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Asymmetrical Binding of Phloretin to the Glucose Transport System of Human Erythrocytes

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Summary. The sidedness of phloretin binding to the glucose carrier has been determined by comparing the type of inhibition produced in zero *trans* entry and zero *trans* exit experiments. Initial rates of zero *trans* entry were measured by the method of R.D. Taverna and R.G. Langdon (*Biochim. Biophys. Acta* **298**:412–421, 1973), which involves pink ghosts loaded with glucose oxidase; this obviates the problem of rapid substrate accumulation inside the cells. With phloretin equilibrated across the membrane, the inhibition of entry was competitive, and the inhibition of exit noncompetitive. The experimental procedures were validated by showing that the inhibition by cytochalasin B, known to bind inside but not outside, was noncompetitive in entry and competitive in exit, as predicted. It was also demonstrated that even after pre-incubation of the cells with a relatively high concentration of phloretin, the phloretin adsorbed in the membrane did not significantly alter the rate of carrier reorientation. The results show that the outward-facing form of the glucose carrier, but not the inward-facing form, bears a phloretin binding site; thus phloretin, as well as cytochalasin B, is bound asymmetrically, phloretin outside and cytochalasin B inside.

Key Words phloretin · glucose transport · asymmetric inhibition · cytochalasin B · inhibition mechanism

Introduction

The Asymmetry of the Glucose Carrier, and Phloretin Binding

That the glucose transport system of erythrocyte membranes has an asymmetrical structure is not in doubt: the affinity for glucose differs on the inner and outer surfaces of the cell membrane, and so does the affinity for certain inhibitors [29]; for example, cytochalasin B binds at the inner but not at the outer carrier site [2, 3, 6]. Phloretin resembles cytochalasin B in having an exceptionally high affinity for the carrier and in binding, reversibly, in competition with the substrate [3, 9, 17]—though neither inhibitor is a structural analog of glucose; but the question of where phloretin binds has not yet been settled.

According to an early report, phloretin does not penetrate the cell membrane [4] and therefore, when added to the suspending medium, could only add to the outer carrier form, regardless of whether the inner form possesses a phloretin binding site. Later studies demonstrated that it actually enters red cells rapidly and is strongly adsorbed both to the cell membrane and to hemoglobin [9, 14]. Hence phloretin could conceivably add to the carrier both inside and outside, and several authors have suggested that it does so. The evidence for this conclusion, however, is weak, as we shall now see.

Taverna and Langdon found that the inhibition of glucose entry by phloretin was competitive in vesicles whose membrane was believed to be inverted (with the inner surface in contact with the external medium) and was also competitive in ghosts, where the orientation of the membrane is the same as in whole cells; from this they inferred that phloretin binds on both sides [26, 27]. The interpretation would be correct if the inhibitor did not penetrate the cell membrane, but it does penetrate, and analysis of the carrier model [21] shows that the inhibition of entry should be noncompetitive if it binds inside. To explain the purely competitive inhibition, it must be assumed that the phloretin binding site was confined to the outer surface of the membrane in both preparations. Whether the membrane orientation was really reversed in the vesicles will be considered later.

Basketter and Widdas, too, have suggested that phloretin binds to both the inner and outer carrier forms [3]. Their conclusion, which can also be questioned, was based on the relative magnitudes of the inhibition constants measured in equilibrium exchange and Sen-Widdas net exit experiments. It had been shown, in the case of cytochalasin B, that a considerably higher concentration was required to

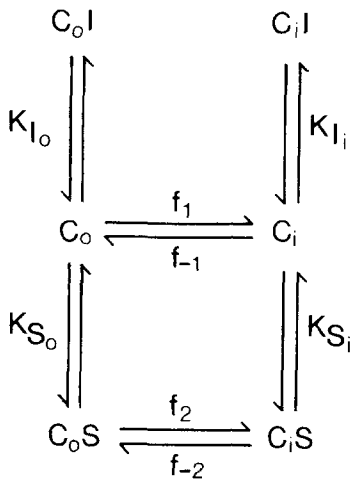

Fig. 1. Transport scheme for the substrate S in the presence of a reversible competitive inhibitor, I. The carrier alternates between an inward-facing conformation, C_i, and an outward-facing conformation, C_o; these forms bind the substrate or inhibitor in the internal and external compartments, respectively

inhibit net exit, where the internal substrate concentration is held constant at a saturating level, than to inhibit exchange, where the substrate concentration is varied; it followed that cytochalasin B binds predominantly inside, in agreement with other experiments on this inhibitor. With phloretin, by contrast, the inhibitory concentrations in the two experiments were similar—0.24 μM in net exit, and 0.4 μM in exchange—which was taken to mean that phloretin binds on both sides. On analysis, however, it is found that the actual result—a somewhat lower figure for exit than exchange—can only be explained if the inhibitor binds exclusively outside. Referring to the transport scheme in Fig. 1, the constants measured in the two experiments are given by $K_{Io}(1 + f_1/f_{-2})$ and $K_{Io}(1 + f_1/f_{-1})$, respectively, where the rate constants f_1 and f_{-1} are for the free carrier, f_2 and f_{-2} for the carrier-substrate complex [21]; in glucose transport, exchange is faster than zero *trans* exit by a factor of 2.3 [23], which indicates that $f_{-2} > f_1$ [13]; accordingly, the inhibition constant in net exit should, from the above expressions, be somewhat smaller than that in exchange, in agreement with the experimental result. This would not be so if phloretin added equally on both sides: here the inhibition constants are given by $K_{Io}(1 + f_1/f_{-2})$ and K_{Io}, respectively, and the former cannot be smaller than the latter.

