Barbiturate Inhibition of GLUT-1 Mediated Hexose Transport in Human Erythrocytes Exhibits Substrate Dependence for Equilibrium Exchange but Not Unidirectional Sugar Flux†

Aida El-Barbary, Joseph D. Fenstermacher, and Howard C. Haspel*

Anesthesia Research, Department of Anesthesiology, Henry Ford Health System, Detroit, Michigan 48202-3450

Received August 14, 1996; Revised Manuscript Received October 3, 1996⊗

ABSTRACT: Barbiturates inhibit GLUT-1 mediated hexose transport both *in vivo* [Gjedde & Rasmussen (1980) *J. Neurochem. 35*, 1382−1387; Otsuka *et al.* (1991) *Am. J. Physiol. 261*, R265−R275] and *in vitro* [Honkanen *et al.* (1995) *Biochemistry 34*, 535−544]. In the present study, the mechanism by which barbiturates inhibit GLUT-1 mediated hexose transport was examined by measuring both unidirectional zero trans and equilibrium exchange fluxes of hexoses in the functionally well-characterized, GLUT-1 rich human erythrocyte system. Unidirectional influx and efflux were both inhibited (>80%) by 10 mM pentobarbital (PB). This symmetrical inhibition of unidirectional flux by PB was virtually independent of cis sugar concentration (2−130 mM) and exhibited an IC_{50} of ∼2 mM. In contrast to unidirectional sugar flux, PB inhibition of equilibrium exchange sugar flux is *attenuated* by increased substrate concentration (e.g., 88% inhibition at 1 mM Glc versus 40% inhibition at 130 mM Glc in the presence of 10 mM PB) and exhibits an IC_{50} of ∼10 mM at 100 mM Glc. Other barbiturates were found to inhibit sugar flux in human erythrocytes in this differential manner. These findings, when viewed with kinetic models proposed for GLUT-1 mediated transport [Carruthers (1990) *Physiol. Rev. 70*, 1135−1176], are consistent with barbiturates being *noncompetitive* inhibitors of Glc translocation and preferentially inhibiting the *unoccupied* form of the carrier protein. We propose, therefore, that barbiturates may prevent or alter the conformational changes associated with the reorientation of the carrier protein within the membrane. Overall, these results imply that barbiturates may more strongly inhibit GLUT-1 mediated Glc flux *in vivo* when the trans Glc is near zero as a result of either metabolism or another transport process.

Previous observations indicate that barbiturates suppress glucose (Glc)[1] transfer across the blood−brain barrier (BBB) in rats (Gjedde & Rasmussen, 1980; Otsuka *et al.*, 1991). The predominant facilitative hexose transporter of the BBB *in vivo* is GLUT-1 [for a recent review, see Maher *et al.* (1994)], and this transporter has been proposed to be a likely target of barbiturates at the BBB (Fenstermacher *et al.*, 1995) and possibly at other cells that express GLUT-1. In support of this suggestion, Honkanen *et al.* (1995) have recently demonstrated that barbiturates inhibit hexose transport in cultured mammalian cells and human erythrocytes, and interact directly with purified GLUT-1.

Both the *in vivo* (Gjedde & Rasmussen, 1980; Otsuka *et al.*, 1991) and *in vitro* (Honkanen et al., 1995) findings report that the barbiturates do not affect the affinity of Glc for GLUT-1 and thereby imply that they act to alter the Glc translocation process mediated by GLUT-1. Kinetic considerations of the system suggest that this alteration could change either the transporter when occupied by substrate and/or the unoccupied carrier and thereby reduce Glc flux. In the present study, the hypothesis that barbiturates inhibit GLUT-1 function by interacting with the occupied and/or unoccupied GLUT-1 transporter is offered and tested. The experiments involve the measurement of both unidirectional zero trans and equilibrium exchange fluxes of radiolabeled hexoses in the functionally well-characterized, GLUT-1 rich human erythrocyte system.

EXPERIMENTAL PROCEDURES

Theory and Interpretation of the Results. The results will be discussed using the single-site alternating conformation model of the GLUT-1 mediated hexose transport system, which was originally presented in a slightly modified form as the "mobile carrier model" by Widdas (1952) and is illustrated by a simple King−Altman kinetic representation (Figure 1). For detailed discussions of the intricacies of the interpretation of this model in relation to the many kinetic reports, the reviews of Baldwin (1993) and Carruthers (1990) are suggested. In its simplest form, the model holds that the carrier possesses a *single* sugar binding site (C) that can be exposed at either the cytoplasmic (C_i) or the extracellular (C_o) membrane surfaces but not simultaneously at both places. Both the unloaded (C_i, C_o) and loaded (C_i·Glc, C_o·Glc) forms of the carrier can undergo conformational changes or reorientations that shift the binding site from one membrane surface to the other. The dissociation constants

† This work was supported by NIH Grant NS-26004 from the NINCDS to J.D.F. and by a grant from the Henry Ford Hospital to H.C.H.

