken by hand and incubated at room temperature for the appropriate times before transport was stopped by addition of 2 ml of ice-cold Krebs-Ringer phosphate buffer. The membranes were then decanted onto Millipore HA (0.45 μm pore size, 25 mm diameter) filters attached to a rotary Doerr pump and quickly washed with 4 ml of ice cold buffer. The filters were soaked in distilled water with three changes for at least 1 h before use. The total time taken to filter and wash the cells was less than 10 s. The filters were dried in air at room temperature and immersed in 4 ml of liquid scintillation fluid containing 4 g/liter of Omnifluor (New England Nuclear) in toluene containing 33% Triton X-100.

Uptake of label is the amount of radioactivity accumulated at a given time minus the radioactivity bound on filters containing membrane to which labeled hexose and 2 ml of cold buffer were added together. Under the conditions of our experiments, these control values represented about 90 to 95% of the total radioactivity obtained from 20-s incubations depending on the amount of membrane present. This method gave highly reproducible results if performed in triplicate. In efflux experiments, 50 μl of membrane suspension was added to a small test tube and preloaded by incubation for 30 min in 0.02 M Hglucose at 37°C. After that time 2 ml of KRP buffer with or without various concentrations of cytochalasin B was added rapidly to the tube and mixed. At the specified times the membrane suspensions were decanted into filters and assayed as described above. The experiments presented in this report were performed on 2 to 5 separate days, and the values are either the results of representative experiments or of the means of several experiments.

"[3H]Cytochalasin B Binding to Fat Cell Membranes – Assay of [3H]cytochalasin B binding to membranes was performed essentially as previously described (13) (which is similar to that for monitoring transport activity as described above). Briefly, the membranes were incubated in 40 to 50 μl of the indicated buffer and 0.5 μl of [3H]cytochalasin B in ethanol was added. After the appropriate times, 2 ml of ice cold buffer were added and the mixture quickly decanted onto EH Millipore filters and the filters washed with 4 ml of ice-cold buffer. It was crucial that the control tubes were run containing buffer and [3H]cytochalasin B, but without membranes, under the exact conditions used for the experimental tubes. The reason is that certain conditions (e.g. elevated temperature) promote aggregation of [3H]cytochalasin B which then itself binds to the filters. The method used was that of Lowry et al. (26) using bovine serum albumin as a standard. Total carbohydrate was estimated with glucose as a standard according to the procedure of Dubois et al. (22). Organic phosphorus was measured by Bartlett’s procedure (30) and the phospholipid content estimated by multiplying the organic phosphorus content by 23. Sialic acid was determined by the method of Warren (31). Sialic acid was measured after hydrolysis of delipitated membranes with 0.1N H₂SO₄ for 1 h and proper corrections made for possible malonyldialdehyde interference.

