yielded similar results (data not shown).

Discussion

The results of this study have unequivocally established that ABOCA is a substrate of system L and is not a substrate or competitor of system A. In addition, the data support our original hypothesis in that ABOCA exhibits a greater affinity for system L than does BCH. The results demonstrating that ABOCA has a greater affinity than does BCH for the L system are consistent with our earlier suggestion of the dominance of flexible apolar interactions over precise steric considerations in selectivity of substrates by the L system (Matthews & Zand, 1977). Steric factors, however, cannot be totally ignored since there is a definite preference for the ABOCA isomer in which the carboxyl function is exo to the methylene bridge. The assignment of structure and absolute configuration of the ABOCA isomers has been reported earlier (Chacko et al., 1978), thereby permitting the present assessment of preference by system L for the exo carboxyl isomer. This result parallels the findings reported for BCH (Christensen et al., 1969) in which the isomer possessing this configuration was favored. The results of the present study suggest that ABOCA may be preferable to BCH as an amino acid analogue for discrimination of amino acid transport systems.

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Equilibria and Kinetics of Ligand Binding to the Human Erythrocyte Glucose Transporter. Evidence for an Alternating Conformation Model for Transport†

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ABSTRACT: Cytochalasin B (CB), *n*-propyl β-D-glucopyranoside (PG), and 4,6-*O*-ethylidene-D-glucose (EG) are known to bind asymmetrically to the human erythrocyte glucose transporter. The first two compounds bind to the inner (cytoplasmic) surface of the transporter, while the latter binds to the outer surface. Equilibrium measurements of the inhibition of CB binding to the glucose transporter reported herein indicate that the ternary complexes of CB transporter with EG, PG, or D-glucose are not formed. Moreover, measurements of CB binding in the presence of both EG and PG or in the presence of high concentrations of D-glucose show that a ternary complex of transporter and sugars bound simultaneously on both sides of the membrane probably does not occur. Finally, the kinetics of dissociation of radiolabeled CB from the transporter in the presence of CB, glucose, PG, and EG have been determined. With the exception of the case of EG, the kinetics fit a simple scheme of rate-limiting unimolecular dissociation, and in no instance do they suggest the existence of a ternary complex of sugar, CB, and transporter. These data are consistent with a model for transport in which the substrate binding site exists alternately at the cytoplasmic and external faces of the membrane, as the result of protein conformational change.

A model for the facilitated diffusion of glucose in erythrocytes, which we call the alternating conformation model, is shown schematically in Figure 1. According to this model, which was described by Vidaver (1966), translocation of the occupied or unoccupied substrate site across the membrane is effected by a conformational change of the transporter. This model accounts for sugar specificities of the transporter that differ at the cytoplasmic and external surfaces of the membrane (Barnett et al., 1975). It also accounts for most of the results from steady-state kinetic studies, including the phenomena of the uphill transport of one sugar induced by a

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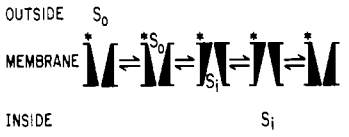


FIGURE 1: Schematic representation of an alternating conformation model for the functioning of the glucose transporter. The asterick depicts carbohydrate linked to the external portion of the transporter (Gorga et al., 1979).

gradient of another and a rate of glucose exchange that is more rapid than the rate of net transport (Geck, 1971; Regen & Tarpley, 1974). Some apparent kinetic inconsistencies remain (Naftalin & Holman, 1977; Foster et al., 1979; Baker & Naftalin, 1979), but it seems possible to us that the basis for these is the failure to consider that the substrate D-glucose is, in fact, a mixture of two substrates, the α and β anomers. Finally, the model is in agreement with the stable transmembrane arrangement of the transporter polypeptide (Gorga et al., 1979; Baldwin et al., 1980).

A key feature of the model, which has not yet been rigorously examined, is the postulate that at any moment there is only a single binding site for the substrate. When the substrate is bound to the conformation with the outward-facing site, the inward-facing substrate site does not exist, and vice versa (Figure 1). Herein, we report equilibrium and kinetic studies of ligand binding to the transporter that directly test this postulate.