* Address correspondence to this author at Anesthesia Research, 1FP-4D, Henry Ford Health System, One Ford Place, Detroit, MI 48202-3450. Telephone: (313)874-5971. Fax: (313)874-3770. E-mail: Howard_Haspel@msn.com.

⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1996.

[1] Abbreviations: BBB, blood−brain barrier; C, sugar binding site; C_i, cytoplasmically-oriented sugar binding site; C_o, extracellularly-oriented sugar binding site; dGlc, 2-deoxyglucose; Glc, glucose; GLUT-1, type 1 isoform of the mammalian facilitative hexose transporters; IC_{50}, concentration resulting in 50% inhibition; MeGlc, 3-*O*-methylglucose; Me_2SO, dimethyl sulfoxide; PB, pentobarbital; TB, transport buffer.

S0006-2960(96)02050-8 CCC: $12.00

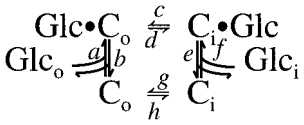

FIGURE 1: Simple King−Altman kinetic representation of the single-site, alternating conformation model of the human erythrocyte Glc transport system. This is a variation on the simple asymmetric carrier model for Glc transport presented in the review by Baldwin (1993). The carrier possesses a *single* sugar binding site, C, that can be exposed at either the cytoplasmic (C_i) or the extracellular (C_o) membrane surfaces, but not simultaneously at both. Both the unloaded (C_i, C_o) and loaded (C_i·Glc, C_o·Glc) forms of the carrier can undergo a conformational change or reorient to either membrane surface. The dissociation constants for sugar binding at the outer surface (*b/a*) and inner surface (*e/f*) can differ, and the rate constants involved in *reorienting* the occupied carrier (*c*,*d*) are greater than those for the unoccupied (*g*,*h)* carrier.

for sugar binding at the outer surface (*b/a*) and inner surface (*e/f*) can differ, and the rate constants involved in *reorienting* the occupied carrier (*c*,*d*) are greater than those for the unoccupied (*g*,*h)* carrier. These concepts help explain, to a first approximation, the observations of "trans acceleration" and the apparent differential affinities for substrate binding at the outer and inner membrane surfaces.

Several alternative models involving two *distinct* sugar binding sites which can or cannot be occupied *simultaneously* and which utilize a *common* translocation state have also been proposed. Despite intensive investigation, the available data and analyses have not clearly resolved which model is most correct. For the purposes of this report, the single-site alternating conformation model (Figure 1) is assumed and used to interpret the results obtained with the three assays of GLUT-1 function employed. When appropriate, we will, however, make reference to interpretations utilizing the alternative "two-site carrier" models (Baldwin, 1993; Carruthers, 1990).

Two of these assays are the unidirectional influx and efflux of radiolabeled hexose. Influx is measured under the conditions of (1) initially zero concentration of sugar inside the erythrocyte (zero trans) and (2) variable sugar concentration outside the cell (variable cis). Efflux is measured under the opposite conditions, namely, zero sugar concentration outside (zero trans) the erythrocyte and appreciable but variable sugar inside the cell (variable cis). From these measurements of influx and efflux, the model (Figure 1) predicts that altered sugar affinity at the cytoplasmic (*b/a*) or extracellular (*e/f*) membrane surfaces, respectively, can be detected. Furthermore, the translocation or reorientation step of the unoccupied carrier's binding sites (*g*,*h*) are probably rate-limiting at high cis substrate concentrations, and modulation of this process can also be assessed from the unidirectional flux data.

The third assay, the measurement of equilibrium exchange efflux, is done with equal, but varying, intracellular and extracellular concentrations of sugar and determines the exit of radiolabeled sugar that has been preloaded into the erythrocytes over time. From these equilibrium exchange data, alterations in sugar affinity from the lower affinity cytoplasmic (*b/a*) membrane surface can be gleaned, and, at high substrate concentrations, alterations in the translocation of occupied carrier (*c*,*d*) can be evaluated. Finally, the three sets of data will be examined collectively to see if they are consistent with barbiturates: (1) altering GLUT-1-mediated sugar flux asymmetrically; (2) competing for sugar binding to the transporter; and (3) interacting either preferentially with the occupied or unoccupied forms of the carrier or equally with both forms.

Materials. Anesthetics, cytochalasin B, phloretin, antifoam emulsion A, and most other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Etomidate was a gift from Janssen Pharmaceutica (Titusville, NJ). [^{3}H]-2-d-Glc, [^{3}H]-D-Glc, and [^{3}H]-MeGlc were purchased from Dupont NEN (Wilmington, DE).