"[3H]Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis – Gel electrophoresis of plasma membranes was performed using polyacrylamide gels prepared as previously described (32) except that 5% acrylamide and half the amount of cross-linker were used. Membranes suspended in 1 mM EDTA, 5 mM Tris, pH 7.8, were diluted with an equal volume of 4% sodium dodecyl sulfate, 8 mM urea, 5 μl of 0.05% bromophenol blue were added, and samples were heated at 100°C for 5 min. The solubilized membranes were applied to columns (100 × 5 mm) of polyacrylamide gels in the absence of glycerol but the urine contained sufficient density to the sample. The reservoir buffer contained 0.1% sodium dodecyl sulfate. Electrophoresis was performed overnight at a current of 4 mA/gel or 8 h at 8 mA/gel. The gels were stained for protein by immersion in Coomassie blue stain (1.25 g of Coomassie blue dissolved in 450 ml of 50% methanol plus 45 ml of glacial acetic acid) for 3 to 6 h. The gels were electrophoretically analyzed in a Cambridge CameraWeb. Gel scanning was performed by the method of Lowry et al. (26) using bovine serum albumin as a standard. Total carbohydrate was estimated with glucose as a standard according to the procedure of Dubois et al. (22). Organic phosphorus was measured by Bartlett’s procedure (30) and the phospholipid content estimated by multiplying the organic phosphorus content by 23. Sialic acid was determined by the method of Warren (31). Sialic acid was measured after hydrolysis of delipitated membranes with 0.1N H₂SO₄ for 1 h and proper corrections made for possible malonyldialdehyde interference.
to various protein extraction procedures which have proved selective in red cells (19, 21, 27) and tested the sedimented membrane residues for \( \alpha \)-glucose uptake after extraction. Fig. 1 shows the remaining membrane polypeptides following extraction of a crude plasma membrane preparation under alkaline conditions using NaOH, hyperosmolar NaI, or dimethylmaleic anhydride. Although all of these substances effected selective elution of membrane proteins, dimethylmaleic anhydride was by far the most effective, followed by NaOH and NaI, respectively. In all three cases the membrane proteins in the 94,000- and 78,000-dalton region of dodecyl sulfate gels remained with the pellet while most other Coomassie blue-staining proteins were selectively eluted to varying degrees. After extraction, the membrane residues were washed, resuspended in Krebs-Ringer phosphate buffer, and dispersed in this buffer using a Brinkmann Polytron apparatus before \(^{3}H\)cytochalasin B binding and \( \alpha \)-glucose uptake were monitored. Although all three preparations exhibited high affinity cytochalasin B binding capacity, only the sodium iodide and dimethylmaleic anhydride-extracted membrane residues exhibited stereospecific \( \alpha \)-glucose uptake (data not shown). Since the dimethylmaleic anhydride extraction was the most selective in eluting membrane proteins, this preparation was used for all subsequent investigations.

Fig. 2 represents densitometric scans of SDS-polyacrylamide gels stained with Coomassie blue or Schiff reagent following electrophoresis of the control membranes and those extracted with dimethylmaleic anhydride. Crude unextracted plasma membranes were resolved into approximately 14 bands on 5% acrylamide gels and resembled the pattern previously observed by several laboratories (34-36) in that two major glycoprotein species (Bands I and II) were observed at 94,000 and 78,000 daltons. The prominence of the 96,000-dalton band (Band III) in this crude plasma membrane preparation relative to purer preparations (29) reflects additional protein contamination in this region from other subcellular fractions (see Ref. 34). In some experiments up to 80% of the total membrane protein was released from the plasma membrane preparation in the presence of dimethylmaleic anhydride. The dimethylmaleic anhydride-extracted pellet consisted chiefly of glycoprotein Bands I and II and a very small amount of the material in the Band III region (Fig. 2B). The values indicated above each region represent the per cent contribution those bands make to the total protein content of the membrane based on Coomassie blue-staining intensity. In the experiment illustrated in Fig. 2, Band I (94,000 daltons) constituted approximately 10% of the plasma membrane preparation, while it composed 69% of the dimethylmaleic anhydride pellet preparation. Similarly, Band II (78,000 daltons) was enriched from 4.4% in control membranes to 25% of the extracted pellet. On the other hand, the major Coomassie blue-staining protein in the control plasma membrane preparation, Band III (56,000 daltons), constituted 37% of the original preparation, but only 5.6% of the extracted pellet. Band III was the only other constituent of this pellet which routinely accounted for greater than 1% of the total staining polypeptides. However, several dimethylmaleic anhydride-extracted preparations have had virtually no detectable protein material in this region of dodecyl sulfate gels (not shown).

Results of analyses of the composition of extracted membranes in three experiments are presented in Table I. While the average total protein recovered in the extracted pellets in the experiments presented was about 25% of the starting material, the relative phosopholipid content increased by 35%. Both neutral sugar and sialic acid content markedly increased on a per mg of protein basis in the dimethylmaleic anhydride-extracted membrane pellet. There was significant solubilization of all three membrane components by dimethylmaleic anhydride.
Purification of the Adipocyte Hexose Transport System

TABLE I
Chemical composition of membrane pellets following extraction with 2,3-dimethylmaleic anhydride

One volume of plasma membranes was combined with 15 volumes of H₂O (at 22°) and 2 mg/ml of solid 2,3-dimethylmaleic anhydride was added, while the pH maintained at 8.0 with 2 n NaOH. After the evolution of acid ceased, the suspensions were centrifuged and the pellet was analyzed as described under “Materials and Methods.” Each value represents a pooled average of three different extractions.