Fannin et al. [9] tended to agree that phloretin may be bound on both sides. They reported mixed competitive and noncompetitive inhibition in zero *trans* entry of galactose, where the cells were pre-incubated with phloretin, but pure competitive inhibition in zero *trans* entry of glucose, where there was no pre-incubation. Zero *trans* exit of galactose was inhibited noncompetitively (glucose was not examined). The mixed inhibition of galactose entry implies that phloretin is bound mainly, but not entirely, on the outer surface, while the competitive inhibition of glucose entry implies that under these conditions it is bound exclusively outside [21].

Experiments reported by Krupka and Devés [19], in which the inhibitions of glucose and xylose exit were compared, indicated that phloretin is bound exclusively to the outward-facing carrier. Because the affinity of xylose for the carrier is considerably lower than that of glucose, a competitive inhibitor which is bound inside is more effective in cells loaded with xylose than with glucose; an inhibitor bound outside is about equally effective with either substrate, for an internal substrate does not compete with an external inhibitor. The results with phloretin and with maltose (which does not enter the cell [22]) were identical and were as predicted for inhibitors bound exclusively outside. The entirely different behavior of cytochalasin B agreed with the predictions for an inhibitor bound only inside, while the intermediate behavior of certain other inhibitors, which are steroids, could be explained if they were bound on both sides [21].

From experiments on ghosts, Beněs et al. [4] suggested that there are phloretin binding sites on both sides, but the observations are puzzling and the argument appears to be faulty: exit of xylose was inhibited by phloretin added to the external medium but not by phloretin trapped inside the ghosts, while entry was inhibited in the opposite way, i.e., by internal but not by external inhibitor. Believing, mistakenly as we now know [9, 14], that phloretin cannot pass through the cell membrane, the authors concluded that transport is blocked only when the inhibitor is bound *trans* with respect to the substrate; but since phloretin would have had time to equilibrate [9], it should have been available on both sides, and therefore transport should have been inhibited in all cases. Indeed, the failure of external phloretin to inhibit entry is in disagreement with Taverna and Langdon's [26] finding, noted above.

Another objection—and as seen below there may be widespread confusion on this point—is that xylose was tested at a concentration of 5 mM, which is well below its half-saturating level, reported to be 14 and 24 mM in entry and exit, respectively. At these low concentrations the substrate could not effectively compete with phloretin, and therefore the extent of inhibition should not have depended on whether the inhibitor adds inside or outside. The inhibition of xylose transport in only one direction,

by phloretin added to either the medium or the ghosts, is therefore an anomaly.

The nature of the difficulty should perhaps be explained further. As inspection of Eqs. (3) and (4), below, shows, the conventional carrier model represented in Fig. 1 predicts that an asymmetrically bound inhibitor has identical effects on entry and exit at low substrate concentrations [21]. This is probably true of any carrier mechanism, no matter how simple or how complex, provided that it is not driven by an external source of energy, as in active transport. The reason is that at sufficiently low substrate concentrations the carrier exists in an equilibrium state, any disturbance of which by the substrate is negligibly small. An inhibitor, regardless of where it binds, acts by sequestering carrier molecules in an inactive form; inhibition results because only a fraction of the system remains to carry out transport, and this fraction does not depend on the location of the substrate. At high concentrations, by contrast, the substrate competes with an inhibitor bound on the same side of the membrane, and partly or completely overcomes the inhibition; but, depending on the details of the transport mechanism [7, 21], it may fail to overcome the inhibition by an inhibitor on the opposite side of the membrane. The result: at saturating substrate concentrations, transport may be blocked in one direction but not the reverse, by an asymmetrically bound inhibitor. These effects, too, are implicit in Eqs. (3) and (4).

The reconstituted transporter has also been studied, and from this work some authors have concluded that the inhibition by phloretin is unsymmetrical, others that it is symmetrical; but neither conclusion can be justified by the evidence. Kahlenberg and Zala [15] and Goldin and Rhoden [10] found inhibition by externally added phloretin but not by phloretin trapped in the vesicles, which suggested asymmetry; but the failure of internal phloretin to inhibit is expected because, as Wheeler and Hinkle [28] pointed out, phloretin would rapidly leak through the membrane and be greatly diluted under the conditions of the assay. On the other hand, Wheeler and Hinkle [28] thought the inhibition symmetrical, since they found that the entry and exit of glucose were inhibited to a similar degree by phloretin added to the external medium; however, the glucose concentration, 0.2 mM, was much lower than the reported half-saturation constants in entry and exit, 1.2 and 0.73 mM, respectively, and, as explained in the preceding paragraph, asymmetry could not reveal itself under these conditions.

But there is an objection to experiments on the reconstituted system that would invalidate any such conclusion even at saturating concentrations of the substrate; for it has been demonstrated by Baldwin et al. [1], as well as by Wheeler and Hinkle [28], that in reconstituted systems the carrier is more or less randomly oriented in the membrane. Consequently, if each transporter molecule is assumed to be asymmetric, some would bind the inhibitor on one side of the membrane, some on the other; and, correspondingly, the substrate could compete, partially, on either side. The system as a whole would then behave in a roughly symmetrical manner.

In sum, where the evidence is not clearly invalid, it suggests that phloretin adds asymmetrically to the carrier, possibly at a site which becomes exposed only on the outer surface. If so, the sites for phloretin and for cytochalasin B would be located at opposite poles of the carrier molecule, which has been shown to span the membrane [1, 11]; and phloretin and cytochalasin B, even though free to diffuse across the cell membrane, could be representative of pairs of inhibitors acting upon the carrier with an opposed orientation. It will be important to decide, beyond question, where phloretin binds, partly because of the physiological role that asymmetric sites could play in the modulation of carrier activity [18], and partly because of the usefulness of asymmetrically bound inhibitors, both in kinetic studies of transport and in physical studies of the isolated carrier molecule [12, 16, 20].

