Unique Cytochalasin B Binding Characteristics of the Hepatic Glucose Carrier†

Jeffrey D. Axelrod and Paul F. Pilch*

ABSTRACT: Cytochalasin B is shown to inhibit uptake of 3-*O*-methylglucose into isolated rat hepatocytes with a K_i = 1.9 μM. The nature of this inhibition was characterized by studies of [^{3}H]cytochalasin B binding to liver plasma membranes. Scatchard analysis of [^{3}H]cytochalasin B binding reveals a complex curvilinear binding pattern. This pattern can be resolved into three components: (1) a high-affinity (ca. 10^{-8} M) cytochalasin E sensitive site unrelated to glucose uptake, (2) a glucose-sensitive site, and (3) a low-affinity site. When 5 μM cytochalasin E is employed to mask the high-affinity site, glucose displaces 40–60% of the remaining [^{3}H]cytochalasin B binding. Analysis of this glucose-sensitive cytochalasin B binding according to Scatchard reveals a K_d = 1.7 μM, indistinguishable from the concentration of cytochalasin B which half-maximally inhibits hepatic glucose uptake. These data identify a glucose-sensitive cytochalasin B binding site in liver plasma membranes which corresponds to the glucose carrier in the intact hepatocyte. The K_i of 1.9 μM for inhibition of hepatic glucose uptake by cytochalasin B and the K_d of 1.7 μM for [^{3}H]cytochalasin B binding to liver plasma membranes are values 1 order of magnitude higher than values for the same parameters determined in all previous studies of facilitated hexose diffusion systems. The hepatic hexose carrier is therefore unique, and this uniqueness may be of regulatory significance with regard to glucose homeostasis.

Efforts aimed at understanding the molecular basis of glucose transport and its regulation by various factors have relied heavily on the use of cytochalasin B. This fungal metabolite is a potent inhibitor of glucose transport in many cell types (Kletzien et al., 1972; Mizel & Wilson, 1972; Estensen & Plagemann, 1972), and [^{3}H]cytochalasin B can be used to characterize the nature of the cytochalasin B–glucose transporter interaction (Lin et al., 1974). The dose of cytochalasin B that inhibits hexose transport half-maximally (0.07–0.04 μM) correlates with the K_d for glucose-sensitive [^{3}H]cytochalasin B binding (0.1–0.5 μM) as measured in human erythrocyte membranes (Jung & Rampal, 1977), rat fat cell membranes (Wardzala et al., 1978), and chick embryo fibroblast membranes (Salter & Weber, 1979). Cytochalasin B binding has been used as the basis for purifying the erythrocyte glucose transporter (Baldwin et al., 1979). The M_r 55 000 protein identified as the cytochalasin B binding protein appears identical with the erythrocyte membrane protein purified on the basis of reconstitution of glucose transport activity (Kasahara & Hinkle, 1977). Recently, the glucose-sensitive cytochalasin B binding protein has been directly identified as an M_r 55 000 species by photocoupling of bound [^{3}H]cytochalasin B to membrane protein(s) in erythrocyte membranes (Carter-Su et al., 1982; Shanahan, 1982) as well as to membranes from other sources (Pessin et al., 1982, Shanahan et al., 1982).

Glucose homeostasis in mammals requires the clearance of blood glucose via glucose transport proteins principally into fat, muscle, and liver and depends as well on the mobilization of glucose from the liver into the circulation. Previous studies of intact liver (Williams et al., 1968) and isolated hepatocytes (Craik & Elliot, 1979) have indicated the presence of a stereospecific glucose transport system in the liver. The rapid kinetics of glucose uptake observed in these studies indicated the likely presence of numerous transporters in the liver consistent with a major quantitative role in glucose homeostasis for this organ. Surprisingly however, in view of the critical nature of liver glucose transport, a detailed analysis of liver glucose transport based on cytochalasin B–liver interactions is lacking.

In the present studies, we have employed cytochalasin B to characterize the glucose transporter of isolated rat hepatocytes and rat liver plasma membranes. We have determined the presence of a glucose-sensitive cytochalasin B binding system in membranes whose dissociation constant does not differ significantly from the concentration of cytochalasin B that inhibits half-maximally 3-*O*-methylglucose uptake by hepatocytes. The cytochalasin B binding characteristics of liver plasma membranes and hepatocytes differ significantly from those observed in previous studies of other cell types.

Materials and Methods

Reagents. Collagenase, type CLS II, was obtained from Worthington, lot 404229. [^{3}H]Cytochalasin B, [^{3}H]-3-*O*-methyl-D-glucose, D-[^{3}H]glucose, L-[^{3}H]glucose, Aquasol, and protosol were obtained from New England Nuclear. Cytochalasin E was purchased from Aldrich. Cytochalasin B was obtained from Sigma. Silicone oil of a specific gravity of 1.06 (550F1) was purchased from Contour Chemical Co. All other chemicals were of reagent grade and were purchased from Sigma.

Isolation of Hepatocytes and Liver Plasma Membranes. Hepatocytes were isolated from the livers of 150–250-g male Sprague-Dawley rats (Charles River Laboratories) by the method of Fehlmann et al. (1979). The rats were anesthetized with sodium pentobarbital, and the livers were perfused via the hepatic protal vein in three stages. Livers were first perfused until thoroughly blanched with buffer A containing 142 mM NaCl, 6.7 mM KCl, and 10 mM Hepes,[1] pH 7.85, to which was added 1.5 units/mL heparin and 0.6 mM EGTA.