| Component         | Plasma membrane | Extracted membrane | Per cent recovered in pellet |
|-------------------|-----------------|--------------------|-------------------------------|
| Protein (mg)      | 3.2             | 0.78               | 24                            |
| Lipid P (µg/mg protein) | 26             | 35                 | 33                            |
| Neutral sugar*    | 0.08            | 0.10               | 30                            |
| Sialic acid*      | 50              | 87                 | 42                            |

* Determined using membranes extracted by the addition of 10 ml of 100% ethanol to 0.4-ml membranes (~1 mg of protein) and occasionally stirring the mixture for 15 min at 22°.

anhydride, however. Interestingly, extraction of membranes with this agent resulted in the solubilization of the low molecular weight PAS-staining glycoprotein designated Band III (Fig. 2), which is suspected of being an endoplasmic reticulum contaminant (34). In these extractions, over 60% of the membrane phospholipid was eluted, but the resulting pellet was still enriched in phospholipids, due to the even greater extraction of proteins from the membrane (70 to 80%). Theoretically, the extraction of all bands other than I and II should result in a recovery of about 13% of the total protein in the membrane pellet based on the gel scan in Fig. 2A. However, since membrane glycoproteins often stain poorly with Coomassie blue, their relative contribution to the total membrane protein may be underestimated. Therefore, it is not feasible to make an exact quantitative comparison between the data in Table I and Fig. 2. While it is possible that other proteins are present in the extracted membrane pellets, the data in Figs. 1 and 2 indicate that if this is the case either these proteins must be present in very small amounts or do not stain with Coomassie blue stain.

FIG. 3. Transmission electron micrograph of dimethylmaleic anhydride-extracted rat adipocyte plasma membranes. The arrow indicates apparent globular structures around the periphery of the vesicles. Preparations were stained for 1 min with 1% phosphotungstic acid (pH 7.4).
electron photomicrograph of these vesicles is presented in Fig. 3. The size distribution of these vesicles ranges from around 0.1 μm to about 0.7 μm. Fig. 3B depicts (arrow) the appearance of apparent globular structures on the outer surface of many of these vesicles. Since some vesicles are present without these structures their presence may represent some type of surface asymmetry with the implication that many of these vesicles may be inverted. Evidence of inverted vesicles in unextracted plasma membrane preparations has been presented by others (37); however, we have no information as to the nature or distribution of these particles in this preparation.

Characterization of Glucose Transport in Dimethylmaleic Anhydride-Extracted Vesicles—Uptake of both D- and L-glucose into dimethylmaleic anhydride-extracted membranes in the presence or absence of cytochalasin B was determined using rapid filtration (Fig. 4). D-Glucose entered the vesicles much more rapidly than L-glucose and often reached equilibrium values by around 10 to 15 min in the absence of cytochalasin B. In the presence of 100 μM cytochalasin B the uptake of D-glucose was markedly decreased and was the same as that of L-glucose. In other experiments D-glucose in the presence and absence of cytochalasin B reached the same equilibrium value at 22° after about 1 h of incubation (not illustrated).

Efflux experiments were also performed in order to test whether this preferential uptake of D-glucose reflected transport activity or rather a binding phenomenon which was inhibited by cytochalasin B. If uptake were due to binding rather than transmembrane flux, then the inhibitory effect of cytochalasin B in the medium should reflect an enhanced rate of dissociation or a decreased rate of association of D-glucose with the vesicles. On the other hand, if the uptake process actually reflects transport, then the presence of cytochalasin B should inhibit efflux as well as influx of hexose. Fig. 5 illustrates the results of an experiment where dimethylmaleic anhydride-extracted membranes were equilibrated with D-[14C]glucose prior to dilution and the loss of labeled glucose assayed at the times indicated. Cytochalasin B significantly retarded this loss of label, consistent with the concept that transport is the process being observed. No transport was observed unless the membranes were dispersed by the Brinkmann Polytron, and transport could be blocked by boiling. Activity also markedly decreased after several hours at room temperature.