An Approach to the Problem

It was shown earlier that the sidedness of inhibitor binding can be determined from the inhibition of equilibrium exchange, of zero *trans* entry, and of zero *trans* exit [21]. As a first step, the nature of the inhibition is established in equilibrium exchange, because here a competitive inhibitor necessarily gives rise to competitive kinetics, whichever side of the membrane it binds on. It has already been demonstrated that the inhibition of exchange by phloretin is purely competitive [3, 9]. It has also been shown that in Sen-Widdas net exit experiments external glucose competes with phloretin [9, 17]. Two facts are thus established: addition of the substrate to the carrier prevents the addition of phloretin, and phloretin adds to the outward-facing carrier. The question remains, does it also add to the inward-facing carrier? If it does, the inhibition of both zero *trans* exit and zero *trans* entry should be noncompetitive, according to the kinetic theory. The reason is as follows: in these experiments the substrate is present on only one side of the membrane, but by hypothesis the inhibitor acts on both sides; the substrate competes with an inhibitor on the same side but not the opposite side, and therefore the inhibition appears to be noncompetitive, in

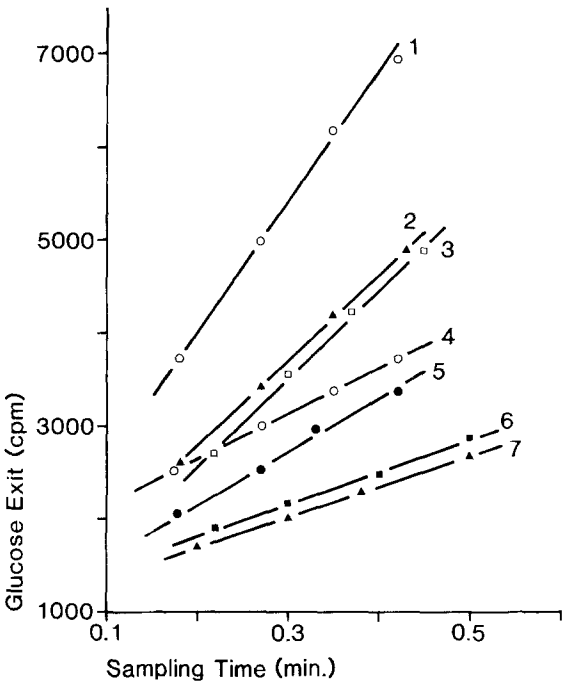

Fig. 2. Exit of ^{14}C-glucose from whole cells, as represented by the radioactivity (cpm) in samples of the suspending medium (*see* Materials and Methods). The cells were preincubated with various concentrations of glucose, as follows. (*1*): 10.42 mM glucose (22.0×10^7 cpm/mmol); (*2, 3, 6* and *7*): 20.25 mM glucose (9.61×10^7 cpm/mmol); (*4* and *5*): 59.6 mM glucose (4.17×10^7 cpm/mmol). In *6* and *7*, 0.37 μM phloretin was present in the exit medium

that it persists at indefinitely high substrate concentrations (i.e., the intercept as well as the slope of a reciprocal plot is increased). If, on the other hand, the inhibitor binds exclusively outside, then the inhibition of substrate entry should be purely competitive (i.e., the inhibition would be reduced at high substrate concentrations, and the intercept in a reciprocal plot would be constant); however, the inhibition of substrate exit would be noncompetitive (with an increase in both the slope and intercept of a reciprocal plot).

There is a technical difficulty in measuring initial rates of zero *trans* entry with a good substrate like glucose: as a consequence of rapid substrate transport into the cell, the internal substrate concentration very soon reaches levels where it begins to saturate the carrier and to be transported outward; substrate movement is then mainly an exchange, and the measured rate is reduced to the difference between influx and efflux. The expected time scale for these events may be estimated from the well established rates of glucose exit. Cells loaded with 100 mM glucose come to equilibrium, when placed in a large volume of buffer at 25°C, in roughly 30 sec [17]; and at this rate, entry of glucose into cells free of substrate could build up an internal concentration of 5 mM in 1.5 sec. The internal half-saturation constant for glucose is reported to be 6–7 mM [5, 23], so that at a time as early as this, return flux of the substrate could have become significant. Indeed, rapid sampling techniques reveal bending in the uptake curve at a time as early as 0.4 sec [24].

The elegant method devised by Taverna and Langdon [26] avoids this difficulty by introducing glucose oxidase into the cells, or rather, into pink ghosts; the enzyme concentration is made high enough to oxidize glucose as it enters, keeping the internal concentration low. The rate of glucose entry is monitored continuously by measuring the rate of oxygen uptake by the system:

$$\beta\text{-D-glucose} + \tfrac{1}{2}\,O_2 \rightarrow \text{gluconic acid}.$$

This method is used in the study described below.

Materials and Methods

Glucose Influx into Ghosts Loaded with Glucose Oxidase

The procedure follows that of Taverna and Langdon [26]. Packed red cells (1.5 ml), which had been washed three times in isotonic saline, were added to 1.2 ml of ice-cold sodium phosphate buffer, 15 mM, pH 7.4, containing 3 mg of glucose oxidase (from *Aspergillus niger*, Type V, supplied by Sigma Chemical Co.), as well as catalase. Lysis occurred upon gentle mixing. The membranes were resealed by adding 414 μl of 1 M sodium phosphate, pH 7.4, and incubating the suspension for 30 min at 37°C. The resulting mixture was chilled on ice and centrifuged for 30 min in the cold at 12,000 × *g*; after removal of the supernatant, the ghost preparation was washed three times in 0.14 M sodium phosphate buffer, pH 7.4, when no further glucose oxidase activity was detectable in the wash. Uptake experiments were performed on the same day the ghosts were prepared.