Solutions. Stock solutions of 250 and 25 mM anesthetics were prepared in 80% (v/v) ethanol or water and kept at 4 °C. Stock solutions of 50 and 0.5 mM cytochalasin B were prepared in Me_2SO and stored in the dark at −20 °C. Phloretin stock solutions (250 mM) were made in ethanol and stored in the dark at 4 °C. Transport buffer (TB) consisted of 140 mM NaCl, 2.5 mM $MgSO_4$, 5 mM KCl, 1 mM $CaCl_2$, and 20 mM HEPES, pH 7.4. Stop solution consisted of iced TB plus 1.25 mM KI, 10 μM $HgCl_2$, 0.1 mM phloretin, and 10 μM cytochalasin B. Stop solution was prepared fresh each day and stored in the dark at 4 °C. Erythrocyte storage solution consisted of 140 mM NaCl, 3 mM $MgSO_4$, 5 mM KCl, 2 mM $CaCl_2$, 5 mM Glc, and 20 mM HEPES, pH 7.4.

Human Erythrocyte Preparation. Fresh human blood (≤15 mL) was drawn by venipuncture from healthy normal volunteers (18−50 years of age) into heparinized vacuum tubes and centrifuged at 1000*g* for 5 min at 4 °C. The plasma, buffy coat, and ∼10% of the packed erythrocytes were removed. The erythrocytes were then washed 3 times by centrifugation and resuspension in iced TB. Erythrocytes were used immediately or kept ≤24 h in storage solution at 4 °C. Stored cells were subjected to three washes with TB prior to use.

Equilibrium Exchange Sugar Efflux. Sugar efflux under equilibrium exchange conditions was assayed essentially as described previously (Honkanen *et al*., 1995). Aliquots of packed erythrocytes (∼500 μL) were incubated ≥1 h at 37 °C) at ∼5% hematocrit in TB containing Glc (1−130 mM). The Glc-loaded cells were centrifuged at 1000*g* for 10 min at 4 °C and resuspended at ∼50% hematocrit in TB with the same Glc concentration as the incubation medium. Aliquots (85 μL) of these Glc-loaded cells were loaded with 16 μCi of [^{3}H]-dGlc (evaporated to dryness) for 15 min at 23 °C. Aliquots of the Glc/[^{3}H]-dGlc-loaded cells (25 μL) were incubated with the indicated agents (evaporated to dryness) for another 15 min at 23 °C.

Efflux was started by the rapid addition of 250 μL of TB (23 °C) containing the indicated agents and Glc concentrations equal to those of the loading medium (1−130 mM) to aliquots (2 μL) of loaded/treated cells. Efflux was terminated at 0−600 s by the rapid addition with mixing of 1 mL of iced stop solution (1 mL). The zero time value was obtained by premixing stop (4 volumes) with initiation (1 volume) solution and adding 1.25 mL to the 2 μL aliquots of load/treated cells. Each experiment was done at least twice, and duplicate determinations were made at each time point in every experiment.

Terminated samples were centrifuged at 1000*g* for 15 min at 4 °C, the supernatants carefully removed, and the cell pellets solubilized in 0.5 mL of 2% (v/v) Triton X-100 containing 0.03% (v/v) anti-foam emulsion A. Duplicate aliquots (200 μL) were taken for determination of cell recovery by hemoglobin absorbance at 540 nm on a micro

titer plate reader. Aliquots (150 μL) from each well were heated (70 °C for 10 min) to inactivate endogenous catalase, cooled, and bleached with 250 μL of 30% (v/v) H_2O_2. Cell-associated radioactivity was then determined by liquid scintillation counting.

The cell-associated radioactivity for each time and condition was normalized to hemoglobin content (i.e., A_{540}), and the duplicates were averaged. The average value for the zero time for each condition was subtracted as extracellular trapping from the corresponding values at the other times. Assuming that the system reaches a steady state by 600 s (Honkanen *et al.*, 1995), the natural logarithm of fractional equilibrium exchange efflux was calculated and plotted versus time. A simple first-order exponential rate equation was used to obtain the apparent first-order rate constant (K_{app}) for each condition and the half-time ($T_{1/2}$) for equilibrium exchange efflux. The percent of control and percent inhibition were calculated for each condition with the corresponding K_{app}'s. Using various Glc concentrations for the incubation and the initiation solutions, the specific efflux was measured at times ranging from 1 s (1 mM Glc) to 10 s (100 mM Glc). Less than 50% of equilibrium was obtained in each case. Efflux rates (nmol/s) were calculated for each concentration, and these rates were similar to the values calculated from the K_{app}'s determined from the simple first-order exponential rate equation. As an internal control, 25 μM cytochalasin B, a potent inhibitor of GLUT-1-mediated Glc transport (Baldwin, 1993), was added to the Glc/[^{3}H]-dGlc-loaded cells (25 μL); at all substrate concentrations, equilibrium exchange sugar efflux was <15% of control (data not shown).