In addition to cytochalasin B, other known inhibitors of D-glucose transport in fat cells were tested for the ability to inhibit D-glucose transport by these dimethylmaleic anhydride-extracted membranes (Table II). In these experiments phlorizin, phloretin, and dipyridamole were potent inhibitors of transport while unlabeled sugars such as 90 mM 3-O-methyl glucose and D-glucose also inhibited, but to a lesser degree. Interestingly, L-glucose itself inhibited D-glucose uptake about 20%. This may reflect an osmotic shrinking of the vesicles rather than inhibition of influx since 90 mM sucrose also exhibited a similar effect (data not shown). Alternatively, this small inhibition may reflect a low affinity of L-glucose for the D-glucose transport system as has been observed in intact fat cells (9).

Characteristics of Cytochalasin Binding—The relationship between cytochalasin B binding and inhibition of glucose transport was studied in these membranes as a function of cytochalasin B concentration (Fig. 6). Half-maximal inhibition of transport by cytochalasin B occurred at about 0.7 μM while maximal inhibition occurred around 10 μM. Binding of [14C]cytochalasin B to dimethylmaleic anhydride-extracted membranes under these conditions was subjected to Scatchard plot analysis (Fig. 7) (38). The shape of the curve obtained suggests that more than one class of sites are involved in binding. The data indicate the existence of at least one class of high affinity binding sites with a dissociation constant of 5 ×
These data indicate that the extracted protein, although we have found this number to be quite variable among experiments.

For binding measurements, 100 μg of protein/tube were incubated in 50 μl of Krebs-Ringer phosphate buffer in the presence of various concentrations of glucose or inhibitor at the appropriate final concentration. Then 1 μl of D-[3H]glucose was added at a final concentration of 0.2 mM and uptake measured in triplicate at 5 min as described under "Materials and Methods."

Table II

| Addition              | Uptake (pmol/mg protein) | Per cent inhibition |
|-----------------------|--------------------------|---------------------|
| **Experiment 1**      |                          |                     |
| None                  | 496 ± 39                | 64                  |
| Cytochalasin B (100 μM) | 155 ± 37            | 21                  |
| l-Glucose (90 mM)     | 333 ± 31                | 37                  |
| D-Glucose (90 mM)     | 286 ± 28                | 55                  |

| **Experiment 2**      |                          |                     |
| Cytochalasin B (100 μM) | 142 ± 5               | 72                  |
| l-Glucose (90 mM)     | 962 ± 43                | 38                  |
| D-Glucose (90 mM)     | 260 ± 14                | 48                  |
| 3-O-Methylglucose (90 mM) | 147 ± 13             | 71                  |
| Phlorizin (1 mM)      | 186 ± 10                | 63                  |
| Phlorizin (1 mM)      | 192 ± 16                | 62                  |
| Dipyridamole (0.2 mM) | 240 ± 8                 | 52                  |

The existence of several classes of high affinity cytochalasin B binding sites is certainly not the only way to interpret these data. It is also possible that the nonlinearity of binding may be due to negative cooperativity. However, a dissociation constant of 5 x 10^-7 M agrees well with previous findings for the intact plasma membrane (13). Furthermore, there is good agreement between this dissociation constant and the value of 7 x 10^-7 M as the concentration of cytochalasin B required to half-maximally inhibit glucose transport activity (Fig. 6). Thus, these results support the notion that the way in which cytochalasin B exerts its potent inhibitory effects on hexose transport is closely associated with its high affinity binding to the membrane.

**DISCUSSION**

Rat adipocyte plasma membrane preparations have previously been shown (12, 13) to exhibit preferential uptake of D-versus L-glucose. In the present study we have demonstrated that this facilitated transport system is retained after extraction of plasma membranes with dimethylmaleic anhydride which results in elution of up to 80% of the total membrane protein in these preparations (13). Furthermore, there is good agreement between this dissociation constant and the value of 7 x 10^-7 M as the concentration of cytochalasin B required to half-maximally inhibit glucose transport activity (Fig. 6).