In the assay for glucose uptake, oxygen consumption was monitored polarographically with a Clark electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio); the reaction mixture (2.05 ml) was stirred magnetically in a thermostated vessel. The rate of oxygen consumption was determined from the linear section of the recording of oxygen loss. The assay suspension was made up as follows. The ghosts prepared from 1.5 ml of cells were suspended in 22.5 ml of 0.14 M sodium phosphate, pH 7.4, and kept on ice; 1 ml of this suspension was added to 1 ml of the same buffer in the reaction vessel and allowed to come to the assay temperature (15°C). When present, the inhibitor was included with the buffer. To start the reaction, 50 μl of a glucose solution was added, giving a final concentration of 5 to 50 mM.

The steady-state concentration of glucose inside the ghosts, during the period when the rates of glucose entry were being measured, can be calculated from the K_m and V values for glucose oxidase. Under the conditions of the assay (pH 7.4, 15°C),

K_m for the enzyme was 24 mM, in close agreement with the value reported by Taverna and Landgon [26], and V was 120 mmol glucose liter^{-1} min^{-1} mg^{-1}. In the course of uptake into ghosts, the measured rate of glucose oxidation, relative to the maximum rate of glucose oxidation by the enzyme enclosed in the ghosts, had a maximum value of 1/50 in the absence of inhibitors, and of 1/100 in the presence of an inhibitor. (It may be noted that glucose oxidase is not inhibited by phloretin [26].) The internal glucose concentration that would produce a given ratio is found from the Michaelis-Menten equation for the enzyme:

$$v = V/(1 + K_m/[S]) \quad (1)$$

or

$$[S] = K_m \left\{\frac{V}{v} - 1\right\}. \quad (2)$$

For present purposes, v is the measured rate of oxidation by the ghosts, and V the maximum rate of oxidation by the enzyme in the ghosts; K_m = 24 mM. If the ratio V/v is 50, $[S]$ = 0.49 mM, and if V/v is 100, $[S]$ = 0.24 mM; either concentration is well below the internal half-saturation constant for glucose transport, which is shown below to be approximately 8 mM. In the transport experiments, therefore, the conditions of zero *trans* entry are closely approached (i.e., entry into cells containing a negligible concentration of substrate).

Efflux of ^{14}C-Glucose from Whole Cells

Washed red cells (1 ml in a total volume of 12 ml) were equilibrated, for 45 min at 37°C, with a given concentration of nonradioactive glucose (10 to 60 mM) in isotonic saline solution. The suspension was centrifuged and the supernatant carefully removed. To the packed cells, 8 μl of a solution of ^{14}C-glucose was added (52.2 mM, 4.8 mCi/m mol, uniformly labeled, supplied by New England Nuclear, Boston, Mass.); the suspension was mixed and incubated at 37°C for an additional 45 min. The cells were then centrifuged under 5 ml of dibutyl phthalate, which is immiscible with water and has a density intermediate between that of saline solution and red cells; the centrifuge tube above the organic layer was carefully washed with saline to remove all radioactivity, after which the dibutyl phthalate was removed by aspiration. The packed cells were then used in the exit assay.

Rates of glucose exit were determined at 15°C. Exit was begun when 100 μl of cells were added to a solution of 5 ml of isotonic NaCl containing mannitol at the same concentration as the glucose in the cells. Samples (1 ml) were taken at intervals, usually over a period up to 30 sec, but sometimes longer when transport was slowed down by an inhibitor; the samples were added to 3 ml of an ice-cold solution of 1.5 mM mercuric chloride and 18 μM phloretin in tubes containing 0.5 ml of dibutyl phthalate. The tubes were quickly centrifuged, and the radioactivity in 1 ml of the supernatant was determined by scintillation counting in 4 ml of Aquasol®. Plots of the radioactivity in the samples against time were found to be linear, as shown in Fig. 2.

To find the specific activity of the glucose in each experiment, which was required to calculate the initial rate of exit in molar units, the exit mixtures were incubated for an additional 15 min at room temperature; this allows the cellular glucose to equilibrate with the external medium. Following this the tubes were centrifuged, and the radioactivity in 200 μl aliquots of the supernatant was counted.

Net Efflux of Glucose from Whole Cells

The light scattering assay, which depends on the osmotic swelling and shrinking of cells in response to changes in the internal glucose concentration, was described before [17]. Cells were loaded with 123 mM glucose at 37°C for 1 hr; they were then packed by centrifugation, and 80 μl was added to 65 ml of isotonic saline solution at a controlled temperature. Initial rates of exit (net exit when glucose was present in the external medium) were calculated from the slopes of the recorder tracings of transmitted light *vs.* time, which were linear.

Results

The phloretin inhibition of glucose entry into ghosts at 15°C is shown in Fig. 3. In one experiment, ghosts were added last, the uptake rate being measured after a period of approximately 15 sec (Fig. 3*A*); in the other, the ghosts were incubated with phloretin for a period of 5 min or more before the addition of glucose (Fig. 3*B*). Either way, the inhibition was competitive. By contrast, the inhibition by cytochalasin B was noncompetitive (Fig. 4).

The inhibition of glucose exit from whole cells at 15°C, with various internal concentrations of glucose, is shown in Figs 5, 6, and 7. The inhibition by phloretin and maltose was noncompetitive, while that by cytochalasin B was competitive.

The effect of preincubation of the cells with phloretin upon the net rate of exit, in a Sen-Widdas experiment, is shown in Fig. 8. Cells (a 20% suspension) were first loaded with 123 mM glucose for 45 min at 37°C, and then incubated with 36 μM phloretin for 1 hr at 25°C; they were packed by centrifugation, and net rates of glucose exit from the cells were determined at 25°C by the light-scattering method, with various concentrations of glucose in the external solution. Exit rates in treated cells were determined with 0.078 μM phloretin added to the external solution, as well as glucose; exit rates in control cells, which were not preincubated with phloretin, were determined in the absence of phloretin. The inhibition is seen to be competitive, which is indicated by parallel lines in such plots of reciprocal rates *vs.* the external glucose concentration.