† From the Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118. *Received September 22, 1982.* This research was supported by a grant from the American Diabetes Association and by Grant AM-30485 from the National Institutes of Health.

[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)-*N*′-2-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

0006-2960/83/0422-2222$01.50/0

This was followed by a short perfusion with buffer A alone (no EGTA or heparin) and finally by buffer A adjusted to 6.3 mM $CaCl_2$ and 0.48 mg/mL collagenase (Worthington type CLS II). The final perfusion was conducted until the livers attained a mottled appearance (5–10 min), and then the livers were excised. The isolated liver was placed in a small amount of buffer B consisting of 120 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 24 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 5% bovine serum albumin, and a trace of phenol red saturated with 95% O_2/5% CO_2, adjusted to pH 7.4. The hepatocytes were dispersed with forceps, filtered through cheesecloth, washed several times by centrifugation in an IEC clinical centrifuge, and filtered through 150-μm nylon mesh. The cells were stored until used in a plastic culture flask under 95% O_2/5% CO_2, and cell viability was 90% or better as determined by phase contrast microscopy and trypan blue exclusion.

Liver plasma membranes were prepared by a modification of the methods of Carey & Hirschberg (1980) and Touster et al. (1970): six to eight rats were decapitated and their livers excised, placed in 4 volumes of ice-cold 10 mM Hepes and 0.25 M sucrose, pH 7.4, and homogenized by using six strokes of a motor-driven Potter-Elvehjem tissue grinder. The homogenate was filtered through cheesecloth and spun at 2000g for 10 min. The supernatants were discarded and the pellets resuspended in a minimum amount of 66% (w/w) sucrose and adjusted to a refractive index of 1.414 (47% w/w sucrose). The suspension was added to 1 in. × 3.5 in. cellulose nitrate centrifuge tubes, overlayed with 37% (w/w) sucrose and with 0.25 M sucrose in 10 mM Hepes, and spun for 3 h at 25 000 rpm in a Sorval AH627 rotor. A light membrane fraction was collected from above the 37% sucrose layer and a heavy fraction from the 37–47% sucrose interface. Each fraction was suspended in 50 mM Hepes, pH 7.4, and pelleted at 40000g for 20 min. The pellets were resuspended in 50 mM Hepes on a tight-fitting Dounce homogenizer, and heavy and light fractions were combined and stored at −20 °C in small aliquots until use. In some preparations, prior to combining with the light fraction, the heavy pellets were subjected to a further purification step as follows: the pellets were resuspended in 66% (w/w) sucrose and adjusted to a refractive index of 1.43–1.44. The suspension was placed in $^5/_8$ in. × 4 in. cellulose nitrate centrifuge tubes, overlayed with 41% (w/v) sucrose and with 0.25 M sucrose in 10 mM Hepes, and spun overnight at 25 000 rpm in a Sorval AH627 rotor. The heavy membranes were collected from above the 41% layer, pelleted at 40000g for 20 min, and then resuspended in 50 mM Hepes. Membranes prepared by using this extra purification step had a whiter appearance and showed lower nonspecific binding of cytochalasin B.

Erythrocyte Membranes. Human erythrocyte ghosts were prepared by the method of Steck & Kant (1974) with several extra final washes. Aliquots were stored at −20 °C until use.

Glucose Uptake Assays. Glucose uptake into isolated hepatocytes was measured by a centrifugation assay as follows: 10-fold concentrated stocks of cytochalasin B in 5% ethanol/95% phosphate-buffered saline were added to test tubes containing isolated hepatocytes [(4–17) × 10^6 cells/mL] in buffer A at 22 °C and allowed to sit for 15 min. Triplicate 100-μL aliquots were transferred to 400-μL Eppendorf microfuge tubes containing 50 μL of 66% (w/w) sucrose covered by 50 μL of silicone oil (specific gravity (1.06). To start the reaction, 100 μL of cocktail containing 2–4 μCi of L-[^{3}H]-glucose or [^{3}H]-3-*O*-methyl-D-glucose in buffer A was rapidly added to give a final concentration of 0.2 mM glucose. Adequate rapid and smooth mixing was assessed by observing the mixing of phenol red in buffer B. At the appropriate time the incubation was ended by spinning in an Eppendorf microfuge for 1 min, whereupon the viable cells banded at the sucrose–oil interface while any dead cells (≤10% of total) remained above the oil. The viable cell band was removed with a razor blade and dissolved in 100 μL of 10% $NaDodSO_4$, 500 μL of Aquasol, and 500 μL of protosol at 37 °C overnight, and the radioactivity was determined by addition of 4 mL of Aquasol and liquid scintillation counting. The data obtained for [^{3}H]-3-*O*-methyl-D-glucose uptake by the methodology described above are essentially identical with those obtained by methodology employing transport stopping solutions (Craik & Elliot, 1979).