Experimental Procedures

Materials. 4,6-*O*-Ethylidene-α-D-glucose (ethylideneglucose; EG)[1] was purchased from Aldrich. The material contained about 1.5% D-glucose and an unknown impurity that absorbed in the ultraviolet. It was purified by chromatography on cellulose (4–8 g on a 7.25 × 12 cm column of cellulose of 38-μm particle size from Sigma) in 1-butanol:ethanol:water (49:11:10 v/v) and by subsequent treatment of an aqueous solution with charcoal [0.15 g of Darco G60 (MCB Manufacturing Chemists) per mmol of sugar]. The final material contained about 0.05% D-glucose, and the absorbance of a 100 mM solution was 0.020 at 280 nm. *n*-Propyl β-D-glucopyranoside (propyl glucoside; PG) was synthesized according to the procedure of Barnett et al. (1975), with the exception that the deacylation was performed with methanol:triethylamine:H_2O (2:1:1 v/v) (Rosevear et al., 1980). Purification was accomplished by recrystallization from ethanol:diethyl ether (twice) followed by chromatography and charcoal treatment as described for ethylideneglucose. The final product contained about 0.03% D-glucose, and the absorbance of a 100 mM solution was 0.022 at 280 nm. Stock solutions of ethylideneglucose and D-glucose in NaP buffer were allowed to stand at room temperature for at least 3 h before use, in order to obtain mutarotatory equilibrium (Bronsted & Guggenheim, 1927).

Erythrocyte ghosts were prepared from outdated blood according to Steck & Kant (1974) and stripped of peripheral proteins by alkali treatment according to the following procedure. Ghosts, at 4 mg of protein/mL in 5 mM sodium phosphate, pH 8, were mixed with 5 volumes of 2 mM $(Na)_2$EDTA/15.4 mM NaOH/0.2 mM dithiothreitol (added just before use). After 10 min at 3 °C, the protein-depleted membranes were pelleted by centrifugation at 48000*g* (max) for 15 min, suspended in 50 mM Tris-HCl, pH 6.8, pelleted again, suspended in the Tris buffer at 4 mg of protein/mL, and stored at −70 °C. Membranes were thawed, sedimented by centrifugation at 27000*g* for 15 min, and resuspended in 100 mM NaCl/20 mM sodium phosphate, pH 7.0, with a glass/Teflon homogenizer for use in the experiments described. The extent to which the resuspended membranes existed as sealed vesicles was determined by assaying the accessibility of sialic acid residues to neuraminidase (Steck & Kant, 1974) and the inactivation of cytochalasin B (CB) binding by trypsin (Baldwin et al., 1980) and by density-gradient centrifugation (Lienhard et al., 1977).

[4-^{3}H]Cytochalasin B (^{3}HCB) was purchased from New England Nuclear. Our initial lot was the result of a custom synthesis and required further purification by thin-layer chromatography to remove an ultraviolet-absorbing impurity (Zoccoli et al., 1978). More recently, this radiochemical has become available as a catalog item. These lots were used without further purification, since each lot exhibited the ultraviolet spectrum of authentic cytochalasin B and was, as shown by chromatography by the manufacturer, radiochemically pure. The manufacturer now purifies this radiochemical by the thin-layer chromatographic procedure referred to above.

Equilibrium Measurements of CB Binding. Equilibrium ^{3}HCB binding to membranes was measured by incubating 100 μL of NaP buffer containing membranes at 1 mg/mL (about 1.5×10^{-6} M in CB sites) and the desired concentrations of ^{3}HCB and sugar in 6 × 50 mm glass tubes for 5 min at 3–4 °C and then pelleting the membranes by centrifugation at 20000*g* for 5 min. The total and free CB concentrations were obtained by determining the radioactivity of 25-μL aliquots taken with a syringe before and after centrifugation, respectively. The concentration of bound CB was calculated by subtracting the free CB concentration from the total. A small fraction of the membranes (3% of the protein) did not pellet under these conditions, and the binding data have been corrected accordingly.

Kinetic Measurements. The kinetics of ^{3}HCB dissociation from the transporter were measured by using the rapid mixing–rapid filtration apparatus described by Boyd & Cohen (1980) in the cold room (3.0–3.8 °C). The reactions were initiated by depressing the common plunger such that 0.5 mL from each syringe was mixed within a fraction of a second and deposited onto a dry GF/F filter (Whatman) in a well of the filtration manifold. Reactions were stopped after the desired interval by opening the port stopcock to the vacuum for 5 s. One syringe contained 4×10^{-8} M ^{3}HCB and 2 mg/mL membrane protein in NaP buffer, and the other syringe contained the competing ligand (CB or sugar) at twice the desired final concentration in NaP buffer.