The half-saturation concentration for phloretin at 15°C was determined with whole cells in 0.14 M sodium phosphate buffer, pH 7.4. Cells were loaded with ^{14}C-glucose (60 mM), and exit rates were determined at various concentrations of phloretin, from 0 to 0.44 μM; the plot of the reciprocal rate against the inhibitor concentration was linear, and from a least

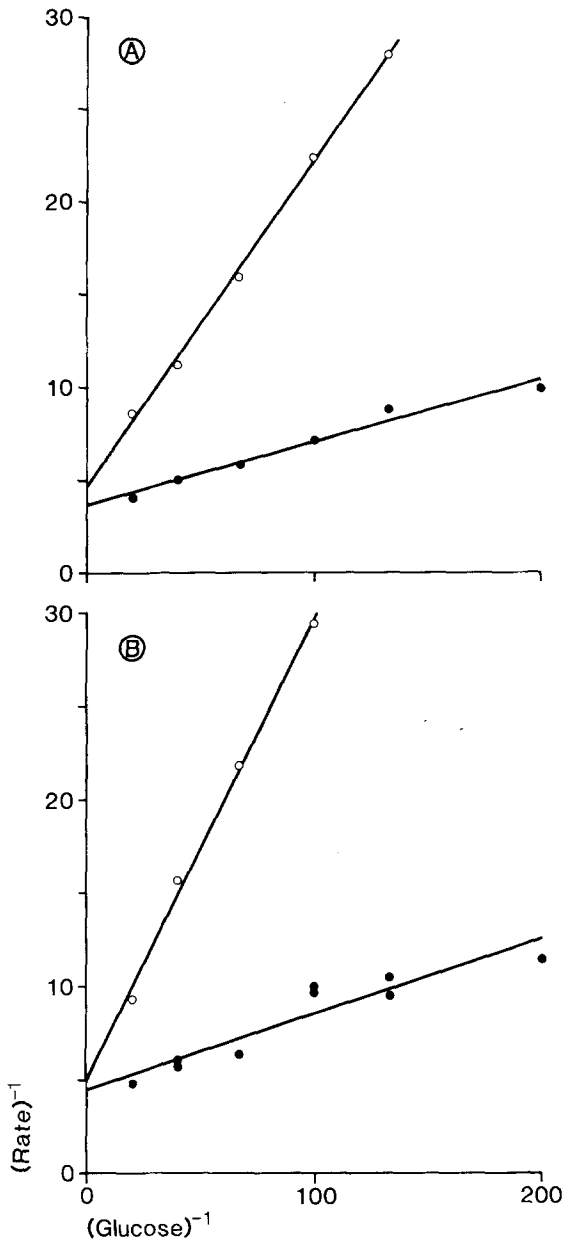

Fig. 3. The inhibition of glucose uptake into pink ghosts by 36.5 μM phloretin (15°C). (*A*): A suspension of ghosts was added to a solution containing glucose and phloretin, and the initial rate of entry was determined after approximately 15 sec. (*B*): The ghosts were pre-incubated with phloretin for at least 5 min before the addition of glucose. In the reciprocal plot of the data, the units of glucose concentration are molar, and the units of rate are mmol glucose per min per liter; in each plot the lower line (solid circles) represents the control rates, the upper line (open circles) the inhibited rates. From a least squares treatment, the ratio of the intercepts on the $1/v$ axis in *A* is 1.27 ± 0.16; in *B* the ratio is 1.12 ± 0.24. (By comparison, the ratio of the slopes is 5.18 and 6.18, respectively). The calculated value of the inhibition constant for phloretin, $\bar{K}_{I_o}$, is 8.73 ± 0.99 μM in *A* and 7.05 ± 1.35 μM in *B*. The half-saturation constant for glucose is 9.25 ± 0.14 mM and 8.89 ± 0.50 mM in *A* and *B*, respectively

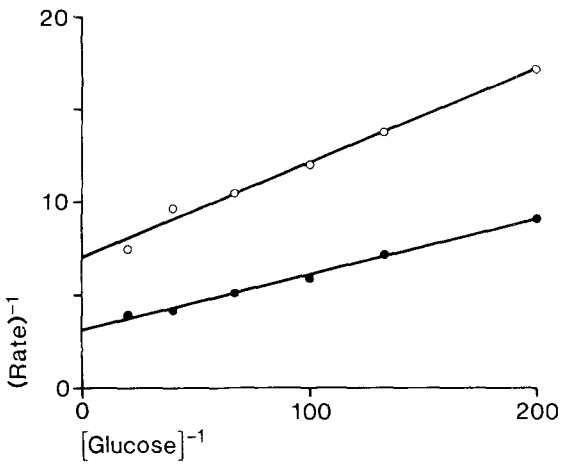

Fig. 4. The inhibition of glucose entry into pink ghosts by 0.208 μM cytochalasin B (15°C). Uptake was begun with the addition of glucose to a suspension of ghosts in a solution of the inhibitor. $\bar{K}_{I_i}^{S}$, the inhibition constant calculated from the ratio of intercepts, is 0.168 ± 0.018 μM, and the half-saturation constant for glucose is 9.41 ± 0.03 mM. Units of glucose concentration, M; units of rate, mmol glucose min^{-1} liter^{-1}. As in all the reciprocal plots, the lower line represents the control rates and the upper line the rates in the presence of an inhibitor

squares analysis the inhibition constant was found to be 0.235 ± 0.003 μM.