Glucose uptake into liver plasma membrane vesicles (Figure 2) was assayed essentially by the rapid filtration assay of Shanahan & Czech (1977) with several modifications. Liver plasma membranes were washed in 10 mM Hepes and 1 mM EDTA, resuspended in Krebs–Ringer phosphate buffer (128 mM NaCl, 5.2 mM KCl, 1.4 mM $CaCl_2$, 1.4 mM $MgSO_4$, and 10 mM Na_2HPO_4, pH 7.4) to 2.5 mg/mL protein, and dispersed with a Tekmar tissuemizer at setting 75 for 5 s. Ninety microliters of membrane suspension at 22 °C was incubated in the absence or presence of 1 μL of an 8.5 mM stock solution of cytochalasin B in ethanol for at least 5 min prior to assaying uptake. Uptake was initiated by the rapid addition of 10 μL of cocktail containing 5 μCi of D-[^{3}H]glucose in Krebs–Ringer phosphate buffer. Uptake was stopped at the appropriate time by addition of 3 mL of ice-cold Krebs–Ringer phosphate buffer containing 2 mM $HgCl_2$ and immediate rapid filtration of the membranes on 0.6-μm, 47 mM polycarbonate filters (Nucleopore) using a vacuum pump. The filters were washed twice with 3 mL of the stop solution. Uptake was determined by scintillation counting of the filters in aquasol.

Cytochalasin B Binding Assays. [^{3}H]Cytochalasin B binding to membranes was determined by a centrifugation assay as follows. Membranes were washed twice with Krebs–Ringer phosphate buffer, suspended in this buffer at the appropriate protein concentrations (see figure legends), and thoroughly dispersed by a Tekmar tissuemizer at setting 75 for 5 s. Cytochalasin E was added from a 10 mM stock solution in ethanol to a final concentration of 5 μM (Figures 5–7) and 0.5% ethanol. L- or D-glucose and various amounts of unlabeled cytochalasin B from stock solution in 5% ethanol were added where appropriate along with 0.2 μCi of [^{3}H]cytochalasin B to a final volume of 1 mL: the final concentration of ethanol was 1% in all cases. Incubation proceeded for 30 or 40 min at 22 °C at which time the liver plasma membranes were pelleted by a 3-min microfuge spin. Liver plasma membranes are isolated from a nuclei-containing fraction of the liver that pellets at 2000g (Carey & Hirschberg, 1980). These membranes are large dense structures[2] that pellet readily by microfuge (ca. 12000g) even after dispersal by a tissuemizer. We recover ≥90% of the added membrane protein in the microfuge pellet. After centrifugation, the medium was quickly and carefully aspirated, and the ends of the tubes containing the pellets were cut off and equilibrated in 4 mL of Aquasol prior to liquid scintillation counting to determine

[2] It should be noted that assay of uptake into liver plasma membrane vesicles required protein concentrations in considerable excess of those required for similar assays in erythrocytes and other tissues. This can be attributed to the relatively large proportion of membrane sheets and concomitantly small fraction of vesicles per milligram of protein, resulting from the homogenization of tissue which has abundant gap junctions and desmosomes (Wibo et al., 1981).

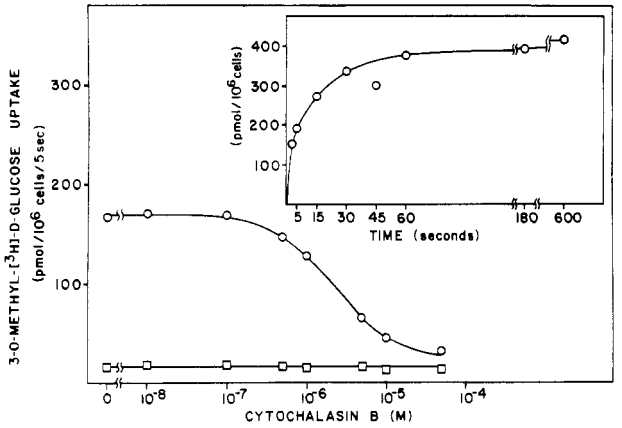

FIGURE 1: Inhibition of 3-*O*-methyl-D-glucose uptake into hepatocytes by cytochalasin B. (Inset) A 100-μL sample of isolated hepatocytes in buffer A at 22 °C was transferred into microfuge tubes (745 000 cells/tube), and 100 μL of [^{3}H]-3-*O*-methyl-D-glucose cocktail was rapidly added. At the appropriate time, the incubation was ended by spinning the cells through a silicone oil layer as described. Cell-associated 3-*O*-methyl-D-glucose is plotted as a function of incubation time (○). To determine the dose–response relationship of transport inhibition by cytochalasin B, 450 μL of hepatocytes was incubated with 50 μL of appropriate concentrations of cytochalasin B stocks in 10% ethanol or buffer with 10% ethanol. Portions of 100 μL (620 000 cells) were taken and assayed for 5 s of 3-*O*-methyl-D-glucose (○) or L-glucose (□) uptake by centrifugation assay described under Materials and Methods.

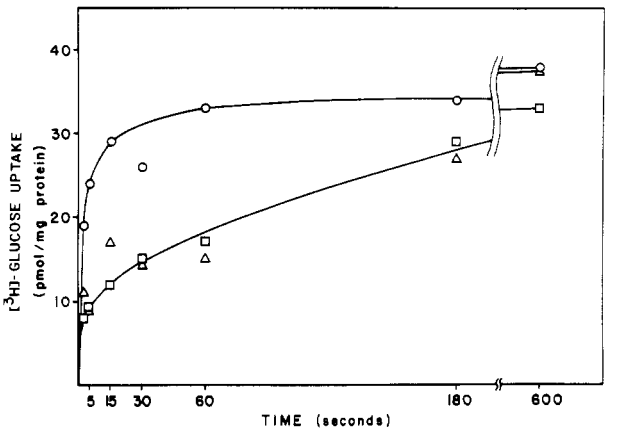

FIGURE 2: Time course of L- or D-glucose uptake into liver plasma membrane vesicles. Ninety microliters of membranes in Krebs-Ringer phosphate buffer, pH 7.4, at 22 °C (0.25 mg of protein) was added to tubes containing 1 μL of 17 mM cytochalasin B in ethanol or 1 μL of ethanol alone. Uptake was initiated by the rapid addition of 10 μL of cocktail containing L-[^{3}H]glucose or D-[^{3}H]glucose and ended by rapid filtration as described under Materials and Methods. Uptake of D-glucose in the absence (○) and presence (□) of cytochalasin B and uptake of L-glucose (△) in the absence of cytochalasin B are plotted as a function of incubation time.

