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PHLORETINYL-3′-BENZYLAZIDE: A HIGH AFFINITY PROBE FOR THE SUGAR TRANSPORTER IN HUMAN ERYTHROCYTES

I. HEXOSE TRANSPORT INHIBITION AND PHOTOLABELING OF MUTAROTASE

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A new phloretin derivative, phloretinyl-3′-benzylazide (PBAz), has been synthesized and compared with phloretin for its ability to inhibit the hexose transporter in human erythrocyte membranes in subdued light. Transport measurements were made using the light scattering (Ørskov optical) method and a Millipore filtration technique with isotopically labeled sugars. Initial rates of sugar flux were measured under four different conditions to test for inhibition asymmetry. In each experimental condition, PBAz is from 6–20-times more potent than phloretin, making it one of the most effective reversible inhibitors known. Although both agents penetrate the cell membrane, they apparently fail to reach inhibitory levels at the inner surface over the time course of our nonequilibrated experiments, because of extensive binding to hemoglobin. The mechanism by which PBAz and its parent phloretin inhibit transport is pure competition with hexose for the carrier which faces the exterior of the membrane. If given time to equilibrate with the cells, the inhibition by both agents converts to a mixed type, i.e., both competitive and noncompetitive. The noncompetitive component could be due to inhibition of those transporter units oriented internally. Alternatively pre-equilibration with the inhibitors may cause them to attain high levels in the lipid membrane and produce nonspecific effects. PBAz and its precursor amine, phloretinyl-3′-benzylamine (PBA), compete with glucose for the sugar binding site on mutarotase at least as well as phloretin. When exposed to long wavelength ultraviolet radiation, PBAz is converted to a reactive intermediate which becomes covalently bound to the enzyme. Both irreversible ligand attachment and mutarotase inhibition are related to dose of the azide and irradiation time, but inactivation is from 5 to 6-times greater than label incorporation. We conclude that PBAz is a potentially useful photoaffinity labeling agent capable of covalently interacting with the transporter site facing the exterior of the red cell.

Introduction

Certain types of ligands, such as sugar analogs [1,2] and general alkylating agents (fluorodinitrobenzene and maleimides) in combination with substrate or competitive inhibitors [3–5], have been tested for their ability to covalently label the glucose transporter in cell membranes. Because of low affinity or lack of specificity for the sugar binding site, the use of these agents has some limitations. Maltosyl isothiocyanate has recently been described by Mullins and Langdon [6] as an effective affinity label, but their conclusions have not been supported by subsequent work [7,8].

Phloretin is a potent inhibitor of the hexose transporter in the human red cell and even the earliest investigators reported that its action was reversible

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Abbreviations. PBAz, phloretinyl-3′-benzylazide; PBA, phloretinyl-3′-benzylamine; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; SDS, sodium dodecyl sulfate.

and competitive [9–11]. Its K_i ranges from 0.5–2.5 μM. Although there is some current controversy about phloretin's specificity [12–14], our impression is that at these low, micromolar levels, the compound interacts primarily with high affinity binding sites on proteins, one of which is the glucose transporter, and not with lipids. We therefore set out to synthesize an azide derivative of phloretin with the expectation that it would be a suitable photoaffinity labeling agent for the sugar carrier in the human red cell membrane.

Mutarotase has been viewed as a model for the sugar carrier after some phloretin-like agents were found to have similar affinity rank as competitors for the glucose binding sites on both proteins [15]. In order to guide our synthetic work, we used the mutarotase assay as a rapid, routine means to determine inhibitory potency of the new compounds. The PBA and PBAz derivatives were found to inhibit the enzyme at least as well as phloretin and act as reversible competitive inhibitors in subdued light. We will also show that the azide can be photoactivated to form an intermediate which covalently binds and irreversible inactivates mutarotase.

Cytochalasin B has heretofore been recognized as the most effective sugar transport inhibitor in the red cell, binding exclusively at the inner membrane surface [5,16–18]. Our results suggest that PBAz is even more potent with a K_i value in the nanomolar range, and interacts reversibly with the transporter units at the exterior membrane surface.

Experimental

Materials and analyses

D-[U-^{14}C]Glucose (190 mCi/mmol) was purchased from ICN, Irvine, CA. D-[1-^{14}C]Mannitol (50.6 mCi/mmol), D-[1-^{3}H]mannitol (2.7 Ci/mmol) and 3-*O*-methyl-D-[^{3}H]glucose (80 Ci/mmol) were obtained from New England Nuclear, Boston, MA. Phloretin was either purchased (K and K Laboratories, Plainview, NY) or prepared by hydrolyzing phlorizin at 80°C in 1 M HCl; the aglycone was crystallized from ethyl acetate/benzene (1·5). Eastman Kodak, Rochester NY, supplied *para*-nitrobenzaldehyde, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) was obtained from Bio-Rad Laboratories, Richmond, CA; monosaccharides from Sigma Chemical Co., St. Louis, MO; Soluene 350 from Packard Instruments, Downers Grove, IL, and Sephadex G-10 and G-75 from Pharmacia Fine Chemicals Inc., Piscataway, NJ.

Protein was measured by the method of Lowry et al [19] using bovine serum albumin as the standard, protein in column effluents was monitored at 280 nm. Radioactivity was determined by conventional liquid-scintillation counting. TLC on Silica Gel GF plates was performed using $CHCl_3/CH_3OH$ (17·3).

Composition of buffers. buffer A, 5 mM Na_2EDTA in 15 mM NaCl, pH 7.4; buffer B, 310 mosM sodium phosphate, pH 7.4, buffer C, 27 mM glycylglycine in 150 mM NaCl, pH 7.8 at 10°C.