Discussion

In ghosts, according to Fannin et al. [9], equilibration of phloretin across the membrane is complete in less than 5 sec. In whole cells, according to Jennings and Solomon [14], the half-time for equilibration at 2% hematocrit is 8.7 sec, which is slower than with ghosts, presumably because of the gradual absorption of phloretin to hemoglobin. In the present experiments, the ghosts contained 39% of the hemoglobin of intact cells (*see* Materials and Methods), and the equilibration time may therefore be longer than with white ghosts prepared by the method of Dodge et al. [8]. In any case the preincubation times with phloretin in the experiments on glucose uptake (more than 5 min in one type of experiment) are sufficiently long to ensure complete equilibration.

The phloretin inhibition of glucose uptake in ghosts is found to be purely competitive (Fig. 3), in agreement with the observations of Taverna and Langdon [26]. It follows that phloretin is not bound, to any significant extent, to the inward-facing carrier site, as the kinetic theory of transport inhibition shows [21]: Thus, the rate of zero *trans* entry of the

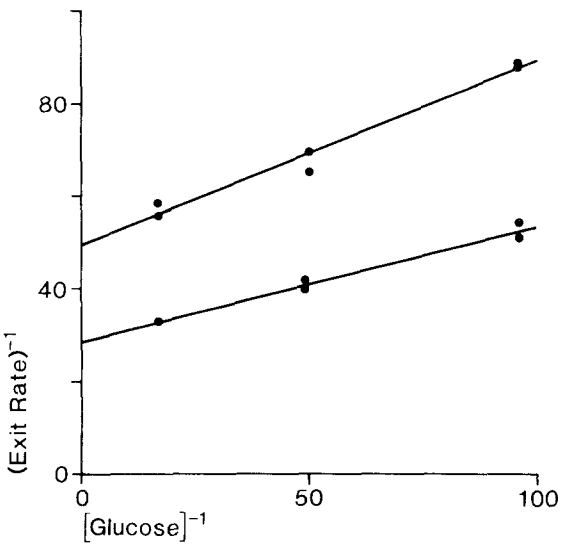

Fig. 5. The inhibition of glucose exit from whole cells by 0.184 μM phloretin (15°C). $\tilde{K}^S_{I_o}$, the inhibition constant calculated from the ratio of intercepts, is 0.253 ± 0.038 μM. The glucose constant, $\bar{K}_{S_i}$, is 8.75 ± 0.32 mM. Units of glucose concentration, M; units of rate, mmol glucose min^{-1} per liter of cell water

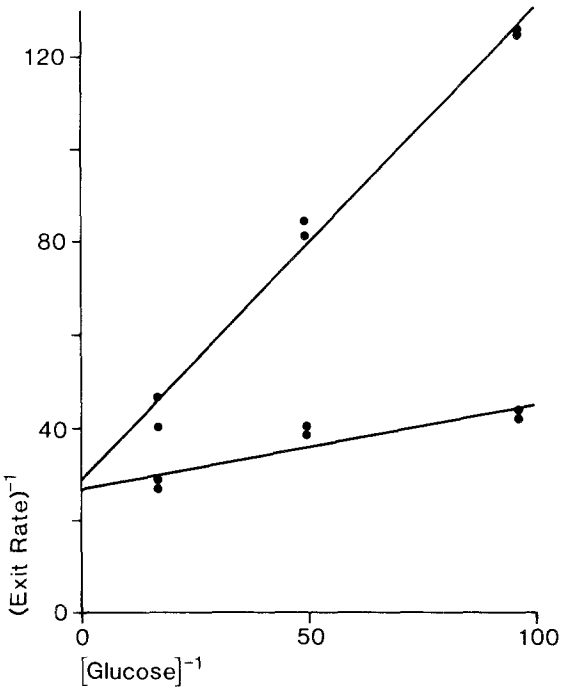

Fig. 6. The inhibition of glucose exit by 0.313 μM cytochalasin B (15°C). The inhibition constant, $\bar{K}_{I_i}$, is 0.067 ± 0.019 μM, and the glucose constant, $\bar{K}_{S_i}$, is 6.75 ± 0.99 mM. Units are as in Fig. 5

substrate S in the presence of an inhibitor I on both sides of the membrane is governed by the following general equation (written in reciprocal form):

$$\frac{1}{v} = \frac{1}{\bar{V}_{S_o}}\left\{1 + \frac{[I]}{\tilde{K}^S_{I_i}} + \frac{\bar{K}_{S_o}}{[S_o]}\left(1 + [I]\left[\frac{1}{\bar{K}_{I_o}} + \frac{1}{\bar{K}_{I_i}}\right]\right)\right\}. \quad (3)$$

In Eq. (3), $\bar{K}_{S_o}$ is the half-saturation constant and $\bar{V}_{S_o}$ the maximum velocity in zero *trans* entry. The inhibitor constants are half-saturating concentrations: $\bar{K}_{I_o}$ is the constant for an externally bound inhibitor measured in zero *trans* entry, and $\bar{K}_{I_i}$ the constant for an internally bound inhibitor measured in zero *trans* exit; $\tilde{K}^S_{I_i}$ is the half-saturating level required at indefinitely high substrate concentrations, i.e., the constant found from the intercept on the ordinate in a reciprocal plot. From a kinetic treatment of the carrier scheme in Fig. 1, the experimental parameters may be written in terms of individual rate constants in the scheme: $\bar{K}_{I_i} = K_{I_i}(1 + f_{-1}/f_1)$, $\tilde{K}^S_{I_i} = K_{I_i}(1 + f_{-1}/f_2)$, and $\bar{K}_{I_o} = K_{I_o}(1 + f_1/f_{-1})$*. It was noted above that the maximum rate of exchange is faster than zero *trans* flux [23], and therefore $f_2 > f_{-1}$ [13]; hence $\tilde{K}^S_{I_i}$ should be smaller than $\bar{K}_{I_i}$. With an inhibitor bound equally well on both sides ($K_{I_i} = K_{I_o}$), the inhibition is necessarily noncompetitive according to the equation, in that