bound [^{3}H]cytochalasin B. Erythrocyte membranes (Figure 7) were pelleted at 28000*g* for 20 min in 16-mL glass centrifuge tubes. The supernatant was carefully aspirated, the sides of the tube carefully blotted, and the pellet dissolved in Aquasol for scintillation counting. In the initial stages of these studies, data points were determined in triplicate, and standard deviations of less than 1% within triplicates were routinely achieved. Consequently, the data points depicted here are the results of duplicate or single determinations. Each experiment was performed on a minimum of three separate occasions with essentially identical results. Depicted are representative experiments.

Binding data were analyzed as follows. Nonspecific [^{3}H]cytochalasin B binding was defined as binding in the presence of 4.2×10^{-5} M unlabeled cytochalasin B. This represents trapped as well as nonspecifically bound ligand. We employed [^{14}C]inulin as a measure of the amount of trapping that occurs during our binding experiments and determined that 95% of the [^{3}H]cytochalasin B in the membrane pellet at 4.2×10^{-5} M cytochalasin B was trapped and the remainder was nonspecifically bound. Where appropriate, binding data were analyzed according to Scatchard (1949). Specifically bound ^{3}[H]cytochalasin B was determined by subtracting nonspecifically bound and trapped ligand from the total cytochalasin B associated with the membrane pellet. Free cytochalasin B was determined by subtracting total bound (but not trapped) cytochalasin B from the total ligand present in the assay. Bound/free ligand vs. bound ligand was then plotted and K_d's were graphically determined by the method of Rosenthal (1967). Cytochalasin B concentrations were verified by their optical density at 2000 Å (Lin et al., 1974). Protein concentrations were determined by the method of Lowry et al. (1951).

Results

Figure 1 illustrates the effects of varying cytochalasin B concentrations on 3-*O*-methyl-D-glucose uptake. The time course of uptake into hepatocytes was first determined (inset of Figure 1). A 5-s uptake point was chosen for subsequent inhibition experiments to assure that uptake was assayed in an essentially linear portion of the curve and reflected initial velocity (Craik & Elliot, 1979). Hepatocytes were then incubated with concentrations of cytochalasin B from 5×10^{-4} to 10^{-8} M, and the velocity of 3-*O*-methyl-D-glucose uptake was determined after 5-s incubations. Cytochalasin B inhibits uptake of this nonmetabolizable sugar into hepatocytes with a K_i of 1.93 (±0.83 SD) μM (n = 5) and is 90% inhibitory at 50 μM. Nonspecific uptake and trapping were determined by measuring cell-associated L-[^{3}H]glucose under identical conditions and are unaffected by cytochalasin B (square symbols).

Glucose uptake into liver plasma membrane vesicles was also assayed for cytochalasin B sensitivity. Figure 2 shows the time course of D-[^{3}H]glucose uptake into liver plasma membrane vesicles in the absence and presence of excess cytochalasin B. Rapid equilibration occurs in the absence of cytochalasin B, while in its presence, equilibration is slow. Uptake in the presence of cytochalasin B essentially equals the uptake of L-[^{3}H]glucose and thus represents leakage into and nonspecific binding to the vesicles. Cytochalasin B therefore completely inhibits specific carrier-mediated uptake in this preparation.[2] By this criterion, cytochalasin B inhibitable D-glucose uptake into hepatocytes (Figure 1) and liver plasma membrane vesicles (Figure 2) closely resembles that of other widely described facilitated glucose diffusion systems such as the human erythrocyte.

[^{3}H]Cytochalasin B binding to liver plasma membranes was assayed in the presence of 500 mM L- and D-glucose as shown in Figure 3 in order to directly demonstrate glucose-sensitive cytochalasin B binding sites. D-Glucose can be seen to markedly inhibit cytochalasin B binding (squares) as compared to L-glucose (circles). However, the complex curvilinear pattern is difficult to analyze quantitatively. A similar complex curvilinear cytochalasin B binding pattern has been observed in erythrocytes (Jung & Rampal, 1977) and adipocytes (Wardzala et al., 1978). In these cases, cytochalasin E was shown to mask binding to a very high affinity class of cytochalasin B binding sites without affecting glucose-sensitive [^{3}H]cytochalasin B binding, thus facilitating quantitative binding analysis.