Syntheses

(a) Phloretinyl-3′-*p*-nitrobenzyl carbinol, (compound I, Fig. 1). Phloretin (10 mmol) and *p*-nitrobenzaldehyde (5 mmol) were dissolved in 75 ml

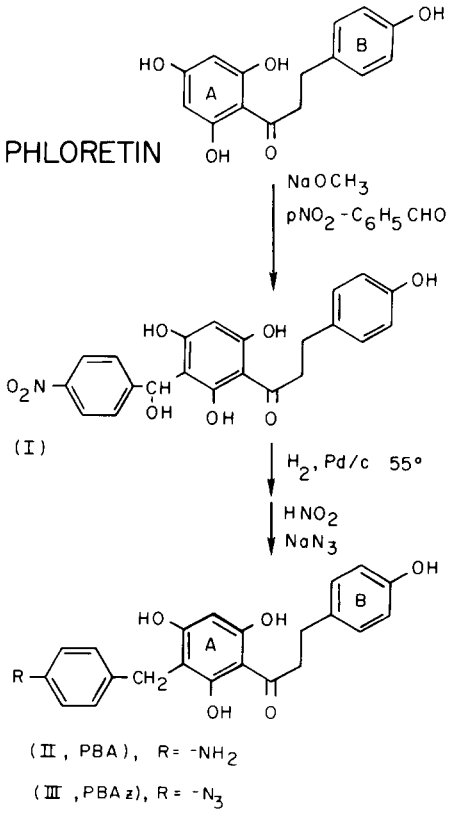

Fig. 1. Route for the synthesis of phloretin derivatives.

0.25 M sodium methoxide in methanol. After 10 min at 0°C, the mixture was acidified with 1.7 M acetic acid and poured into ice water. The dried yellow product (6.6 g) was chromatographed on a Sephadex G-10 column (5 X 16 cm) using 40% ethanol as eluant. Unreacted phloretin (2.1 g) was eluted after 0.9–1.6 L, whereas compound I was obtained in the 3–5.5 L fraction. The product crystallized as pale yellow needles (2.2 g, 46%) from methanol/water. m.p. > 240°C (dec.). R_f = 0.75 (phloretin R_f = 0.70). The product still contained a contaminant (approx. 5%) which could not be easily separated to obtain the analytical sample. It was, therefore, quantitatively acetylated (theor. – OH content, 20.0%; found, 20.6%) to form the pentaacetate which was obtained as white plates (m.p. 154–156°C) from methanol R_f = 0.94. Anal. Calcd. for $C_{32}H_{29}O_{13}N$: C, 60.47; H, 4.60; N, 2.20 Found: C, 60.6, H, 4.54; N, 2.16.

(b) Phloretinyl-3′-benzylamine (PBA, compound II). Compound I (2.4 mmol in 200 ml methanol) was catalytically hydrogenated with 10% Pd on charcoal at 55°C, 4 h at 30 cm H_2O pressure. At lower temperatures, reduction was incomplete and yielded an unstable orange product. Crude compound II was separated from the catalyst, dissolved in 160 ml benzene/methanol (7·1) and then chromatographed on a Silica Gel SC-32 column (2.5 cm X 36 cm) using benzene/methanol (5:1) as eluant. Purified amine (in the 200–360 ml eluate) was crystallized from 60% aqueous ethanol (–15°C). Yield. 0.5 g (53%) off-white crystals, m.p. 219–220°C. R_f = 0.46. Anal. Calcd. for $C_{22}H_{21}O_5N$: C, 69.65; H, 5.58; N, 3.69. Found: C, 69.6; H, 5.46; N, 3.55.

The butyramide of compound II, prepared by coupling butyric acid and the amine in methanol with EDAC, was crystallized from ethyl acetate/benzene as yellow prisms (m.p. 216°C, R_f = 0.40). Anal. Calcd. for $C_{26}H_{27}O_6N$: C, 69.5, H, 6.06; N, 3.12 Found: C, 69.5; H, 6.10; N, 2.98.

The structure proposed for compound II (and III) was confirmed by comparing the proton NMR spectra with that of phloretin. The A_2B_2 pattern at 2.82 and 3.26 ppm arising from the $-CH_2CH_2CO-$ moiety in phloretin also appears at 2.84 and 3.32 ppm in the spectra of PBA and PBAz. The new CH_2 group in both derivatives is seen as a 2H singlet at 3.85 ppm, and there is a concommitant decrease in the intensity of the singlet signal for the A ring from 2H in phloretin to 1 H in compounds II and III. The spectra show the expected AA′BB′ patterns for the *p*-substituted aromatic rings.

The Amersham Corporation, Arlington Heights, IL, incorporated tritium into PBA by their TR-7 procedure which is thought to catalytically exchange benzylic hydrogens. Radiochemically pure compound II (80 mCi/mmol) was obtained after repeated silica gel column chromatography

(c) Phloretin-3′-benzylazide (PBAz, compound III) Since compound III is photolabile, it was synthesized and isolated in subdued light. Cold 0.5 M HCl (16 mequiv.) was added slowly with magnetic stirring to 0.8 mmol compound II in 25 ml acetone (–2°C), followed by dropwise addition of 1.6 mmol $NaNO_2$ (in 2 ml H_2O). The reaction mixture was stirred for 5 min and NaN_3 (8 mmol in 10 ml cold water) was added. After 10 min, excess urea was added with stirring. PBAz was precipitated by addition of H_2O. The dried product was chromatographed on a Silica Gel 60 column, 230–400 mesh, 2.5 X 37 cm, equilibrated in MeOH/$CHCl_3$ (1:19). The azide was eluted with MeOH/$CHCl_3$ (1:9) in the 0.3–1.0 L fraction and was recrystallized from aqueous methanol as pale yellow needles, m.p. 155–157°C, R_f = 0.62. Infra-red spectra had the sharp 2120 cm^{-1} and 1290 cm^{-1} absorption peaks, characteristic of the azide group. Radiochemically pure [^{3}H]PBAz, prepared from the tritiated PBA, was isolated by these same procedures.

Ghost preparation

Human blood was either freshly drawn or obtained from local blood banks and used within two weeks. Ghosts were prepared by the method of Dodge et al. [20] and resealed as described by Mueller and Morrison [21]. Concentrations of erythrocytes and ghosts were estimated from hematocrits, hemocytometer counts or by use of a Coulter counter.

Sugar transport assays

Carrier activity was assessed either by measuring radioactively labeled sugar flux or by an analysis of red cell turbidity changes caused by transport-dependent osmotic effects. An advantage of the latter method is that low cell densities can be used to minimize nonspecific inhibitor binding. All incubations were performed under subdued light in buffer B unless otherwise indicated.

(a) Influx and efflux by the Ørskov technique [22]

Red cell volume changes were monitored at 700 nm with an Aminco DW-2a Recording Spectrophotometer. The optical-cell compartment was adapted to hold a small stirring paddle connected to the shaft of a variable speed motor through a flexible cable to keep erythrocytes suspended in the cuvette. Addition of cells or substrates was made through a permanently attached, light-tight needle adapter.