Filtration of the 1 mL of membrane suspension was complete in approximately 1 s, and 98.0% of the applied protein was retained on the filter. Time points as short as 1 s could be accurately obtained with the help of an assistant and the use of a metronome. The filtrates were collected in glass tubes held in a rack within the filtration manifold, and the concentration of unbound CB was determined from the radioactivity in aliquots of the filtrates. The total CB concentration was determined by depositing 0.5 mL from each syringe directly into a glass tube and measuring the radioactivity in an aliquot. A control experiment, in which solutions of CB alone were filtered, showed that the percentage of the CB absorbed by the filter and manifold was constant over the range of unbound CB concentrations observed during the kinetic ex-

[1] Abbreviations used: CB, cytochalasin B; ^{3}HCB, [4-^{3}H]cytochalasin B; EDTA, ethylenediaminetetraacetic acid; NaP buffer, 100 mM NaCl/0.4% ethanol/20 mM NaP_i, pH 7.0; PG, *n*-propyl β-D-glucopyranoside; EG, 4,6-*O*-ethylidene-D-glucose.

periments, and amounted to 21 ± 4.4% (mean ± standard deviation of four determinations).

Other Methods. Ethylideneglucose was assayed by the colorimetric method of Park & Johnson (1949), and propyl glucoside was determined by the anthrone reaction (Spiro, 1966). D-Glucose was measured by means of hexokinase and glucose-6-phosphate dehydrogenase (diagnostic kit 15 from Sigma Chemical). Protein was assayed by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Results and Discussion

Characteristics of the Protein-Depleted Erythrocyte Membranes. For this investigation, in which the experimental approach has been to examine effects upon CB binding to the transporter (see below), a preparation with the following characteristics was needed. First, the only sites for CB present in a significant amount should be the high-affinity sites for CB located on the glucose transporter, so that the complication of CB binding to other sites is avoided. Second, the membranes should exist almost entirely in unsealed form, so that in the kinetic studies the potential complication of rate-limiting membrane permeation by a ligand is avoided. The erythrocyte membranes stripped of peripheral proteins with dilute alkali (see Materials) have previously been shown to fulfill the first requirement (Baldwin et al., 1979), and the evidence for their unsealed state is given below. In contrast, ghosts contain several types of high-affinity sites for CB (Lin & Snyder, 1977; Jung & Rampal, 1977), and preparations of the purified transporter (Sogin & Hinkle, 1978; Baldwin et al., 1979), which also contain lipid, have been shown to consist largely of sealed vesicles (Baldwin et al., 1980, and unpublished experiments).

The unsealed character of the protein-depleted membranes was established by the use of three methods (see Experimental Procedures). The sialic acid residues, which are located on the external membrane surface, were found to be 98% accessible to the enzyme neuraminidase. The CB sites on the transporter are destroyed by proteolysis only at the cytoplasmic surface. Less than 15% of the sites remained after trypsin digestion of the membranes. Thus, both surfaces of the protein-depleted membranes are accessible to macromolecules. Finally, the behavior of these membranes on density-gradient centrifugation in dextran T70 gradients containing 100 mM NaCl/20 mM sodium phosphate, pH 7.0, indicated their unsealed nature. The membranes sedimented as a broad band with a density of 1.050–1.060 g cm^{-3}. On dextran gradients, protein-depleted erythrocyte membranes have been found to band at 1.01 g cm^{-3} or less when they are sealed and 1.025 g cm^{-3} or greater when they are unsealed (Zoccoli & Lienhard, 1977; Lienhard et al., 1977).

Binding Equilibria for CB and a Single Sugar. The prediction, based upon the alternating conformation model, of a single substrate binding site (Figure 1) cannot be tested by direct measurements of substrate binding, since under normal conditions the affinities of the various sugars are too low (see below). However, CB is a ligand with sufficiently high affinity that its binding can be measured directly. Evidence in the literature suggests that the binding site for CB overlaps with that of the inward-facing substrate site (Jung & Rampal, 1977; Basketter & Widdas, 1978; Devês & Krupka, 1978; Zoccoli et al., 1978; Sogin & Hinkle, 1980a), although an allosteric site for CB at the inner surface is also possible (Krupka & Devês, 1980). On the other hand, ethylideneglucose is a nontransported derivative that preferentially, by a factor of about ten, forms an outward-facing complex with the substrate