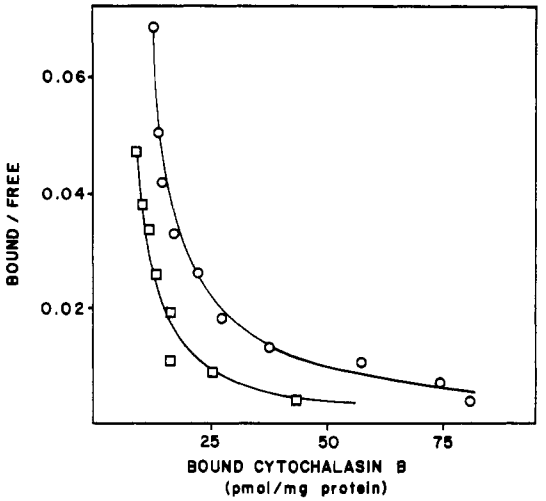

FIGURE 3: Scatchard plot of cytochalasin B binding to liver plasma membranes in the absence of cytochalasin E. Liver plasma membranes (0.25 mg of protein/assay point) were incubated in the presence of 500 mM L- or D-glucose, [^{3}H]cytochalasin B, and various amounts of unlabeled cytochalasin B in a total volume of 1 mL for 30 min at 22 °C. The membranes were pelletted, the supernatants were removed, and the radioactivity in the pellets was determined as described under Materials and Methods. The data are plotted according to Scatchard for incubation with 500 mM L-glucose (○) or 500 mM D-glucose (□).

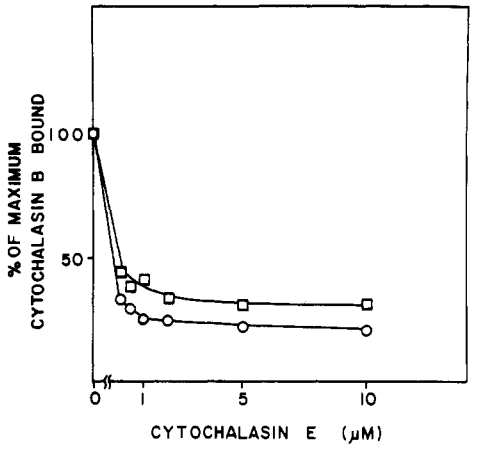

FIGURE 4: Effect of cytochalasin E on cytochalasin B binding to liver plasma membranes. Liver plasma membranes (0.25 mg of protein) were incubated in the presence of various concentrations of cytochalasin E and either 2×10^{-8} M (○) or 5×10^{-8} M (□) [^{3}H]cytochalasin B in a volume of 1 mL at 22 °C for 30 min. Binding of [^{3}H]cytochalasin B was determined as described under Materials and Methods, and the results are plotted as a percentage of [^{3}H]cytochalasin B binding observed in the absence of cytochalasin E.

Cytochalasin E was tested for its ability to block cytochalasin B binding to non-glucose-sensitive sites as shown in Figures 4 and 5. Membranes were incubated with various concentrations of cytochalasin E, and binding of two different concentrations of [^{3}H]cytochalasin B was assayed. As can be seen in Figure 4, binding of 5×10^{-8} M [^{3}H]cytochalasin B is inhibited by approximately 70% at 5 μM cytochalasin E, and binding of 2×10^{-8} M [^{3}H]cytochalasin B is inhibited by almost 80% at the same concentration of cytochalasin E. Concentrations of cytochalasin E greater than 5 μM result in no further reduction in cytochalasin B binding. Since cytochalasin E does not block glucose transport in hepatocytes (data not shown), it appears that it inhibits cytochalasin B binding to a site other than the glucose transporter. To verify that cytochalasin E does not affect the glucose-sensitive sites, the glucose sensitivity of [^{3}H]cytochalasin B binding in the presence of 5 μM cytochalasin E was determined (Figure 5).

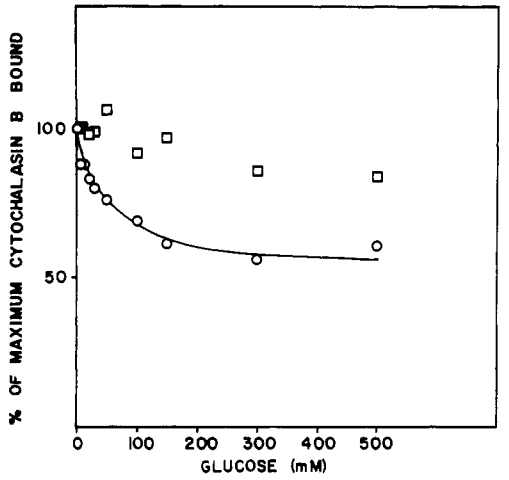

FIGURE 5: Glucose displacement of [^{3}H]cytochalasin B binding to liver plasma membranes in the presence of cytochalasin E. Liver plasma membranes (0.21 mg of protein) were incubated with 5 μM cytochalasin E, 2×10^{-8} M [^{3}H]cytochalasin B, and various concentrations of L-glucose (□) or D-glucose (○) for 30 min at 22 °C. [^{3}H]Cytochalasin B binding was determined as described under Materials and Methods, and the data are plotted as a percentage of [^{3}H]cytochalasin B binding in the absence of glucose.

[^{3}H]Cytochalasin B binding was measured in the presence of 5 μM cytochalasin E and varying concentrations of L- or D-glucose, and the data are plotted as a percentage of binding in the absence of glucose. In the presence of cytochalasin E, approximately 40% (and as much as 60% in other experiments) of the remaining cytochalasin B binding sites are D-glucose displaceable. The ED_{50} for inhibition of binding is approximately 40 mM glucose, indicating that cytochalasin E displaces [^{3}H]cytochalasin B from a class of sites distinct from those which are glucose sensitive. In subsequent [^{3}H]cytochalasin B binding experiments, therefore, 5 μM cytochalasin E was routinely present.