Unidirectional Zero Trans Sugar Efflux. Experimental procedures for unidirectional zero trans sugar efflux measurements were similar to those of the equilibrium exchange efflux measurements with the following modifications. To ensure near-complete efflux for these determinations, erythrocytes were loaded with MeGlc (1−100 mM), a nonmetabolizable Glc analog, instead of Glc. Aliquots (110 μL) of MeGlc-loaded cells were then loaded with 22 μCi of [^{3}H]-MeGlc (evaporated to dryness) and treated with the indicated agents (evaporated to dryness). Efflux was started by the rapid addition of (250 μL) TB containing the indicated agents and mannitol at concentrations equal to that of the Glc in the loading medium (1−130 mM) to the aliquots (2 μL) of loaded/treated cells. The stop solution also contained mannitol concentrations equivalent to that of the loading medium. The mannitol maintains osmotic balance and prevents rapid cell volume changes. Further analysis and calculations were nearly identical to those described for equilibrium exchange efflux. The same internal control with 25 μM cytochalasin B, number of experiments, and determinations per time point as given for equilibrium exchange efflux were used.

Unidirectional Zero Trans Sugar Influx. Packed erythrocytes were diluted to 33% hematocrit with TB. Appropriate quantities of the indicated agents (evaporated to dryness) were dissolved in 190 μL of TB. Aliquots (50 μL) of the diluted cells were added and allowed to incubate for 15 min at room temperature. Influx was started by adding a 10 μL aliquot of TB containing 0.05−3.5 M Glc and 4 μCi/mL [^{3}H]-dGlc. The final concentrations of components during influx were 6.6% cells, 2−130 mM Glc, 0.16 μCi/mL [^{3}H]-dGlc, and 0−10 mM of the indicated agent. Influx was terminated at the specified time by the rapid addition of 1 mL of iced stop solution with mixing. Each experiment was done at least twice, and duplicate determinations were made at each time point in every experiment.

Aliquots (100 μL) of each terminated sample were reserved for the determination of total radioactivity. Terminated samples were centrifuged at 1000*g* for 5 min at 4 °C and the supernatants removed. The cell pellets were washed by resuspension in 1.0 mL of iced stop solution and recentrifuged for 20 min, and the supernatant was again carefully removed. Cell pellets were solubilized with 750 μL of 1% (w/v) sodium dodecyl sulfate and heated at 70 °C for 15 min to inactivate endogenous catalase. Triplicate 200 μL aliquots of each sample were bleached with 100 μL of 30% (v/v) hydrogen peroxide for 15 min, and 25 μL of glacial acetic acid was added to prevent autofluorescence. Cell-associated radioactivity was then determined by liquid scintillation counting. Aliquots of the samples reserved for the determination of total radioactivity were treated and analyzed similarly. The remaining detergent-solubilized material was diluted 1:10 with water and used for estimating cell recovery and cell number by the hemoglobin absorbance at 540 nm on a micro titer plate reader. The hemoglobin content (i.e., A_{540}) values were compared with the absorbance of a standard solution of cells of known hematocrit and cell count. Cell counting of standards was performed with a hemocytometer.

Cell-associated radioactivity for each time and condition was normalized to cell number (i.e., A_{540}), and the triplicates were averaged. These values were normalized to the specific activity (dpm/pmol) determined from the amount of radioactivity in the totals. The average value for the zero time for each condition was subtracted as extracellular trapping from the corresponding values at the other times. Results are expressed as pmol of Glc influx per 10^6 cells. The early linear portions of the time course are used to determine initial rates of influx. The same internal control with 25 μM cytochalasin B as done for equilibrium exchange efflux was run.

RESULTS AND DISCUSSION

Pentobarbital Inhibits Unidirectional Zero Trans Sugar Influx. The normally rapid rate of unidirectional zero trans sugar influx into erythrocytes was greatly reduced by 10 mM pentobarbital (PB) (Figure 2). Simple first-order kinetic analysis of the extended time course of influx at 2 mM Glc (Figure 2, left panel) and direct rate determinations (Figure 2, middle panel) indicated that (1) the equilibrium level is the same in the presence or absence of 10 mM PB and (2) the initial rate of sugar influx was lowered by 88%. At 130 mM Glc, 10 mM PB reduced the initial rate of sugar influx by 81% (Figure 2, right panel). The K_m of Glc for zero trans sugar influx has been reported to be ~2 mM (Baldwin, 1993; Carruthers, 1990); the reduction in Glc influx by 10 mM PB found in the present experiments was, therefore, nearly the same at both subsaturating (2 mM) and saturating (130 mM) Glc concentrations.

Pentobarbital Inhibits Unidirectional Zero Trans Sugar Efflux. The initial rate of unidirectional zero trans sugar efflux from erythrocytes decreased by exposure to 10 mM PB by approximately 95% at 2 mM MeGlc (Figure 3, left panel) and 90% at 100 mM MeGlc (Figure 3, right panel).