Thus, these results support the notion that the way in which cytochalasin B exerts its potent inhibitory effects on hexose transport is closely associated with its high affinity binding to the membrane.
bands are observed in SDS-gel electrophoresis does not imply that only two homogenous proteins remain in these residues. On the contrary, work in this laboratory indicates that the 94,000-dalton band exists as two high molecular weight dimers of around 200,000 in the absence of reducing agents such as mercaptoethanol or dithiothreitol. In addition, isoelectric focusing of these proteins in the presence of Triton X-100 results in a number of PAS-staining bands of different isoelectric points. However, dimethylmaleic anhydride extraction does provide a convenient single step procedure for significant purification of membrane components which still retain transport activity and a high affinity cytochalasin B binding capacity. Analysis of these extracted membranes reveals enrichment of phospholipid content, total glycoprotein, and sialic acid residues when compared to plasma membrane controls (Table I).

The evidence for the persistence of a hexose transport system operating in these extracted membranes is unequivocal. First, the existence of such a system in intact adipocytes, as well as in plasma membrane preparations, is well documented (5, 6). Secondly, electron micrographs reveal the presence of vesicular structures in the extracted preparations (Fig. 3). These vesicles display glucose uptake phenomena similar to the more intact systems, namely saturation, inhibition by known inhibitors of the intact transport system, and apparent substrate specificity. Furthermore, transport activity is abolished under denaturing conditions in which transport would not exist in intact cells or plasma membrane preparations. All of these conditions indicate that the adipocyte hexose transport system survives the extraction procedure and resides in the membrane fraction used for these experiments.

The apparent resistance to dimethylmaleic anhydride extraction by both the hexose transport system and high affinity cytochalasin B binding is also documented in the red cell (19, 21). This persistence of both phenomena in the rat adipocyte, as presented here, represents an additional model for isolating and purifying plasma membrane hexose transport components. The evidence in this study is consistent with the idea that both cytochalasin B binding and glucose transport activity reside in one or both of these major glycoprotein bands. Although some minor bands appear in this extraction, they account for less than 1% of the Coomassie blue-staining protein while Band III (54,000 daltons) contributes less than 5%. Bands I and II, however, constitute over 90% of the stained protein. The correlation of increased protein in these two bands with increased specific activity of cytochalasin B binding is consistent with their possible involvement in these two events, especially since the relative amounts of the other protein bands appear decreased during the extraction procedure. Nevertheless, the possibility cannot be discounted that some minor band which stains poorly with Coomassie blue remains refractory to this extraction and is involved in one or both of these phenomena.

Evidence for the involvement of glycoproteins in both cytochalasin B binding as well as hexose transport in the red cell has been accumulating in recent years (17-25). Initial studies using various extraction procedures implicated Band III (about 100,000 daltons as designated by (39)) as the band containing proteins responsible for hexose transport and cytochalasin B binding (19-21, 22). This band has already been shown to contain the anion transport system (38). More recently however, several investigators (17, 18, 24, 25) have implicated the glycoprotein region known as Zone 4.5 (55,000 to 70,000 daltons) as containing both these capacities. Kasamara and Hinkle (24) as well as Kahlenberg and Zala (25) have been able to reconstitute transport activity in liposomes from Zone 4.5 protein fractions. This type of reconstitution of preferential D-glucose uptake is a valuable tool in ultimately identifying transport-associated proteins since it is the only method available for monitoring transport activity once extraction has become selective enough to release the carrier.

While this manuscript was in preparation, Brunner et al. (40) reported that dimethylmaleic anhydride extractions of human adipocyte membranes resulted in increased specific activity of D-glucose uptake but decreased insulin binding. Although two major glycoprotein bands resisted extraction, this preparation contains significant amounts of protein from many other major bands, making localization of carrier-associated components difficult. These results may be due to the difficulty of obtaining a plasma membrane fraction from human fat cells relatively free of contamination from other subcellular fractions. In addition, it may be that human adipocyte plasma membranes are much more resistant to selective dimethylmaleic anhydride extraction than rat adipocytes. In any case, the results presented herein indicate that the hexose transport system in rat adipocytes is firmly associated with the plasma membrane and is extremely resistant to extraction by protein perturbants. The removal of all but two major membrane glycoproteins, combined with the persistence of transport activity, make this preparation an ideal model for further purification and isolation of this transport system.

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Purification of the Adipocyte Hexose Transport System

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