Figure 6 shows the results of a Scatchard analysis of [^{3}H]cytochalasin B binding in the presence of 5 μM cytochalasin E and 50 mM L- or D-glucose. By comparison of the curves generated in the presence of L-glucose and no cytochalasin E (Figure 3) or 5 μM cytochalasin E (Figure 6A), it is evident that the cytochalasin E sensitive cytochalasin B binding sites represent the high affinity portion of the Scatchard plots shown in figure 3. Thus, in the presence of 5 μM cytochalasin E (Figure 6), these high-affinity sites are masked, and the intermediate-affinity, glucose-sensitive sites are easily seen. With the cytochalasin E sites masked, a shift in the glucose-sensitive portion of the curve is more readily analyzed. D-Glucose (50 mM) produces the expected shift in K_d. That is, in the control condition in the presence of 50 mM L-glucose, the K_d of the glucose-sensitive site is determined to be 1.47 μM, while in the presence of 50 mM D-glucose the apparent affinity, of K(app), is found to be 2.92 μM, as determined graphically by the method of Rosenthal (1967). This procedure is illustrated in Figure 6B where the data in Figure 6A have been analyzed by the Rosenthal (1967) procedure for a two-component situation. The low-affinity site is the same for both the D-glucose condition and the L-glucose condition. By use of these values the inhibition constant for glucose inhibition of cytochalasin B binding can be calculated from the equation

$$K(\text{app}) = K_d\left(1 + \frac{[I]}{K_i}\right)$$

where [I] is the inhibitor concentration (50 mM D-glucose)

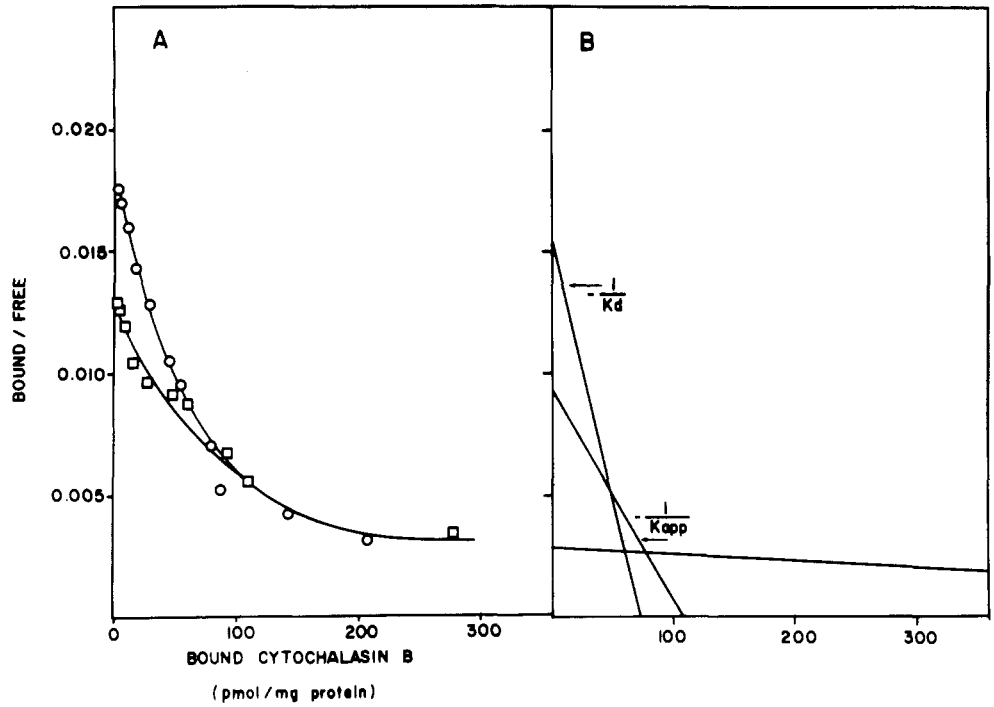

FIGURE 6: Scatchard plot of cytochalasin B binding to liver plasma membranes in the presence of cytochalasin E and L-glucose or D-glucose. Liver plasma membranes (0.2–0.35 mg/tube) were incubated in the presence of 50 mM L-glucose (○) or 50 mM D-glucose (□), 5 μM cytochalasin E, [^{3}H]cytochalasin B, and various amounts of unlabeled cytochalasin B in a total volume of 1 mL for 40 min at 22 °C. Binding was determined as described under Materials and Methods, and the data are plotted according to Scatchard (A). These data were graphically resolved into a model representing two classes of binding sites by using the method of Rosenthal (1967) (B). The slopes of the high-affinity region correspond to the parameters in the figure (see the text).

and the values of K_d and K(app) are taken from Figure 6B. The K_i is found to be 51 mM glucose, a value in excellent agreement with the ED_{50} of 40 mM for glucose displacement of cytochalasin B binding determined in the experiment shown in Figure 5.