Zero-trans uptake of galactose. A cell suspension (3 6 ml; 0.1% Ht) was preincubated for 10 min at 20°C in the presence or absence of inhibitor. At zero time, D-galactose was added as a concentrated stock solution to given a final concentration of 20, 40, 80 or 240 mM. Cell shrinkage due to water efflux lasted a few seconds, thereafter, a linear sugar uptake rate (mM/min) was determined from the first 20% of the continuous tracing of absorbance decrease. The absorbance scale was calibrated to known changes of intracellular galactose concentrations occurring between zero time and equilibrium.

Zero-trans efflux of galactose. Erythrocyte suspensions (5% Ht) were equilibrated with varying concentrations of D-galactose for 30 min at 37°C. The cells were then centrifuged and supernatant was removed to yield a 40% Ht. Efflux was initiated by the rapid addition of 10 μl of these cells into 4 ml of buffer B at 20°C containing varying amounts of inhibitor plus mannitol at a concentration matching that of galactose used for preloading. Mannitol prevented abrupt cell swelling and intracellular galactose dilution due to osmotic differences. Sugar efflux rate (exit time) was determined from the record of absorbance increase as described by Sen and Widdas [23]

Variable-trans glucose efflux. Sen-Widdas exit experiments were carried out as in zero-*trans* efflux (above) except that the cells were preincubated to contain 100 mM D-glucose, and efflux buffer B contained variable levels of glucose, with or without inhibitor, but no mannitol.

(b) Isotope flux studies

Zero-trans [^{14}C]glucose uptake. Our method represents a consolidation of those used by others [24–26] To a magnetically stirred suspension of either red cells (3% Ht) or ghosts (7.5% Ht) at 20°C, [^{14}C]glucose solutions (containing inhibitor when indicated) were added such that the final 1.2 ml volume contained from 1 to 25 mM labeled hexose. Transport was terminated at selected intervals by rapidly mixing 0.1-ml aliquots of the suspension with 0.9 ml of ice-cold stop solution (2 mM $HgCl_2$ and 1.25 mM KI in 342 mM NaCl) in an Eppendorf tube; in the ghost experiments, the stop also contained 0.1 mM phloretin. Tubes were centrifuged, the supernatants were removed and spun again for 2–3 s to collect any residual liquid. Radioactive glucose associated with packed ghosts was extracted with 50% ethanol, whereas with erythrocytes, an equal volume of 12.5% trichloroacetic acid was used In parallel experiments, extracellular volume in cell pellets was determined with tritiated mannitol.

Preliminary tests indicated that by the time the third incubation sample could be taken (9 s), glucose concentration in ghosts had already attained about 45% of the equilibrium value and uptake was no longer linear. Therefore, to approximate initial rate, data were subjected to a computerized nonlinear curve-fitting procedure in which an arbitrary point near the origin was included to account for the condition that the initial concentration of glucose in the cell was zero. Initial rate values were calculated as the intracellular glucose attained in mM/s.

Equilibrium exchange of 3-O-methylglucose. Freshly drawn red cells, washed four times in buffer C, were equilibrated for 1 h (20°C) with 3.5, 7 or 21 mM 3-*O*-methyl-[^{3}H]glucose in buffer C. Each cell suspension was then centrifuged twice for 5 min at 3000 × *g* and all possible supernatant was removed A 20-μl aliquot of these packed loaded cells was rapidly delivered to 10 ml of magnetically stirred buffer C (10°C) containing the same unlabeled 3-*O*-methylglucose concentration, with or without inhibitors added as ethanol solutions (final ethanol concentration, 0.5%). Consecutive samples were withdrawn at about 7-s intervals using a Swinnex filter assembly with a 3-μm SS filter and AP prefilter (Millipore Corporation) attached to a 10-ml plastic syringe. Each cell-free filtrate was assayed for the appearance of 3-*O*-methyl-[^{3}H]glucose to determine the initial rate of carrier efflux.

Noncovalent binding of phloretin and derivatives to erythrocytes, ghosts and hemoglobin. At the outset of the transport studies, we noticed that the concentration of the phloretin derivatives in the incubation medium fell sharply after the addition of red

cells or ghosts. Since free inhibitor concentration must be known in order to estimate its K_i, concentration was determined after the final sample was taken in the experiments. Cells or ghosts were centrifuged and inhibitor binding was assessed as loss from the supernatant of either radioactivity or ultraviolet absorbance. The spectrophotometric assay of unlabeled agents involved dilution of an aliquot with Tris-HCl or borate buffer, pH 9.0–9.3; at this pH, the absorbance maxima of the compounds are shifted to higher wavelengths [27] where interference from other materials is minimized When supernatants contained traces of hemoglobin, its contribution at 325–330 nm was found by multiplying the absorbance at 578 nm by the A_{230}/A_{578} ratio for hemoglobin; the polyphenolic compounds do not absorb at 578 nm. Their molar extinction coefficients (ϵ in cm^{-1} M^{-1}) and absorbance maxima in 0.025 M $Na_2B_4O_7$ are: phloretin, 25 500 at 325 nm; PBA, 21 000 at 332 nm; PBAz, 19 000 at 250 nm and 21 000 at 332 nm.

Binding of ligands to hemoglobin was assessed by the chromatographic method of Hummel and Dreyer [28]. A 20 000 × g supernatant of lysed erythrocytes (approx 15 mg Hb) was made to contain 50 μM ligand in buffer B. This sample was added to a Sephadex G-75 column (1.5 × 25 cm) equilibrated in 50 μM ligand in buffer B, which also served as eluting solution. Absorbance in the eluate was monitored at 285 nm and the quantity of each agent bound to protein was assessed from the area of the depletion trough in the elution profile.

Mutarotase inhibition. The mutarotase used in these experiments was obtained from bovine kidney cortex and was partially purified by affinity chromatography [29]; it was about 30% pure based upon densitometry scans of polyacrylamide gels stained with Coomassie Blue G. An automatic-recording polarimeter was used to measure activity [30,31], a unit (U) being the amount of enzyme in buffer A at 20°C that will convert 0.255 μmol α-D-glucose (at 25 mM) to the β-anomer in 1 min. All procedures were done in subdued light and substrate was used at 5, 10, 20, 40 and 100 mM in buffer A.