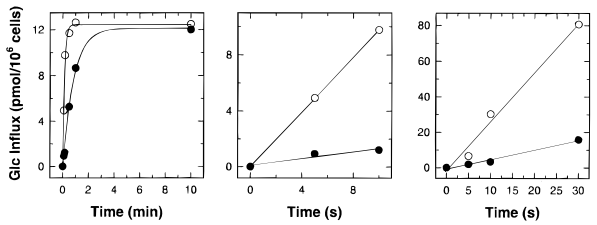

FIGURE 2: Pentobarbital inhibits unidirectional sugar influx by human erythrocytes. Isolated human erythrocytes were preincubated with (●) or without (○) 10 mM PB, and unidirectional zero trans sugar influx (pmol of Glc influx/10^6 cells) was assayed in the continued presence of the indicated agents at 2 mM (left and middle panels) or 130 mM (right panel) Glc. Linear regressions of the early time data (middle and right panel) yielded initial rates (K_{app}); correlation coefficients (r^2) were >0.95. Percent inhibition, calculated as $100 \times [1 - ({}^{PB}K_{app}/{}^{control}K_{app})]$, was 88% at 2 mM Glc and 81% at 100 mM Glc. The lines shown in the left panel are fit by nonlinear regression using a simple first-order exponential rate equation. The data are from individual experiments and are representative of the entire set. Note that the scale of the axes is not the same in the three panels.

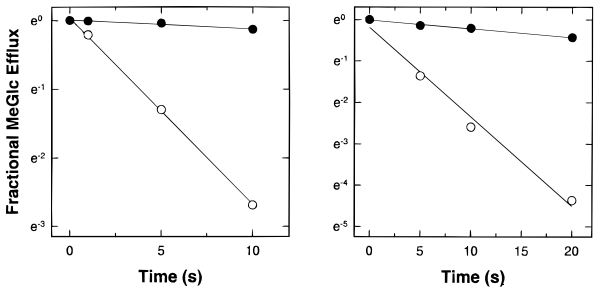

FIGURE 3: Pentobarbital inhibits unidirectional sugar efflux by human erythrocytes. Isolated human erythrocytes were loaded with 1 mM (left) or 100 mM (right panel) MeGlc, radioloaded with [^{3}H]-MeGlc, and preincubated with (●) or without (○) 10 mM PB. Unidirectional zero trans sugar efflux was assayed in the continued presence of the indicated agents with equivalent external concentrations of mannitol replacing the loaded Glc. The K_{app}'s were calculated from the first-order exponential fits of the data. Percent inhibition, ~95% in the left panel and ~90% in the right panel, was calculated as $100 \times [1 - ({}^{PB}K_{app}/{}^{control}K_{app})]$. The data are from individual experiments and are representative of the entire set. Note that the scale of the axes is not the same in the two panels.

The K_m of MeGlc for unidirectional zero trans sugar efflux is reportedly ~5 mM (Cloherty *et al.*, 1996); in view of this, the rate of unidirectional zero trans sugar efflux was reduced by 10 mM PB to the same extent at both subsaturating (2 mM) and saturating (100 mM) Glc concentrations. It appears, therefore, that inhibition by 10 mM PB of both unidirectional sugar influx and efflux is virtually unaffected by substrate concentration.

Pentobarbital Inhibits Equilibrium Exchange Sugar Efflux. Treatment of erythrocytes with 10 mM PB decreased equilibrium exchange sugar efflux by about 88% at 1 mM Glc (Figure 4, left panel) and 54% at 100 mM Glc (Figure 4, right panel). The K_m of Glc for equilibrium exchange sugar flux is ~20 mM (Baldwin, 1993; Carruthers, 1990), and the two Glc concentrations used in these experiments broadly span the K_m for this process. In contrast to the concentration-independence of the inhibition of unidirectional fluxes, inhibition of equilibrium exchange efflux of Glc by PB (10 mM) is dependent on substrate concentration.

Differential Sensitivity of Unidirectional Zero Trans and Equilibrium Exchange Sugar Efflux to Pentobarbital Concentration. In experiments where the Glc concentration was

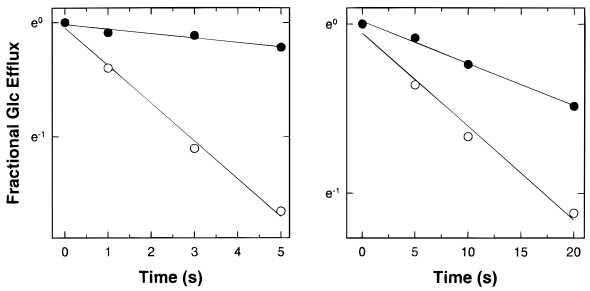

FIGURE 4: Pentobarbital inhibits equilibrium exchange sugar efflux by human erythrocytes. Isolated human erythrocytes were loaded with 1 mM (left) or 100 mM (right panel) Glc, radioloaded with [^{3}H]-dGlc, and preincubated with (●) or without (○) 10 mM PB. Equilibrium exchange sugar efflux was assayed in the continued presence of the indicated agents with external Glc concentrations equal to the loaded Glc concentration. The K_{app}'s were calculated from the slopes of the fitted lines. The percent inhibition was 88% at 1 mM and 54% at 100 mM Glc. The data are from individual experiments and are representative of the entire set. Note that the scale of the axes is not the same in the two panels.