In a series of experiments of [^{3}H]cytochalasin B binding to liver plasma membranes in the presence of 5 μM cytochalasin E, the dissociation constant of the ligand as determined by Scatchard plots was found to be 1.74 (±0.47 SD) μM (n = 8). This value does not differ significantly from the K_i for cytochalasin B inhibition of hepatocyte 3-*O*-methylglucose uptake [1.93 (±0.83 SD) μM] from Figure 1, suggesting a 1/1 stoichiometric correspondence between glucose-sensitive cytochalasin B binding and glucose transport inhibition. Therefore, it is highly likely that cytochalasin binding parameters determined in membranes reflect the interaction of this compound with the hepatic glucose carrier in the intact cell. Cytochalasin B has been reported to have a K_i of 0.07–0.4 μM for inhibiting transport and an essentially identical K_d for transporter–ligand interaction in erythrocytes (Jung & Rampal, 1977; Zoccoli et al., 1978), adipocytes (Wardzala et al., 1978; Cushman & Wardzala, 1979), and chicken embryo fibroblasts (Salter & Weber, 1979; Pessin et al., 1982). The order of magnitude difference in the concentration of cytochalasin B required to inhibit transport in liver vs. these other cells is a novel and interesting observation. One possibility is that this difference is due to metabolic activity in liver plasma membranes that degrades cytochalasin B. We sampled the supernatant after a typical cytochalasin B binding assay with liver plasma membranes and compared this supernatant to untreated cytochalasin B by thin-layer chromatography (Lin et al., 1974). All the applied material migrated with the standard [^{3}H]cytochalasin B, and no degradation was observed (data not shown). To verify differences in K_d's with respect to liver membranes and erythrocyte membranes, we directly compared [^{3}H]cytochalasin B binding in the same experiment. Figure 7 shows a Scatchard plot of cytochalasin B binding to liver and erythrocyte membranes in the presence of 5 μM cytochalasin E. The abscissa is plotted as nanomolar to facilitate comparison of the respective affinities. In this experiment the K_d for the [^{3}H]cytochalasin B–transporter interaction is 0.20 μM in red cell membranes while the K_d for liver membranes is 1.67 μM. It should be noted that the protein concentration for liver plasma membranes in this assay was 0.23 mg/mL while, for erythrocytes, 0.015 mg/mL protein was required in order to achieve similar bound/free ratios. If the total numbers of binding sites in the

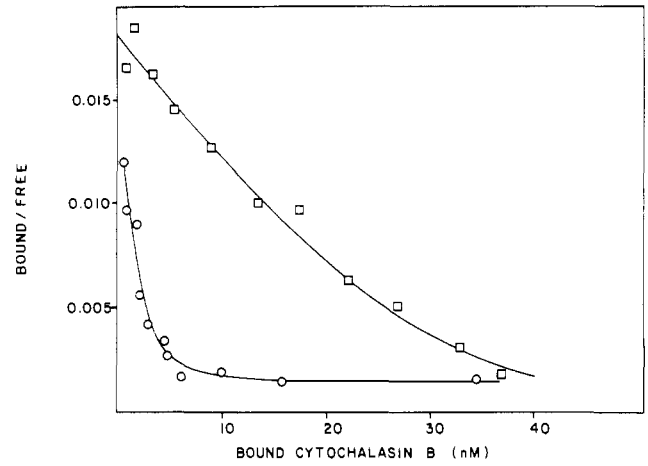

FIGURE 7: Scatchard plot of cytochalasin B binding to liver plasma membranes and erythrocyte ghosts in the presence of cytochalasin E. Liver plasma membranes (0.23 mg of protein) (□) or erythrocyte ghosts (0.015 mg of protein) (○) were incubated in the presence of 5 μM cytochalasin E, [^{3}H]cytochalasin B, and various amounts of unlabeled cytochalasin B in a total volume of 1 mL for 40 min at 22 °C. Binding was determined as described under Materials and Methods, and the data are plotted according to Scatchard.

glucose-sensitive region of each curve (B_{max}) are determined by using the above protein values, they are found to be nearly equal: B_{max} = 121 pmol/mg of protein in liver membranes, and B_{max} = 133 pmol/mg of protein in red cell membranes. Therefore, while the affinity for the erythrocyte glucose sensitive sites is nearly 10-fold greater than that of liver, they have approximately the same abundance on a per milligram of membrane protein basis. It should also be noted that the liver plasma membrane preparation we employed (Carey & Hirschberg, 1980) and other liver plasma membrane preparations [reviewed by Evans (1980)] represent an enrichment of the plasma membrane contaminated to a variable degree with intracellular membranes. Thus, our value of the maximal cytochalasin B binding capacity of liver plasma membranes is likely to be an underestimate.

Discussion

Studies of glucose uptake into intact rat liver (Williams et al., 1968) and isolated hepatocytes (Craik & Elliot, 1979) indicated that rat liver possesses a very active carrier system for the facilitated uptake of hexose. These previous studies have compared the carrier system of the hepatocyte to that of human erythrocyte on a quantitative basis. That is, the rate of glucose uptake by liver is very rapid and approximately half that of the human erythrocyte when normalized to intracellular water space (Williams et al., 1968). In the human red cell the carrier comprises 2–5% of the membrane protein (Zoccoli et al., 1978; Kasahara & Hinkle, 1977). It has been reported that cytochalasin B is capable of inhibiting hepatocyte glucose uptake (Bauer & Heldt, 1977) and that [^{3}H]cytochalasin B binds to liver plasma membranes (Riordan & Alon, 1977). However, detailed quantitive analysis of liver–cytochalasin B interactions has been lacking until the present report.