Photoinactivation of mutarotase with PBAz. In a typical experiment, 400 U of the enzyme (150 μg protein) and variable amounts of [^{3}H]PBAz (added in 50% ethanol) were mixed in a 30 ml beaker in subdued light. The final volume (5 ml buffer A) was never greater than 4% in ethanol, including the controls without azide. At zero time, the beaker at 20°C in a Dubnoff shaking incubator was irradiated by a Sylvania Sunlamp (120 V, 250 W) positioned 15 cm above the solution surface. At 0, 7, 14 and 21 min, a 1-ml aliquot was taken for the determination of mutarotase activity or covalent inhibitor binding to the protein In preliminary tests under these conditions, a significant amount of the azide was found to be photolyzed within 5 min (loss of its 250 nm absorbance peak) to a mixture of products. Therefore, after the 7 and 14 min samples were removed, additional [^{3}H]PBAz was added to restore the inhibitor concentration to pre-illumination levels.

After irradiation, any irreversible inhibition of mutarotase was assessed by first removing noncovalently bound residual PBAz and inhibitory photolytic products. Aliquots (1 ml) of the incubation were washed with buffer A through Sephadex G-50 columns (0.5 × 50 cm) having an overlying 4-cm layer of Sephadex G-10. The G-10 bound PBAz (and PBA) even more avidly than phloretin [27], whereas the G-50 separated the protein from most of the ^{3}H-labeled photolytic products which escaped G-10 binding. Mutarotase was quantitatively recovered in a 6-ml fraction after the void volume; the entire fraction was diluted to 20 ml with buffer A and assayed for activity with 25 mM α-D-glucose. In control experiments, PBAz and the enzyme were irradiated separately and mixed immediately before the column fractionation

To determine the extent of covalent labeling of the enzyme, aliquots from the incubation were processed through Sephadex columns equilibrated in water. The protein-containing eluate was either analyzed directly or electrophoretically concentrated in an ISCO Electrophoretic Sample Concentrator (Instrumentation Specialities Co., Lincoln, NB) and duplicate samples were subjected to SDS-polyacrylamide gel electrophoresis (8.5% gel rods) according to the procedure described by Fairbanks et al. [32]. One gel rod was stained with Coomassie blue G and analyzed with an ISCO gel scanner at 590 nm; the duplicate was sliced into 1-mm disks which were treated with Soluene 350 and then assayed for tritium by the method of Ward et al. [33]

Results

Noncovalent binding of ligands to cells, ghosts and hemoglobin

Phloretin's extensive binding to the erythrocyte [34] was shown by Jennings and Solomon [35] to be primarily due to its unlimited association with hemoglobin; about 10% was bound to the cell membrane which possesses both saturable and nonsaturable binding sites Since PBA and PBAz are more hydrophobic than phloretin, we expected even greater nonspecific binding of these agents. At a red cell density of 0.1% in buffer B at 20°C (conditions of the Ørskov procedure), the bound fraction of each inhibitor, mean ±S.D. was: phloretin, 0.13 ± 0.03; PBA, 0.28 ± 0 003; PBAz, 0.69 ± 0.10. The amount bound was a linear function of the nominal concentration over the range tested, namely 1–20 μM for phloretin and PBA, and 0.1–4 μM for PBAz. The presence of 100 mM D-glucose had no effect on this nonspecific binding.

With hemoglobin-free ghosts (0 1% Ht), less than 2% loss of phloretin from the medium occurred, whereas at 10 μM PBA, as much as 15% binding was found. About 50% of PBAz was bound to 0 1% ghosts at 1–2 μM. Whereas all three agents required about 1.5–2 min to reach an equilibrium binding to intact cells, their interaction with ghosts was complete at the earliest sampling time (5 s). Our findings resemble those of Jennings and Solomon [35]; not only is the binding of phloretin and its analogs to the intact cell greater than to the ghost, but it is also time dependent. Apparently, the compounds interact rapidly with the membrane, penetrate it and then undergo an association with hemoglobin which serves as a binding sink

Direct measurement of phloretin and PBA binding to hemoglobin supports this idea Although the agents were tested at only one concentration, the results of our Hummel and Dreyer chromatography showed that PBA interacts at least 10-times more extensively than phloretin (the elutogram data are not presented); ligand/hemoglobin molar binding ratios were 3.4 and 0.28, respectively. For phloretin, this represents the equivalent of $1.4 \cdot 10^6$ molecules bound to the hemoglobin in one erythrocyte, which is within the range reported by Jennings and Solomon if appropriate corrections are made for pH differences and ligand concentration. Even though the PBAz interaction was not quantitated, its greater binding to the intact cell than ghost suggests that it also is extensively bound to hemoglobin. Furthermore, in preliminary photolabeling experiments of intact red cells, with [^{3}H]PBAz, a high degree of irreversible tritium binding to hemoglobin was found

Potency of PBA and PBAz as sugar transport inhibitors.

(a) Zero-trans galactose and glucose uptake. PBAz is a powerful inhibitor of the influx of galactose into the intact red cell, as measured by the photometric method (Fig. 2). Its K_i based on the concentration remaining free in the medium, is 0.077 μM, making

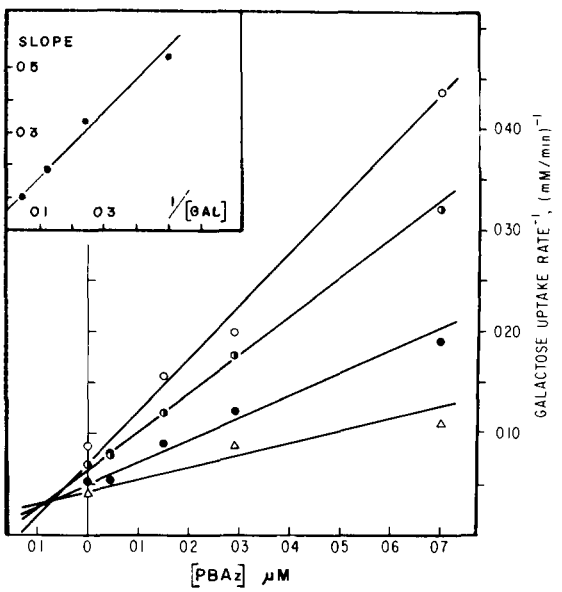

Fig 2. Dixon plot showing PBAz inhibition of zero-*trans* galactose uptake by intact erythrocytes in the dark. Cells at a hematocrit of 0 1% were preincubated in the presence of varying amounts of inhibitor. The uptake reaction was started by the addition of sugar so that final galactose concentration was (○) 20, (◑) 40, (●) 80, or (△) 240 mM Points represent the mean values of 3–7 determinations of initial uptake rate at 20°C by the Ørskov method PBAz concentration has been corrected for 69% binding under these conditions. K_i = 77 nM, calculated from the mean of the intersection points. The inset panel shows the slopes of these lines plotted against the inverse of the galactose concentration. This secondary plot indicates that the inhibition is of a mixed type