* While Eq. (3) is general, and applies whether substrate dissociation is a fast or a rate-limiting step, $\tilde{K}^S_{I_i}$ is written here for the case of rapid substrate dissociation, a condition which holds for glucose transport, as shown by the fact that exchange is faster than zero *trans* flux [13, 23]. However, conclusions regarding the sidedness of inhibitor binding do not depend on this condition, but only on the condition that transport is inhibited competitively in one direction and noncompetitively in the other. The complete expression for the inhibition constant is:

$$\tilde{K}^S_{I_i} = \{k_{-2}(f_{-1} + f_2) + f_{-1}(f_2 + f_{-2})\}/(k_{-2}f_2)$$

(where $K_{S_i} = k_{-2}/k_2$). The ratio of this constant to $\bar{K}_{I_i}$ is:

$$\frac{\tilde{K}^S_{I_i}}{\bar{K}_{I_i}} = \frac{(1 + f_{-1}/f_2) + (1 + f_{-2}/f_2)f_{-1}/k_{-2}}{(1 + f_{-1}/f_1)}.$$

Inhibition is of the noncompetitive type (i.e., both the slope and intercept of a reciprocal plot are increased, though not necessarily by the same factor) if the ratio is not too far from unity. On the other hand, the ratio becomes very large, and the inhibition therefore competitive, if $f_{-1} \gg k_{-2}$—i.e., if substrate dissociation is the sole rate-limiting step in transport; in this case no information about the sidedness of inhibitor binding can be obtained. The inhibition can be noncompetitive, and the site of inhibitor binding deduced, even if $f_{-1} \simeq k_{-2}$, with dissociation as slow as the carrier reorientation steps.

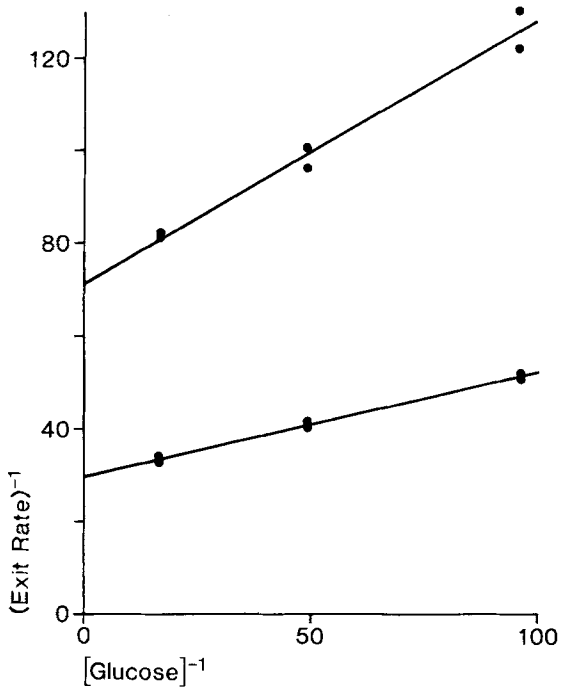

Fig. 7. The inhibition of glucose exit by 20 mM maltose (15°C). $\bar{K}^S_{I_o}$, the inhibition constant calculated from the ratio of intercepts, is 14.3 ± 0.97 mM, and $\bar{K}_{S_i}$ for glucose is 7.58 ± 0.07 mM. Units are as in Fig. 5

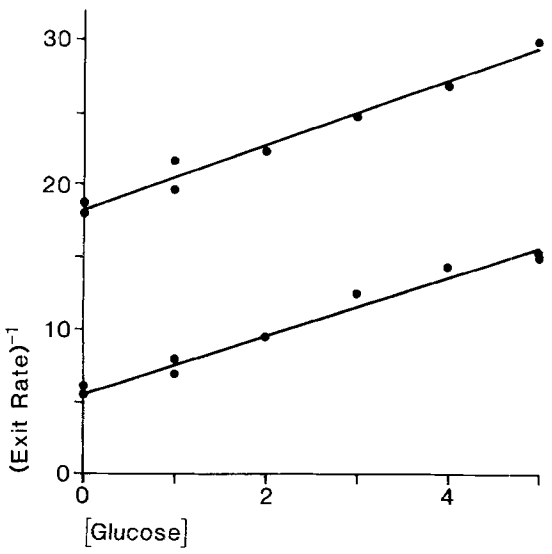

Fig. 8. Sen-Widdas net exit experiment (25°C) involving cells pre-incubated with phloretin. *Lower line:* rates of exit from control cells loaded with 123 mM glucose, with varying concentrations of glucose in the external solution. *Upper line:* rates of exit from cells treated with 36 μM phloretin for 1 hr at 25°C and assayed in a solution containing 0.078 μM phloretin

both the slope and intercept in a reciprocal plot ($1/v$ versus $1/[S_o]$) increase in the presence of the inhibitor. That there is no significant increase in the intercept in the experiment (Fig. 3) implies that $\bar{K}^S_{I_i} \gg \bar{K}_{I_o}$, (since, as was already noted, $\bar{K}^S_{I_i} < \bar{K}_{I_i}$). It follows that $K_{I_i} \gg K_{I_o}$; in other words, the inhibitor does not bind to the inward-facing carrier.