Our detailed investigations of the interaction between the liver glucose transporter and cytochalasin B revealed a pattern of binding and of hexose transport inhibition by this compound similar to those observed for the human erythrocyte (Jung & Rampal, 1977; Zoccoli et al., 1978), the rat adipocyte (Wardzala et al., 1978), and chick embryo fibroblasts (Salter & Weber, 1979) with one striking the unexpected difference. The quantitative parameters representing the affinity of cytochalasin B for the glucose transporter and its ability to block hexose uptake are an order of magnitude greater than in the other tissues studied. Furthermore, while [^{3}H]cytochalasin B can be photolytically incorporated into an M_r 45 000–60 000 band from erythrocyte ghosts (Carter-Su et al., 1982; Shanahan, 1982), as well as into the membranes of chick embryo fibroblasts (Pessin et al., 1982; Shanahan et al., 1982) and rat adipocytes (Shanahan et al., 1982), no appreciable label was specifically incorporated into liver plasma membranes upon photolysis under conditions of equal saturation of the transporter with ligand (data not shown). Implicit in the differences between the hepatic glucose transporter and these other carrier systems with respect to transport inhibition, binding affinity, and susceptibility to photolabeling is a possible structural difference. This putative difference may be of significance with respect to our understanding of the molecular basis of transport and its regulation. A complete understanding of the repsonse of hexose transport systems to factors such as insulin (Cushman & Wardzala, 1980) and to cellular states such as transformation (Salter & Weber, 1979; Pessin et al., 1982) may depend on further characterization of the differences in these transport systems on a molecular level. To date, the erythrocyte carrier is the only glucose transporter to be purified (Kasahara & Hinkle, 1977; Baldwin et al., 1979). Because the liver transporter is nearly as abundant in the membrane as that of the red cell (Figure 7), it should also be feasible to purify the hepatic transport protein and to examine its molecular features. Attempts at purification of the hepatic glucose transporter are currently under way in our laboratory.

Registry No. Cytochalasin B, 14930-96-2; D-glucose, 50-99-7; 3-*O*-methyl-D-glucose, 146-72-5; cytochalasin E, 36011-19-5.

References

Baldwin, S. A., Baldwin, J. M., Gorga, F. R., & Lienhard, G. E. (1979) *Biochim. Biophys. Acta 552*, 187–197.

Bauer, H., & Heldt, H. W. (1977) *Eur. J. Biochem. 74*, 397–403.

Carey, D. J., & Hirschberg, C. B. (1980) *J. Biol. Chem. 255*, 4348–4354.

Carter-Su, C., Pessin, J. E., Mora, R., Gitomer, W., & Czech, M. P. (1982) *J. Biol. Chem. 257*, 5419–5425.

Craik, J. D., & Elliot, K. R. F. (1979) *Biochem. J. 182*, 503–508.

Cushman, S. W., & Wardzala, L. J. (1980) *J. Biol. Chem. 255*, 4758–4762.

Estensen, R. D., & Plagemann, P. G. W. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1430–1434.

Evans, W. H. (1980) *Biochim. Biophys. Acta 604*, 27–64.

Fehlmann, M., LeCam, A., & Freychet, P. (1979) *J. Biol. Chem. 254*, 10431–10437.

Jung, C. J., & Rampal, A. L. (1977) *J. Biol. Chem. 252*, 5456–5463.

Kasahara, M., & Hinkle, P. C. (1977) *J. Biol. Chem. 252*, 7384–7390.

Kletzien, R. F., Perdue, J. F., & Springer, A. (1972) *J. Biol. Chem. 247*, 2964–2966.

Lin, S., Santi, D. V., & Spudich, J. A. (1974) *J. Biol. Chem. 249*, 2268–2274.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 266–275.

Mizel, S. B., & Wilson, L. (1972) *J. Biol. Chem. 247*, 4102–4105.

Pessin, J. E., Tillotson, L. G., Yamada, K., Gitomer, W., Carter-Su, C., Mora, R., Isselbacher, K. J., & Czech, M. P. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2286–2290.

Riordan, J. R., & Alon, N. (1977) *Biochim. Biophys. Acta 464*, 547–561.

Rosenthal, H. E. (1967) *Anal. Biochem. 20*, 525–532.

Salter, D. W., & Weber, M. J. (1979) *J. Biol. Chem. 254*, 3554–3561.

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.

Shanahan, M. F. (1982) *J. Biol. Chem. 257*, 7290–7293.

Shanahan, M. F., & Czech, M. P. (1977) *J. Biol. Chem. 252*, 6554–6561.

Shanahan, M. F., Olson, S. A., Weber, M. J., Lienhard, G. E., & Gorga, J. C. (1982) *Biochem. Biophys. Res. Commun. 107*, 38–43.

Steck, T. L., & Kant, J. A. (1974) *Methods Enzymol. 31*, 172–180.

Touster, O., Aronson, N. N., Dulaney, J. T., & Hendrickson, H. (1970) *J. Cell Biol. 47*, 604–618.

Wardzala, L. J., Cushman, S. W., & Salans, L. B. (1978) *J. Biol. Chem. 253*, 8002–8005.

Wibo, M., Thines-Sempoux, D., Amar-Costesec, A., Beaufay, H., & Godelaine, D. (1981) *J. Cell Biol. 89*, 456–474.

Williams, T. F., Exton, J. H., Park, C. R., & Regen, D. M. (1968) *Am. J. Physiol. 215*, 1200–1209.

Zoccoli, M. A., Baldwin, S. A., & Lienhard, G. E. (1978) *J. Biol. Chem. 253*, 6923–6930.