it at least 20-times as effective as phloretin, whose K_i was confirmed to be about 2 μM (Fig. 3) These data in the form of Dixon plots indicate that the inhibitors act either as competitive or as mixed inhibitors. Secondary plots, in which the slopes of the Dixon lines are plotted against the inverse of the galactose concentration, distinguish between these two types of inhibition; a straight line through the origin should be obtained for strictly competitive inhibition. Our results show rather that the inhibition is mixed for both compounds. In these Ørskov experiments, erythrocytes were preincubated with the inhibitors which equilibrated in the cell interior. When experiments were performed in which zero-trans uptake of [^{14}C]glucose was measured, no pre-equilibration period was allowed. Results (not shown) obtained under these conditions indicated that phloretin and PBA (PBAz was not tested) acted as pure competitive inhibitors, both agents having K_i values of about 2 μM. In a limited number of similar experiments using ghosts with no preincubation period, phloretin, PBA, and PBAz were all competitive inhibitors with K_i (±S.D.) values of 1.05 ± 0.07, 1.09 ± 0.11 and about 0.3 μM, respectively. The K_m (2.5 mM) and V (1.0 mM/s) values derived from Hofstee plots of control glucose influx data (not illustrated) were within the range of published values [24,36,37]

(b) Variable-trans glucose efflux. In this Sen-Widdas exit experiment (Fig. 4), cell shrinkage due to glucose efflux under saturated carrier conditions is monitored in relation to variable amounts of glucose in the efflux medium. Externally added sugar acts as an inhibitor of the osmotic change by interacting with the exterior face of the transporter. The half-saturation constant for glucose at the outer surface was confirmed to be about 4 mM [23,38]; exit time under these conditions was 0.7 min. Both of these parameters are increased equally by phloretin and PBAz, indicating that they act at the outward-facing transporter competitively with glucose. The apparent K_i for phloretin, 0.87 μM, is in the range found by others [17,34,35,38,39], while the azide is much more potent with a K_i of 0.07 μM.

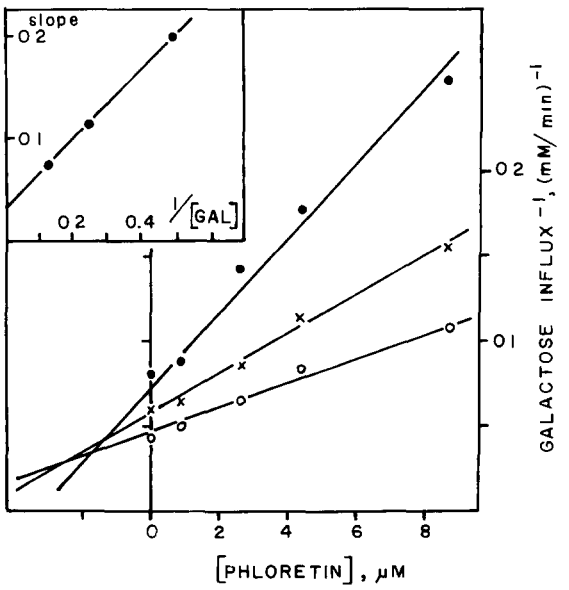

Fig. 3. Dixon plot showing phloretin inhibition of zero-*trans* galactose uptake. Conditions of these experiments are as indicated in Fig. 2. Galactose concentrations: (•) 20, (X) 40, and (○) 80 mM. Phloretin concentration has been corrected for 13% binding. K_i = 1.8 μM.

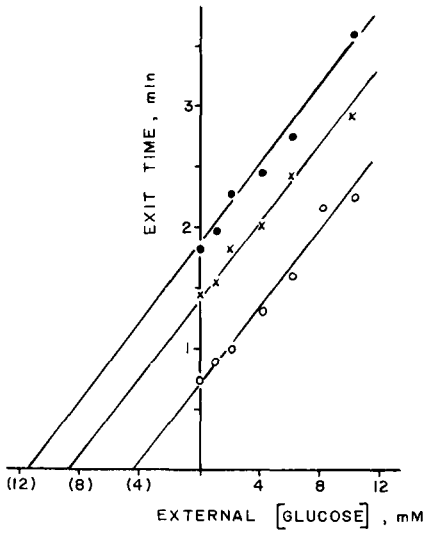

Fig 4. Inhibition of D-glucose transport by phloretin and PBAz in a variable *trans* (Sen-Widdas) experiment conducted in the dark. Initial rate of net glucose loss from erythrocytes preloaded at 100 mM was determined by the Ørskov procedure and is expressed as exit time, in min. The incubation medium contained varying levels of D-glucose with no inhibitor (○) or with phloretin (X) at 0.87 μM or PBAz (•) at 0.087 μM (final free concentrations). Both inhibitors act competitively and PBAz is more than 10-times as potent as phloretin Each point represents the mean of 3–8 observations.

(c) Zero-trans galactose efflux. Whereas phloretin and PBAz compete with hexose entering the cell, they behave as noncompetitive inhibitors of galactose efflux. Efflux data, presented as a Hunter-Downs plot in Fig. 5, indicate that the level of galactose in the pre-loaded cells has no effect on the fractional inhibition (i) caused by a given inhibitor concentration (I). In this plot, a horizontal line indicates noncompetitive blockade, and K_i values are obtained directly from the intercept on the ordinate, 0.27 μM for phloretin and 0.04 μM for the azide. Since initial efflux was estimated when presumably neither inhibitor had attained a significant concentration in the cell interior, hexose interacting with the carrier's cytoplasmic face does not displace externally bound inhibitors. We also found noncompetitive inhibition for phloretin and its azide on the zero-*trans* efflux of variable amounts of 3-*O*-methyl-[^{3}H]glucose in a series of experiments to be published elsewhere.