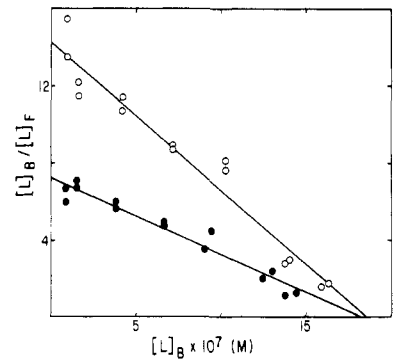
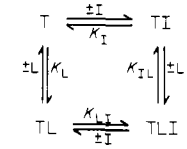


FIGURE 2: Scatchard plots for the binding of CB to the glucose transporter in protein-depleted membranes in the absence (○) and presence (●) of 25 mM ethylideneglucose. The lines are the best fits according to linear least-squares analysis. The value of the dissociation constant for CB is 1.3×10^{-7} M, and there are 1.8 nmol of sites per mg of membrane protein.

site (Baker et al., 1978; Devês & Krupka, 1978). Consequently, we have examined the effect of ethylideneglucose upon the binding of CB.

The binding of CB (L) to the glucose transporter (T) in the presence of a reversible inhibitor (I) can be described by the following general scheme:



where K_L, K_I, K_{IL}, and K_{LI} are the dissociation constants for the various equilibria. The Scatchard equation (Scatchard, 1949) is then

$$\frac{[L]_B}{[L]_F} = \frac{1}{K_L}[[T]_T - [L]_B]\frac{1 + [I]/K_{LI}}{1 + [I]/K_I} \qquad (1)$$

where the subscripts B, F, and T refer to bound, free, and total, respectively. Figure 2 presents Scatchard plots for the binding of CB to the transporter in the absence and presence of 25 mM ethylideneglucose. The finding that the value of the x intercept is the same for both plots shows that the scheme given above applies, and the finding that the value of the slope is less with ethylideneglucose present means that K_I is less than K_{LI} (eq 1).

The results in Figure 2, however, do not permit a decision as to whether or not a ternary complex of ethylideneglucose, CB, and transporter has sufficient stability to allow its detection. In order to answer this question, we measured the binding of CB as a function of the concentration of ethylideneglucose, at a single CB concentration much less than that of the transporter. Under these conditions, eq 1, in reciprocal form, reduces to

$$\frac{[L]_F}{[L]_B} = \frac{K_L}{[T]_T}\frac{1 + [I]/K_I}{1 + [I]/K_{LI}} \qquad (2)$$

According to eq 2, a plot of $[L]_F/[L]_B$ against [I] will curve toward a plateau if [I] approaches the value of K_{LI}. On the other hand, the plot will be linear if no significant amount of ternary complex forms over the range of [I]. As Figure 3 shows, the inhibition data give a linear plot up to 150 mM ethylideneglucose. The dotted line in this figure is the curve predicted from eq 2 on the assumption that the value of K_{LI} is 20 times larger than K_I; its deviation from the data shows that K_{LI} must be greater than K_I by at least this factor. Thus,

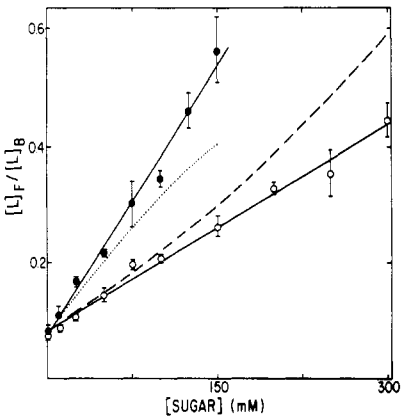


FIGURE 3: Inhibition of CB binding to the glucose transporter by ethylideneglucose (●) and D-glucose (○), at a single low concentration of CB (5×10^{-8} M). The mean ± the standard deviation of three determinations is given. The dotted line is calculated from eq 2 using the intercept and K_I values for ethylideneglucose on the assumption that K_{LI} equals $20K_I$. The dashed line is calculated from a simplified version of eq 3 $[[L]_F/[L]_B = (K_L/[T]_T)[1 + [I]/K_I + [I]^2/(K_IK_I')]]$ using the values of the intercept and K_I for D-glucose, on the assumption that K_I', the constant for the first dissociation from the ternary complex, equals $10K_I$.