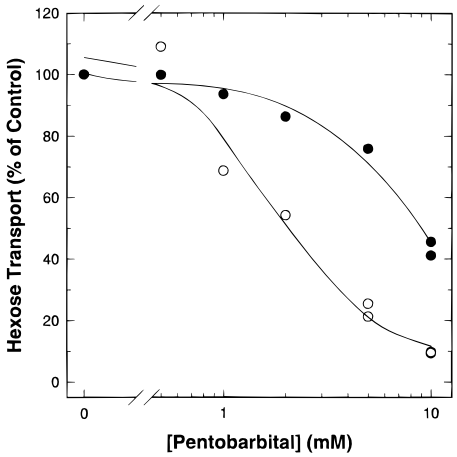

FIGURE 5: Pentobarbital inhibits unidirectional sugar efflux more than equilibrium exchange sugar efflux by human erythrocytes. The effects of different concentrations of PB (0−10 mM) on unidirectional zero trans (○) and equilibrium exchange (●) sugar (100 mM Glc) efflux by human erythrocytes were assayed (see the legends of Figures 3 and 4 for some further details). Percent of control values were calculated from the K_{app}'s at each PB concentration as follows: $100 \times [{}^{PB}K_{app}/{}^{control}K_{app}]$. The lines shown are only to guide the eye of the reader. The data are a composite of several experiments.

100 mM, the rates of unidirectional zero trans sugar efflux and equilibrium exchange sugar efflux depended strongly on the concentration of PB and resembled typical dose−response curves (Figure 5). The PB concentrations yielding 50% reductions in rate (IC_{50}) were ~2 mM for unidirectional sugar efflux and ~10 mM for equilibrium exchange sugar efflux. When the Glc concentration was lowered to 20 mM, the IC_{50} of PB for equilibrium exchange sugar efflux was reduced to ~5 mM (data not shown). In contrast, as it was at 100 mM Glc, the IC_{50} of PB was ~2 mM for both unidirectional sugar efflux and influx (data not shown) at 20 mM Glc. Thus, PB is a less potent inhibitor of equilibrium exchange sugar efflux than unidirectional sugar efflux.

Dependence of Pentobarbital Inhibition of Sugar Flux on Substrate Concentration. The reduction of unidirectional sugar efflux and influx by 10 mM PB was virtually independent of substrate concentration (2−130 mM Glc or MeGlc, respectively) (Figure 6). In contrast, equilibrium

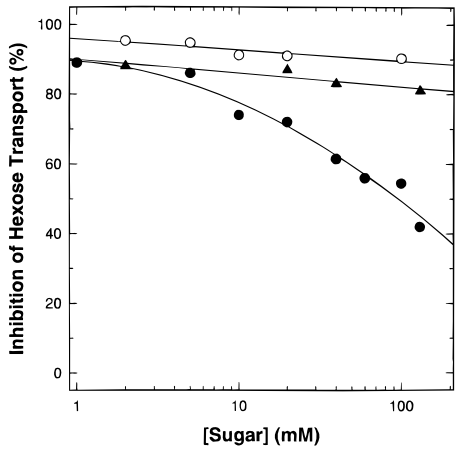

FIGURE 6: Dependence of equilibrium exchange sugar efflux and unidirectional sugar influx and efflux on hexose concentration in pentobarbital-treated human erythrocytes. PB (10 mM) inhibition of equilibrium exchange sugar efflux (●), unidirectional zero trans sugar efflux (○), and influx (▲) was determined at hexose concentrations of 1−130 mM Glc, 2−100 mM Glc, and 2−130 mM MeGlc, respectively. Assays and calculations were described in the legends of Figure 2, 3, and 4. The lines shown are only to guide the eye of the reader. The data are a composite of several experiments.

exchange sugar efflux in PB (10 mM) treated erythrocytes was clearly dependent on substrate concentration (Figure 6). For example, this efflux rate decreased from 88% of control at 1 mM Glc to 42% at 130 mM Glc. These findings are most consistent with barbiturates being *noncompetitive* inhibitors of Glc translocation and preferentially inhibiting the *unoccupied* form of the carrier protein.