(d) Equilibrium exchange of 3-O-methylglucose. Entry and exit experiments conducted under zero-*trans* conditions will yield information about the side of the membrane to which an inhibitor binds. However, an equilibrium exchange experiment is the only unambiguous test to establish a competitive (vs. non- or uncompetitive) mechanism. We therefore determined the effect of phloretin and PBAz on the exchange flux of nonmetabolizable 3-*O*-methyl-[^{3}H]glucose. A Dixon plot of the exchange rates found at two nominal azide concentrations is displayed in Fig. 6. Its K_i value, corrected for nonspecific azide binding, is 0.12 μM. The results clearly show PBAz to be a powerful and strictly competitive inhibitor of the transporter. Under identical conditions, phloretin also behaved as a purely competitive inhibitor (data not shown) but had a much higher K_i value (1.5 μM).

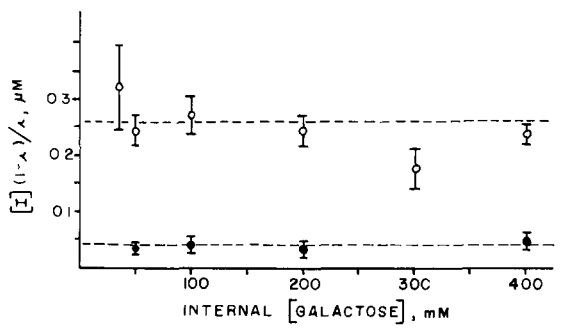

Fig 5. Inhibition of zero-*trans* D-galactose efflux. Erythrocytes were equilibrated with galactose at concentrations of 35–400 mM. These loaded cells were then rapidly discharged into a galactose-free medium and cell shrinkage due to hexose efflux was measured by the light-scattering technique. Phloretin (○) was tested at 0.2, 0.4, 1.0 and 2.0 μM; PBAz (●) was 0.02, 0.045, 0.095 and 0.15 μM. Neither inhibitor was preincubated with cells. [I] represents the concentration of inhibitor and i is the fractional inhibition of flux and is equal to $1-(v_i/v)$. A straight line with no slope in this Hunter-Downs method of plotting indicates that the inhibition is noncompetitive. The intercepts on the ordinate (0.27 and 0.04 μM) are the respective K_i values for phloretin and PBAz and are based on the final free concentration in the incubation. Fractional inhibition values ±S.D. were calculated from initial efflux rates which were the means of 3–11 observations at each galactose concentration.

Phloretin and derivatives as mutarotase inhibitors.

Results of mutarotase inhibition experiments per-

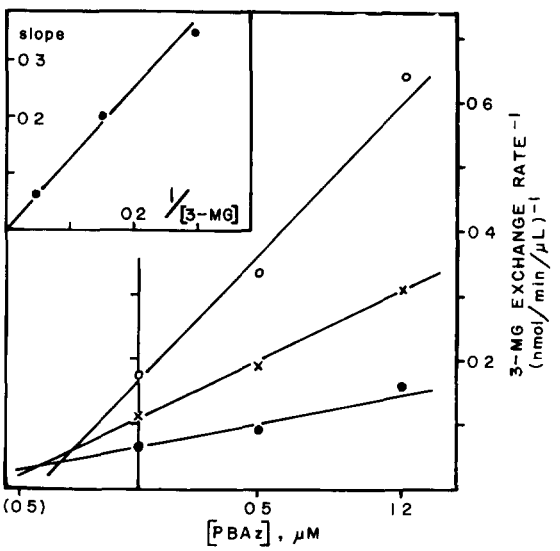

Fig 6. Effect of PBAz on the equilibrium exchange of 3-*O*-methylglucose. Cells were preloaded with tritiated 3-*O*-methylglucose at 3.5 (○), 7 (X) or 21 mM (●). The exchange rate with unlabeled sugar at equal outside concentrations was measured when 0, 0.5 or 1.2 μM PBAz was initially present in the efflux medium. However, 60–65% of the azide at each concentration is nonspecifically bound under these experimental conditions. The K_i value, based on final free concentration is 0.12 μM. The inset is a secondary plot which shows that the inhibition is strictly competitive. The K_m of 3-*O*-methylglucose was 10 mM and V was 22.2 nmol/min per μl cells. 3-MG, 3-*O*-methylglucose.

formed in subdued light were analyzed as Dixon plots and indicated that PBA and PBAz (see Fig. 7) are similar to phloretin in potency. Secondary plots showed that all three agents acted strictly competitively having K_i values of 24, 20 and 26 μM, respectively.

Photoinactivation of mutarotase with [^{3}H]PBAz.

Exposure of mutarotase to long wavelength ultraviolet radiation in the presence of [^{3}H]PBAz caused a dose/time related inactivation of the enzyme which could not be reversed by Sephadex G-10/G-50 chromatography. To prepare for this study, several types of control experiments were performed (in subdued light, unless indicated otherwise) in order to establish the effectiveness of the Sephadex column fractionation.

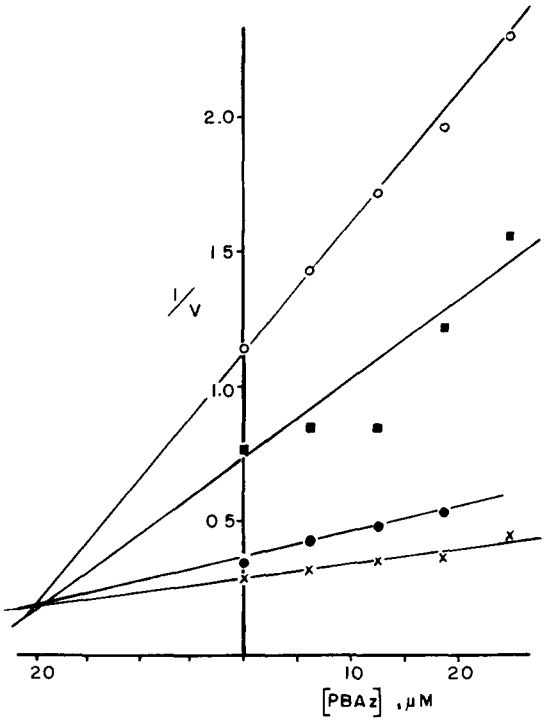

Fig. 7. Inhibition of mutarotase by PBAz. The inhibitor was tested in subdued light at 6.25, 12.5, 18.75 and 25 μM with α-D-glucose at 5 (○), 10 (■), 20 (●) and 40 (X) mM. V was measured as chart units/min per mM glucose [31]. The apparent K_i of PBAz is 20 μM and from the secondary plot of these data (not shown), the inhibition is strictly competitive. Phloretin and PBA were tested under similar conditions.