Table I: Inhibition Constants for Sugars

compound	K_I (mM) from $[L]_F/[L]_B$ vs. Scatchard plots[a]	K_I (mM) from $[L]_F/[L]_B$ vs. [I][a]	K_I (mM) from transport[b]
ethylideneglucose	26	24	15[c]
D-glucose	59	70	
propyl glucoside	23	24	15[d]

[a] See text for details. [b] From data for the inhibition of transport, calculated according to the kinetic treatment of Devés & Krupka (1978). [c] Data from Baker et al. (1978). [d] Data from Barnett et al. (1975).

in agreement with the prediction from the alternating conformation model, there is no indication of a ternary complex.

The binding of CB to the transporter in the presence of D-glucose and of propyl glucoside was investigated in the same way as described for ethylideneglucose. D-Glucose, a substrate, would be expected to have significant affinity for both the inward- and outward-facing sites. Propyl glucoside is a nontransported derivative that has been shown to form preferentially, by a factor of at least 10, a complex with the inner facing substrate site (Barnett et al., 1975; Devés & Krupka, 1978). The results for these two compounds were similar to those for ethylideneglucose; there was no indication of ternary complex formation at concentrations of D-glucose and propyl glucoside up to 300 and 100 mM, respectively (data not shown, except for D-glucose in Figure 3). Table I presents the values of K_I for the three sugars, derived from the plots according to eq 1 and 2. The values agree reasonably well with each other and with literature values for the inhibition of transport. While this work was in progress, Sogin & Hinkle (1980a) reported similar results for the binding of D-glucose and propyl glucoside to purified glucose transporter.

Binding Equilibria for CB and Two Sugars. If CB binds at an allosteric site rather than at the inward-facing substrate site, then the results with ethylideneglucose described above allow no conclusion concerning the simultaneous binding of substrate at both membrane surfaces. A second approach to this question involves determining the combined effect of the inhibitor that binds to the outward-facing substrate site (ethylideneglucose, I_o) and the inhibitor that binds to the inward-facing substrate site (propyl glucoside, I_i). The general scheme for the equilibria under these conditions is

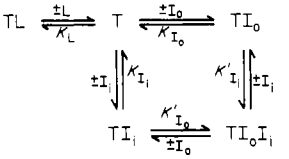


Table II: CB Binding in the Presence of Propyl Glucoside and Ethylideneglucose[a]

[propyl glucoside] (mM)	[ethylidene-glucose] (mM)	$[L]_B/[L]_F$ measured[b]	$[L]_B/[L]_F$ calculated[c]
0	0	13.24 ± 0.64[d]	
40	0	5.55 ± 0.05	5.28
80	0	3.44 ± 0.27	3.29
160	0	1.64 ± 0.11	1.88
0	40	3.70 ± 0.23	3.70
0	80	2.12 ± 0.19	2.15
0	160	1.18 ± 0.02	1.17
40	40	2.34 ± 0.09	2.60,[e] 1.47[f]

[a] The concentration of ^{3}HCB was 5×10^{-8} M. [b] Mean ± standard deviation of three determinations. [c] Each ratio measured in the presence of only one inhibitor was used to obtain a value of K_I for that inhibitor, according to eq 4. The average of the three K_I values was then used in this equation to reckon the calculated ratios. The mean K_I values for ethylideneglucose and propyl glucoside in the experiment were 16 and 27 mM, respectively. [d] Equals $[T]_T/K_L$; see eq 4. [e] Calculated from the average K_{I_o} and K_{I_i} values obtained in the presence of only one sugar, according to eq 4. [f] Calculated according to eq 3, assuming that the K_I' values equal the K_I values.

The binding of CB, at a concentration much less than $[T]_T$, is described by

$$\frac{[L]_B}{[L]_F} = \frac{[T]_T}{K_L[1 + [I_i]/K_{I_i} + [I_o]/K_{I_o} + [I_i][I_o]/(K_{I_o}K_{I_i}')]} \quad (3)$$

If the ternary complex (TI_oI_i) does not occur (that is, K_{I_o}' and K_{I_i}' are very large), then the last term in the denominator of eq 3 is negligible, and eq 3 simplifies to

$$\frac{[L]_B}{[L]_F} = \frac{[T]_T}{K_L[1 + [I_i]/K_{I_i} + [I_o]/K_{I_o}]} \quad (4)$$