Effects of Other Barbiturates on Hexose Fluxes. Secobarbital, phenobarbital, and barbital also reduced both equilibrium exchange sugar efflux and unidirectional sugar influx (data not shown). Like PB, these barbiturates more potently inhibited unidirectional sugar influx than equilibrium exchange sugar efflux. The order of potency for the barbiturates tested is secobartial > PB > phenobarbital ≈ barbital for both measurements, and this loosely correlates with barbiturate hydrophobicity (i.e., octanol:water partition coefficients; Firestone *et al.*, 1986).[2]

Conclusions and Possible Mechanisms for Barbiturate Inhibition of GLUT-1. In agreement with our earlier observations (Honkanen *et al.*, 1995), the current data demonstrate that barbiturates inhibit GLUT-1 mediated hexose transport in human erythrocytes and that this inhibition obeys the Meyer−Overton rule, namely, that potency is proportional to hydrophobicity (Franks & Lieb, 1994). The rates of zero trans sugar influx and efflux were similarly reduced by barbiturates, and the size of this reduction was independent of substrate concentration (Figures 2, 3, and 6). This agrees with our preliminary kinetic analysis of barbiturate inhibition of GLUT-1 in murine fibroblasts (Honkanen *et al.*, 1996). Barbiturate inhibition is, thus, apparently symmetrical with respect to sugar binding to GLUT-1 for both the cytoplasmic and extracellular orientations of the transporter. Equilibrium exchange sugar efflux was also decreased by barbiturates (Figure 4) and was most inhibited when Glc concentration and carrier occupancy were low andthe level of unoccupied GLUT-1 was high. PB (0−10 mM) inhibited unidirectional sugar flux more than equilibrium exchange flux (Figure 5).

With the assumption of the single-site alternating conformation model for GLUT-1 mediated hexose transport (Figure 1), our results with erythrocytes suggest that barbiturates are *noncompetitive* inhibitors of Glc translocation and preferentially inhibit the *unoccupied* form carrier. If barbiturates were competitive with Glc binding to the inner or outer orientations of GLUT-1, then some substrate dependence of barbiturate inhibition of zero trans sugar efflux and/or influx should have been observed; such dependency was not found (Figures 2, 3, and 6). Clearly, these interpretations are not at variance with the alternative "two-site carrier" models of glucose translocation (Baldwin, 1993; Carruthers, 1990).

For unidirectional flux to occur when the trans concentration of sugar is zero, reorientation of unoccupied carrier is an obligatory step for substrate translocation. For equilibrium exchange flux, the content of unoccupied GLUT-1 found in either the outer or the inner orientation is a function of substrate concentration. Although initially appearing competitive, both the diminished sensitivity to barbiturates of equilibrium exchange sugar efflux, relative to unidirectional sugar flux, and the dependence of barbiturate inhibition of equilibrium exchange on substrate concentration might be explained by the action of these drugs exclusively and directly on unoccupied GLUT-1. Substrate might therefore be *protecting* GLUT-1 from barbiturate inhibition by limiting the availability of unoccupied carrier. Similar complex and apparently competitive effects of cytochalasin B derivatives (Cloherty *et al.*, 1996; Jung & Rampal, 1977, 1975; Taylor & Gagneja, 1975; Bloch, 1973), steroids (Jung *et al.*, 1971; Lacko *et al.*, 1975; Krupka & Deves, 1980; LeFevre & Marshall, 1959; LeFevre, 1959), phloretin (LeFevre & Marshall, 1959; LeFevre, 1959), and local anesthetics (Baker & Rogers, 1973) have also been reported and might also be interpreted, at least in part, in this manner. Effects of these agents on substrates interacting with GLUT-1 have been observed, but the state of the carrier interacting with them has yet to be fully defined. We are presently pursuing this issue by continuing our initial biophysical examinations (Honkanen *et al.*, 1995) of the direct interaction of barbiturates with purified GLUT-1 using fluorescence spectroscopy.

In these earlier biophysical studies (Honkanen *et al.*, 1995), the concentration dependence of quenching by PB of the intrinsic fluorescence of purified GLUT-1 was not affected by substrate occupancy. However, the effects of PB on the concentration dependence of quenching by Glc of the intrinsic fluorescence of purified GLUT-1 have not been ascertained. More explicitly, Glc does not seem to alter PB binding, but it is not clear if PB alters substrate binding. In contrast, our present kinetic studies suggest that for inhibition of GLUT-1 *function* barbiturates prefer the unoccupied carrier. This is consistent, as we have suggested, with *noncompetitive* inhibition but does not distinguish if barbiturates can *bind* to *both occupied* and *unoccupied* forms of the carrier.[3]

The present findings that in erythrocytes barbiturates inhibit GLUT-1 mediated unidirectional sugar influx and

[2] Etomidate (IC_{50} ~2 mM) and ketamine (IC_{50} ~1 mM), but not ethanol (IC_{50} ≥68 mM), also inhibit erythrocyte hexose transport (Haspel *et al.*, manuscript in preparation).