(1) Mutarotase, in buffer A with or without azide, was recovered quantitatively and fully active in the 6-ml eluate following the void volume of the mixed-bed Sephadex column.

(2) Unphotolyzed [^{3}H]PBAz, present in an aliquot applied to the column at the highest level tested (40 μM), was quantitatively absorbed in the presence or absence of mutarotase.

(3) An azide blank at 30 μM (no mutarotase initially) was irradiated according to the program described in Experimental. A 1-ml aliquot taken at the 7, 14 or 21 min sampling times was rapidly mixed with 200 U mutarotase in the dark and immediately applied to a Sephadex column. Elution of the enzyme was carried out as described, and recovery of activity in the 6-ml eluate was found to be 100 ± 5% at each time period. Less than 0.2% of the total tritium originally present appeared in the eluate containing mutarotase. Subsequent buffer wash removed only an additional 4% from the column. These traces of radioactivity were not identified

Irreversible inhibition of mutarotase occurred when the enzyme was irradiated in the presence of 30 μM PBAz; 7, 16 and 20% of enzyme activity could not be recovered from the Sephadex columns at the three time periods, whereas a progressive loss of only 2, 4.5 and 6% was found in the absence of the azide. The result indicated that either the azide had catalyzed the photodestruction of the enzyme or had formed a ligand which covalently interacted and irreversibly inhibited it. In order to distinguish these two possibilities, we performed the same experiments with [^{3}H]PBAz except that the protein from the Sephadex column was subjected to gel electrophoresis. Fig. 8 displays the results of one of these photolysis experiments in which the densitometric tracing of the gel is superimposed on a bar graph of the radioactivity distributed throughout the rod. The data are from a sample taken after a 14-min irradiation and shows three major peaks of radioactivity, one remains at the origin of the gel rod. It represents either polymeric photogenerated material (it is present in azide blanks as well, but to a lesser extent) and/or is associated with denatured enzyme or protein which does not penetrate the gel. A second peak, also appearing in azide blanks, probably represents highly charged photolytic products which migrate only slightly slower than the tracking dye, there is little Coomassie

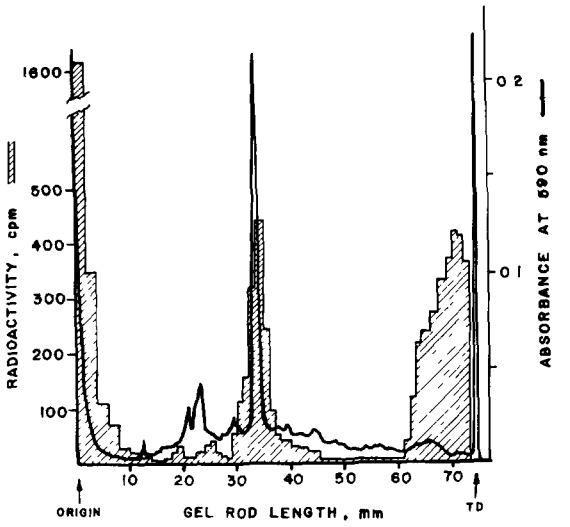

Fig 8 SDS-polyacrylamide gel electrophoresis of mutarotase photolabelled with [^{3}H]PBAz. Experimental details are described in the text The electrophoresis was carried out until the tracking dye (TD) migrated 75 mm The gel rod was sliced into 1 (or 2)-mm disks which were analyzed for tritium. The bar graph represents the difference in radioactivity distribution from a sample taken after 14 min irradiation and an azide blank (no enzyme) treated identically A duplicate gel rod containing a mutarotase blank (14 min irradiated enzyme, no PBAz, fractionated on Sephadex) and processed under identical conditions, was then stained with Coomassie blue and scanned with a densitometer. The superimposed absorbance plot at 590 nm is a photographic enlargement of the densitometry tracing of the stained gel. Mutarotase migrates 33–35 mm.

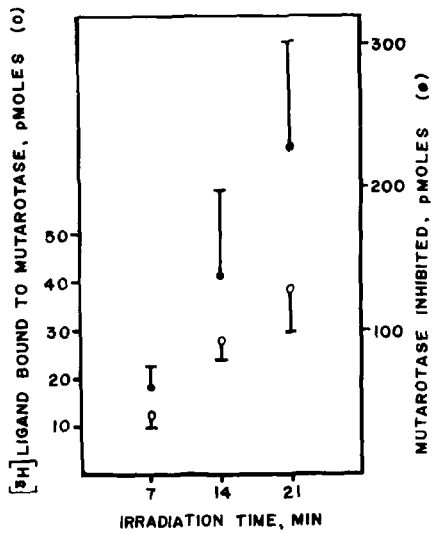

Fig 9. Lack of correlation between photoinactivation and covalent ligand binding of mutarotase by [^{3}H]PBAz. Experimental details of the experiment are given in the text. Both the irreversible ligand attachment (○) and mutarotase inhibition (●) are proportional to the irradiation time (and PBAz dose) but inactivation is 5–6-times greater than ligand binding at each time period. A total of 150 nmol [^{3}H]PBAz was present at zero time. Immediately after taking the 7 and 14 min samples, the incubation medium was recharged with 100 and then 70 nmol, respectively. The points represent the means ±S.D. of three experiments in which the total mutarotase originally present was about 1200 pmol (150 μg protein, 30% mutarotase, M_r = 37 kdalton). The means represent the difference between the experimental and respective blank values

blue staining material associated with this radioactivity Of greatest interest is the third tritium-containing area. It migrates with the major protein component of the enzyme preparation previously shown to be mutarotase [29].

The data summarized in Fig. 9 are from a series of photoinactivation experiments in which samples taken at the three irradiation periods were analyzed for irreversible mutarotase inactivation as well as for covalent [^{3}H]-labeled ligand binding by the electrophoretic procedure. A time (dose) dependent loss of enzyme activity occurs reaching about 20–25% after 21 min irradiation but the inactivation cannot be correlated stoichiometrically with the degree of [^{3}H]-labeled ligand incorporation. After each irradiation period, inhibition is from 5 to 6-times greater than that attributable to irreversible ligand binding. It appears that some inactivation is catalyzed by a photogenerated azide intermediate which fails to covalently bind. Based upon the amount of ligand present in the incubation medium at zero time (150 μmol), the efficiency of label incorporation during the first irradiation period was extremely low, less than 0.01%.