The values of the constants on the right side of eq 4 are known from measurements in the presence of one or no inhibitor. Thus, existence of a ternary complex would be revealed by a measured value for the ratio of bound to free CB in the presence of the two sugar derivatives that is significantly less than the value calculated from the known constants and eq 4. The results in Table II show that the measured value (2.34) is not significantly less than the calculated one (2.60). If the dissociation constants for the terrnary complex (K_{I_o}' and K_{I_i}') had been equal to those for the binary complexes (K_{I_o} and K_{I_i}), the value of the bound to free ratio in the presence of both sugars would have been reduced to 1.47 (Table II). It can be estimated from these data that the values for the dissociation constants of the ternary complexes must be at least 4 times greater than those for the binary complexes.

The treatment given in this section is also applicable to the question of whether a ternary complex of the transporter with D-glucose bound simultaneously at the inward- and outward-facing substrate sites exists. If such were the case, then in accord with the reciprocal of eq 3, a plot of $[L]_F/[L]_B$ against the concentration of D-glucose should curve upward, due to the increasing contribution of the $[I]^2$ term. Since the plot is linear (Figure 3, open circles), there is no significant formation of this ternary complex. The dashed line in the figure shows the curve expected for the case in which the affinity of the hypothetical ternary complex for glucose is one-tenth that of the binary complex.

We emphasize that the conclusions in this section are valid regardless of whether CB binds at the inward-facing substrate site or at an allosteric site. They depend only on the assumption that the binding of CB is competitive with sugar binding at both the inward- and outward-facing substrate sites (see the above scheme). The validity of this assumption is established by the results in the previous section.

Kinetic Measurements of CB Dissociation. The equilibrium binding data presented above strongly suggest that substrate binding sites on opposite sides of the membrane cannot be occupied simultaneously. They do not, however, rule out the possibility that the binding of a second ligand to the already liganded transporter may be too weak to detect. It seemed possible that such weak associations, if they did occur, might substantially perturb the rate of CB dissociation from the transporter. For example, ethylideneglucose and D-glucose at high concentrations might increase the rate of CB dissociation by binding to an outward-facing substrate site of low affinity. Moreover, if the CB site were separate from the inner facing substrate site, propyl glucoside could alter the rate of dissociation as well. Consequently, we have investigated the kinetics of CB dissociation.

The simplest possible scheme for the dissociation of ^{3}HCB (L) from the transporter (T) in the presence of a competing ligand (I) is

$$TL \underset{k_a}{\overset{k_d}{\rightleftharpoons}} L + T \underset{K_I}{\overset{\pm I}{\rightleftharpoons}} TI$$

where k_d and k_a are the first- and second-order rate constants for dissociation and association, respectively. This scheme includes no weak interaction between the competing ligand and the transporter/CB complex and assumes that the binding of the competing ligand to the transporter is rapid relative to the dissociation of ^{3}HCB. When, as is the case here, the total concentration of transporter is much larger than the total concentration of ^{3}HCB, the integrated rate equation for CB dissociation according to this scheme is

$$\ln\left(\frac{[L]_E - [L]}{[L]_E - [L]_0}\right) = (k_d + k_a[T]_F)t = \left(k_d + k_a\frac{K_I}{K_I + [I]}[T]_T\right)t \quad (5)$$

where $[L]_E$, $[L]_0$, and $[L]$ are the concentrations of unbound ^{3}HCB at equilibrium, initially, and at intermediate times, respectively. Thus, the dissociation of CB should be a first-order process of approach-to-equilibrium, and a plot of the observed first-order rate constant (k_{obsd}) against the function $K_I/(K_I + [I])$ should be linear, with the y intercept equal to the true first-order rate constant for dissociation of CB (k_d).

Figure 4 presents data for the dissociation of ^{3}HCB that occurs upon mixing ^{3}HCB/transporter with an excess of unlabeled CB. The first-order plots are linear, and the value of k_{obsd} approaches a lower limit (k_d) at the higher concentrations

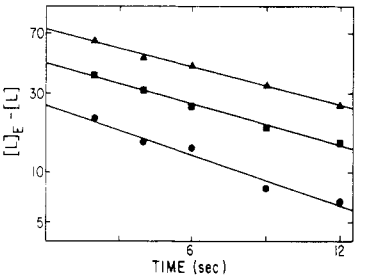


FIGURE 4: Kinetics of ^{3}HCB dissociation from the transporter in the presence of 2.5 (●), 5.0 (■), and 20 (▲) μM CB. $[L]_E$ and $[L]$ (see text) have been expressed as percentages of the total amount of radiolabeled CB, and the difference was plotted on the logarithmic scale against time.