For zero *trans* exit the corresponding equation is

$$\frac{1}{v} = \frac{1}{\bar{V}_{S_i}}\left\{1 + \frac{[I]}{\bar{K}^S_{I_o}} + \frac{\bar{K}_{S_i}}{[S_i]}\left(1 + [I]\left[\frac{1}{\bar{K}_{I_i}} + \frac{1}{\bar{K}_{I_o}}\right]\right)\right\}. \quad (4)$$

An inhibitor which is bound only inside (and for which $\bar{K}_{I_o}$ and $\bar{K}^S_{I_o}$ are very large relative to $\bar{K}_{I_i}$) should obviously give purely competitive kinetics. But if the inhibitor is bound outside the inhibition should be noncompetitive, irrespective of whether it also binds inside, for both the slope and intercept of a reciprocal plot then increase in the presence of the inhibitor. In the experiment, phloretin produced noncompetitive inhibition in zero *trans* exit (Fig. 5). Thus the two experiments—zero *trans* entry and exit—combine to show that phloretin binds to the outward-facing but not the inward-facing carrier, in agreement with previous evidence from this laboratory [19].

The observations on cytochalasin B, shown in earlier studies to bind inside but not outside [2, 3, 6], corroborate the experimental methods: as expected, the inhibition of glucose entry is noncompetitive and of exit, competitive, exactly the reverse of the behavior for phloretin (Figs. 4 and 6). Again, maltose, included in the study because of the virtual certainty that it does not enter the cell [22], binds exclusively on the outside and therefore inhibits glucose exit noncompetitively (Fig. 7) just as phloretin does. Maltose inhibition was not examined in entry experiments because of a low affinity in ghost preparations; however, it has been shown to compete with external glucose in Sen-Widdas net exit experiments [17].

As noted in the Introduction, the observations of Taverna and Landgon [26, 27] indicate that in ghosts and membrane vesicles a phloretin binding site was exposed on the surface of the membrane in contact with the suspending medium, but not on the internal surface (otherwise the inhibition of entry would have been noncompetitive instead of competitive). As no direct evidence was provided regarding the orientation of the membrane, it is likely that in both preparations the orientation was the same as in whole cells. The method used to produce vesicles was derived from that of Steck et al. [25], but Taverna and Langdon deviated from the method by adding $MgSO_4$ to the membrane suspension prior to the homogenization step; Steck et al.

showed that in the presence of $MgSO_4$, endocytosis did not occur and the vesicles were right-side-out. Even though endocytosis was observed by Taverna and Langdon prior to their addition of $MgSO_4$, the final product could have been right-side-out vesicles.

An observation still unexplained is the partially noncompetitive inhibition of galactose uptake reported by Fannin et al. [9], though the technical difficulty of measuring true initial rates of zero *trans* entry in whole cells, discussed in the Introduction, could have led to error. It may be noted that the entry of phloretin into the cell is sufficiently rapid to have given rise, even without pre-incubation with the inhibitor, to a significant noncompetitive component in the glucose entry experiment as well (assuming that phloretin added to internal carrier sites), but instead pure competitive inhibition was seen by these authors.

Fannin et al. [9] have suggested a possible alternative explanation for their finding of partially noncompetitive inhibition of galactose entry (which was observed after a 10-min incubation of the cells with phloretin); namely, a modification of the membrane structure by adsorbed phloretin, with a secondary effect on the mobility of the carrier. The hypothesis has now been tested but is not supported by the results: preincubation with 36 μM phloretin for 1 hr at 25°C did not give rise to a noncompetitive component in the inhibition, as determined in a Sen-Widdas net exit experiment (Fig. 8). The internal concentration of glucose in this experiment is saturating (123 mM compared with an internal half-saturation constant of approximately 7 mM [5, 19, 23]; and with this condition, the general rate equation for net transport, in the presence of an inhibitor at equilibrium across the cell membrane, is as follows [21]:

$$v = \bar{V}_{S_i} \Big/ \left\{ 1 + \frac{[I]}{\tilde{K}^S_{I_o}} + \frac{[S_o]}{\tilde{K}^S_{S_o}} \right\} \tag{5}$$

where $\bar{V}_{S_i}$ is the maximum rate of zero *trans* exit. If phloretin, when adsorbed into the membrane, reduces the rate of carrier reorientation (governed by the rate constants f_1, f_{-1}, f_2, and f_{-2} in Fig. 1) by the factor $g(I_m)$, then Eq. (5) becomes

$$v = g(I_m)\bar{V}_{S_i} \Big/ \left\{ 1 + \frac{[I]}{\tilde{K}^S_{I_o}} + \frac{[S_o]}{\tilde{K}^S_{S_o}} \right\}. \tag{6}$$

(The expression $g(I_m)$ denotes some function of the membrane-bound inhibitor concentration.) According to the first of these equations, plots of $1/v$ against $[S_o]$, the external glucose concentration, should give parallel straight lines at increasing inhibitor concentrations; according to the second equation (Eq. (6)), the lines should diverge: i.e., the plot should exhibit a noncompetitive component, $g(I_m)$. From a least squares analysis, the ratio of the slopes of the lines in Fig. 8 is 1.126 ± 0.087, while the ratio of intercepts is 3.234 ± 0.177. Thus, any noncompetitive effect is small enough to be neglected.

Finally, it may be noted that the inhibition constant for phloretin found in zero *trans* entry experiments with ghosts—9 μM—is different from that in zero *trans* exit experiments with whole cells—0.24 μM. The constant for whole cells is the same whether it is measured in isotonic saline solution or in 0.14 M sodium phosphate buffer, the medium used in experiments with ghosts. A similar discrepancy was noticed by Taverna and Langdon [26]. The zero *trans* entry experiment gives a constant, $\bar{K}_{I_o}$, which in theory [21] is equal to $K_{I_o}(1 + f_1/f_{-1})$, while zero *trans* exit gives $\tilde{K}^S_{I_o}$, equal to $K_{I_o}(1 + f_1/f_{-2})$. It was shown above that with glucose as substrate, $f_{-2} > f_{-1}$, and therefore $\tilde{K}^S_{I_o}$ should be somewhat smaller than $\bar{K}_{I_o}$; but it is unlikely that this could explain the magnitude of the experimental difference, involving a factor of 35. The cause of the discrepancy is not known.

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