efflux more than equilibrium exchange sugar efflux also have implications for our previous *in vivo* observation of PB-reduced Glc transfer across the blood−brain barrier (Otsuka *et al.*, 1991). When Glc flux is essentially unidirectional, such as in neurons and other highly metabolically active cells, barbiturates may more potently retard Glc uptake than in systems where Glc concentration is appreciable on both sides of the membrane and equilibrium exchange is sizable. For the brain where Glc concentrations in the interstitial fluid and endothelial cell cytoplasm are apparently >50% of plasma Glc concentration, equilibrium exchange is almost certainly significant for both the luminal (blood-side) and abluminal (brain-side) membranes of cerebral endothelial cells, which comprise the blood−brain barrier. Testing of this hypothesis *in vivo* has some merit since it impacts on the use of barbiturates in the operating room and in the treatment of cerebral injury, ischemia, edema, and other brain lesions.

ACKNOWLEDGMENT

We acknowledge the expert technical assistance of Ms. Keli Stephenson in the preparation of the manuscript. We thank Dr. Kenneth Davies for his help in obtaining several of the anesthetic agents. Helpful discussions with Drs. Rebecchi, Patlak, and Marsh are also acknowledged.

[3] As discussed in the now classic kinetics text of Segel (1975), concerning noncompetitive inhibition, it is difficult to kinetically distinguish between inhibitor interacting exclusively with free enzyme or interacting with *both* free enzyme and enzyme−substrate complex (i.e., the "open" versus "free" enzyme concept). Although by definition noncompetitive inhibition involves two *distinct*, but possibly *not independent*, sites for inhibitor and substrate, the potential for substrate binding restricting access to the inhibitor site cannot be excluded. The potential complexities for a system in which the enzyme (i.e., carrier) has two "spatially distinct" sites or conformations which can interact with substrate (i.e., sugar) are clearly even greater.

REFERENCES

Baker, G. F., & Rogers, H. J. (1973) *J. Physiol. 232,* 597−608.
Baldwin, S. A. (1993) *Biochim. Biophys. Acta 1154,* 17−49.
Bloch, R. (1973) *Biochemistry 12,* 4799−4801.
Carruthers, A. (1990) *Physiol. Rev. 70,* 1135−1176.
Cloherty, E. K., Heard, K. S., & Carruthers, A. (1996) *Biochemistry 35,* 10411−10421.
Fenstermacher, J. D., Wei, L., Acuff, V., Lin, S.-Z., Chen, J.-L., Bereczki, D., Otsuka, T., Nakata, H., Tajima, A., Hans, F.-J., Ghersi-Egea, J.-F., Finnegan, W., Richardson, G., Haspel, H., & Patlak, C. (1995) in *New Concepts of a Blood−Brain Barrier* (Greenwood, J., Begley, D. J., & Segal, M. B., Eds.) pp 89−101, Plenum Press, New York.
Firestone, L. L., Miller, J. C., & Miller, K. W. (1986) in *Molecular and Cellular Mechanisms of Anesthetics* (Roth, S. H., & Miller, K. W., Eds.) Appendix, pp 455−470, Plenum Press, New York.
Frank, N. P., & Lieb, W. R. (1994) *Nature 367,* 607−614.
Gjedde, A., & Rasmussen, M. (1980) *J. Neurochem. 35,* 1382−1387.
Honkanen, R. A., McBath, H., Kushmerick, C., Callender, G. E., Scarlata, S. F., Fenstermacher, J. D., & Haspel, H. C. (1995) *Biochemistry 34,* 535−544.
Jung, C. Y., & Rampal, A. L. (1975) *Fed. Proc. Fed. Am. Soc. Exp. Biol. 34,* 238.
Jung, C. Y., & Rampal, A. L. (1977) *J. Biol. Chem. 252,* 5456−5463.
Jung, C. Y., Carlson, L. M., & Whaley, D. A. (1971) *Biochim. Biophys. Acta 241,* 613−627.
Krupka, R. M., & Deves, R. (1980) *Biochim. Biophys. Acta 598,* 134−144.
Lacko, L., Wittke, B., & Geck, P. (1975) *J. Cell. Physiol. 86,* 673−680.
LeFevre, P. G. (1959) *Science 130,* 104−105.
LeFevre, P. G., & Marshall, J. K. (1959) *J. Biol. Chem. 234,* 3022−3026.
Maher, F., Vannucci, S. J., & Simpson, I. A. (1994) *FASEB J. 8,* 1003−1011.
Otsuka, T., Wei, L., Bereczki, D., Acuff, V. R., Patlak, C., & Fenstermacher, J. D. (1991) *Am. J. Physiol. 261,* R265−R275.
Segel, I. H. (1975) in *Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems,* Chapter 3, pp 100−160, Wiley-Interscience Publishers, New York.
Taylor, N. F., & Gagneja, G. L. (1975) *Can. J. Biochem. 53,* 1078−1084.
Widdas, W. F. (1952) *J. Physiol. (London) 118,* 23−39.

BI962050F