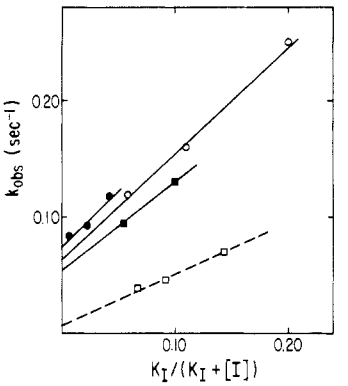


FIGURE 5: Observed first-order rate constants for the dissociation of ^{3}HCB from the transporter, in the presence of unlabeled CB (●), D-glucose (○), propyl glucoside (■), and ethylideneglucose (□). The values of $K_I/(K_I + [I])$ were calculated from the constants given in the legend of Figure 2 and in Table I. Each point is the average value from duplicate determinations of the rate constant; these agreed to within ±15% or less of the average value.

of CB (Figure 5, closed circles). Thus, as one might expect, with unlabeled CB as the competing ligand, the kinetics are consistent with the scheme. The value of k_d obtained from the secondary plot (Figure 5) is 0.075 s^{-1}. Since the equilibrium dissociation constant for cytochalasin B is 1.3×10^{-7} M (legend of Figure 2), the value of the second-order rate constant for the association of CB is 5.8×10^5 M^{-1} s^{-1}.

The rates of dissociation of ^{3}HCB from the transporter in the presence of several concentrations of D-glucose and of propyl glucoside were also first order (data not shown). The secondary plots of k_{obsd} gave limiting values for k_d that do not differ significantly from that found in the case of unlabeled CB (Figure 5, open circles and closed squares). Thus, there is no indication of a weak interaction between these sugars and the transporter/CB complex.

The results with ethylideneglucose, however, differ from those obtained with glucose and propyl glucoside. Although the first-order plots were approximately linear (data not shown), the values of k_{obsd} are only about 50% of those expected on the basis of the rates with the other ligands (Figure 5, open squares). Since the equilibrium effect of ethylideneglucose is the displacement of bound CB, one might expect that a weak association between the transporter/CB complex and ethylideneglucose would lead to an increase in the rate of CB dissociation. Thus, an alternative explanation for the observed decrease seems more likely to be correct. We suggest that a step in addition to the unimolecular dissociation of CB is partially rate limiting and that this step is the conformational change of the unoccupied transporter from the form with the inward-facing substrate site (T_i) to that with the outward-facing site (T_o):

$$T_iL \underset{k_a}{\overset{k_d}{\rightleftharpoons}} T_i + L \overset{k_{-1}}{\rightleftharpoons} T_o \overset{\pm I}{\underset{K_I}{\rightleftharpoons}} T_oI$$

Since ethylideneglucose binds to the outward-facing substrate site, the reduction in the concentration of T_i that leads to the release of bound ^{3}HCB necessarily requires the conversion of T_i to T_o. In support of this explanation is the fact that the value of the rate constant for the conformational change (k_{-1}) that can be estimated from steady-state parameters for glucose transport in cells at 0 °C (0.5 s^{-1})[2] is of roughly the same magnitude as k_d. Clearly, further experimentation will be needed to substantiate this explanation.

Conclusion. Ternary complexes of the glucose transporter with CB and either ethylideneglucose, D-glucose, or propyl glucoside, with ethylideneglucose and propyl glucoside, and with two molecules of D-glucose cannot be detected. This finding is consistent with the alternating conformation model for transport. Although it does not exclude models in which there is simultaneous occupancy by two or more molecules of substrate (Holman, 1980), it does place upon them the restriction that stability of the ternary complex must be low.

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[2] Since the value of the maximal flux for equilibrium exchange of D-glucose at 0 °C is much greater than that for entry (Lacko et al., 1972), the maximal flux for entry approximates $k_{-1}[T]_T$ (Geck, 1971), where $[T]_T$ is the moles of transporter in that number of cells with an internal volume of 1 L. $[T]_T$ is obtained from values in the literature for the amount of transporter per cell (Jung & Rampal, 1977; Sogin & Hinkle, 1980b).

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