Discussion

Mutarotase as a model system

It was not surprising that the degree of irreversible mutarotase inactivation and the extent of covalent labeling of the enzyme were unequal. The efficiency of photolabeling a soluble protein at the temperature we used with an agent having a low apparent affinity (K_I about 20 μM) is predictably low. Furthermore, our experimental design was not

optimized to detect a stoichiometric interaction even if it could occur. For example, the azide concentration, only slightly greater than its K_i, varied during the experiment, requiring us to spike the incubation with fresh reagent after each time period. Finally, only the covalently bound tritium migrating with mutarotase to the middle of the gel rod was used to make the correlation shown in Fig. 9. The amount of protein-bound tritium remaining at the origin was not determined. Much of this could represent labeled, inactivated enzyme which aggregated during the SDS-mercaptoethanol heat treatment of the sample prior to electrophoresis. In any case, it was not the purpose of this study to address these points more extensively than we have. The mutarotase model was used to determine whether PBAz can be photoactivated and form an intermediate (nitrene?) which would covalently interact with the active center of the enzyme. Our results are clearly consistent with this view and encourage us to examine the reaction as it relates to the photolabeling of the sugar transporter in the erythrocyte membrane.

A final remark should be made in relation to the use of mutarotase as a model system. All of the agents tested inhibit the enzyme in a strictly competitive manner and are 5–10-times more potent than the parent glycoside, phlorizin, which also acts competitively. We have previously discussed how aglycones may react more effectively than a ligand possessing a glucose moiety [15]; competitive kinetics cannot rule out reciprocal allosteric interaction as the mechanism for the inhibition by an aglycone. The same arguments apply to the mechanism of transport inhibition. In any event, our earlier impression that the receptor site on mutarotase may resemble that of the glucose transporter in the red cell membrane is not supported by our current findings. Not only are all of these agents over 10-times as potent as carrier inhibitors, but there is also a discrepancy in their relative activities. PBAz, for example, is up to 20-fold more powerful than phloretin as a transport poison, while the two are about equipotent as mutarotase inhibitors.

The results of all the transport experiments using intact red cells are summarized in Table I for easy comparison. We have estimated kinetic parameters which are within the range previously reported by others working at our conditions (20–25°C, pH 7.4–

TABLE I

SUMMARY OF THE TRANSPORT INHIBITION STUDIES

All apparent K_i values cited are based on inhibitor concentrations that have been corrected for nonspecific binding. The equilibrium exchange study was the only series performed at 10°C (instead of 20–22°C).

Type of erythrocyte transport experiment	K_t^i or K_t^o (mM)	Maximum rate (mM/min)	Type of inhibition and apparent K_i (μM)	
			Phloretin	PBAz
Zero-*trans* influx				
galactose	18	31	Mixed	
			1.8	0.08
glucose	3.7	32	Competitive	
			2.0	–
Zero-*trans* efflux				
galactose	53	146	Noncompetitive	
			0.27	0.04
Sen-Widdas exit				
glucose	4	143	Competitive	
			0.87	0.07
Equilibrium exchange				
3-methoxyglucose	10	20	Competitive	
			1.5	0.12

7.8, 0.1–0.2% Ht). For example, the half-saturation constant, K_t^o, for D-galactose reacting with the outside transporter is about 5-times larger than that for D-glucose (K_t = about 4 mM) as estimated from either zero-*trans* influx or Sen-Widdas exit experiments [34,36,38–41] Furthermore, the maximum influx rate for both galactose and glucose were, as expected, identical even though this parameter was determined by entirely different methods (optical and isotopic flux measurements)

Most of the results compiled in Table I are best explained by and support the idea that the carrier system in the red cell is asymmetric ([42,43], and see Ref. 44 for a review) and that the inhibitors act only on the transporter units facing the outside. Findings consistent with this model include. (1) K_t and V for zero-*trans* entry are lower than the respective values for zero-*trans* exit, (2) inhibition kinetics were strictly competitive for both phloretin and PBAz in the influx and the Sen-Widdas exit experiments (not pre-equilibrated with inhibitors), (3) both agents acted noncompetitively and exerted their greatest effect on the sugar efflux process as compared to influx, and (4) the ligand concentrations required to decrease the equilibrium exchange rate are greater than those needed in the Sen-Widdas exit study, a final characteristic expected of an outside inhibitor acting on an asymmetric system (see Ref. 17)

Our kinetic analysis of the equilibrium exchange inhibition caused by phloretin and PBAz meets the criterion proposed by Devés and Krupka [45] to establish a strictly competitive mechanism of action. From their kinetic survey, these authors also concluded that sidedness of the inhibition can be discriminated from results of zero-*trans* influx and efflux studies Basketter and Widdas [17] argued that experiments of the Sen-Widdas type will also identify inhibitors that act only on the outside of the membrane. Under either of these conditions, our results indicate that when phloretin and PBAz are initially present only at the cell exterior, the mechanism by which they inhibit sugar flux is strictly a reversible and competitive binding to those carrier units oriented externally. This is not to say that they cannot act on the cytoplasmic side of the system, if a significant free concentration is attained intracellularly. Phloretin has been previously described as an effective bilateral inhibitor of sugar flux [17,37], and our results in the zero-*trans* galactose influx work, in which cells were pre-equilibrated with the inhibitors, showed that the inhibition was of the mixed type. This suggests that both conformations of the transporter were blocked, the one on the inside being affected noncompetitively because internal sugar concentration was initially zero. Alternatively, pre-equilibration with the agents may have allowed them to reach high levels in the lipid membrane matrix and influence other parameters [12] including nonspecific inhibitory effects on transport of compounds other than hexoses [46,47].

Since Jennings and Solomon [35] have clearly demonstrated that phloretin penetrates the red cell membrane, and we have results indicating that the azide is even more membrane-permeable, how can our studies with cells not pre-equilibrated with inhibitors, give purely competitive (or noncompetitive) inhibition kinetics? Apparently, appreciable internal concentrations of inhibitor were not attained during the linear phase of the uptake process, possibly due to binding to hemoglobin which has a high and unsaturable affinity for the ligands. This may be a major reason why others have stated that phloretin does not react with the carrier on the inner surface of the membrane

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