Barbiturates Inhibit Hexose Transport in Cultured Mammalian Cells and Human Erythrocytes and Interact Directly with Purified GLUT-1†

Robert A. Honkanen,‡ Heather McBath,‡ Christopher Kushmerick,‡ Gordon E. Callender,‡ Suzanne F. Scarlata,‡ Joseph D. Fenstermacher,‡,§,|| and Howard C. Haspel*,‡,||

The Department of Anesthesiology, Henry Ford Hospital, Detroit, Michigan 48202 and The Departments of Physiology & Biophysics and Neurological Surgery, State University of New York, Stony Brook, New York 11794

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ABSTRACT: Barbiturates reduce cerebral blood flow, metabolism, and Glc transfer across the blood−brain barrier. The effect of barbiturates on hexose transport in cultured mammalian cell lines and human erythrocytes was studied. Pentobarbital inhibits [^{3}H]-2-dGlc uptake in 3T3-C2 murine fibroblasts by ~95% and ~50% at 10 and 0.5 mM, respectively. Uptake of [^{3}H]-2-dGlc is linear with time in the presence or absence of pentobarbital, and the percent inhibition is constant. This suggests that hexose transport, not phosphorylation, is inhibited by barbiturates. Inhibition by pentobarbital of hexose transport in 3T3-C2 cells is rapid (<1 min), is not readily reversible, is not altered by the presence of albumin [1% (w/v)], and is independent of temperature (4−37 °C) and the level of cell surface GLUT-1. The IC_{50}'s for inhibition of hexose transport in 3T3-C2 cells by pentobarbital, thiobutabarbital, and barbital are 0.8, 1.0, and 4 mM, respectively. This is consistent with both the Meyer-Overton rule and the pharmacology of barbiturates. Neither halothane (≤10 mM) nor ethanol [≤0.4% (v/v)] significantly inhibits hexose transport. Inhibition by pentobarbital (0.5 mM) of [^{3}H]-2-dGlc uptake by 3T3-C2 cells decreases the apparent V_{max} (~50%) but does not alter the apparent K_m (~0.5 mM). Inhibition of hexose transport by barbiturates, but not ethanol [≤0.4% (v/v)], is also observed in human erythrocytes and four other cultured mammalian cell lines. Pentobarbital quenches (Q_{max} ~75%) the intrinsic fluorescence of purified and reconstituted GLUT-1 (K_d ~3 mM). Quenching is independent of Glc occupancy, is unchanged by mild proteolytic inactivation, and does not appear to directly involve perturbations of the lipid bilayer. We propose that barbiturates can interact directly with GLUT-1 and inhibit the intrinsic activity of the carrier. Glc crosses the blood−brain barrier primarily via the GLUT-1 of the endothelial cells of cerebral capillaries. Partial inhibition of this process by barbiturates may be of significance to cerebral protection.

Barbiturates are commonly utilized as anesthetics for neurosurgery in an effort to prevent sequelae associated with elevated intracranial pressures (Shapiro, 1975; Michenfelder, 1978; Michenfelder *et al.*, 1976; Richter & Holtman, 1982; Willow & Johnston, 1983). In this regard, barbiturates are thought to act by limiting cerebral blood flow and metabolism (Nilsson & Seisjo, 1975; Saija *et al.*, 1989; Todd & Warner, 1992). Studies of cerebral blood flow and Glc transfer across the blood−brain barrier (BBB)[1] in rats have established that barbiturates reduce both of these processes in most brain areas (Gjedde & Rasmussen, 1980; Ingvar *et al.*, 1980). Recently, we have completed a detailed reexamination of pentobarbital-induced changes in cerebral blood flow, Glc metabolism, and Glc transfer (Otsuka *et al.*, 1991a,b; Wei *et al.*, 1993). One of the central conclusions of these studies was that the maximal velocity for Glc transfer (T_{max}) across the BBB is lowered by barbiturates. This decrease in T_{max} for Glc appeared to be due to a direct effect of barbiturates on the Glc carriers of the endothelial cells of cerebral microvessels. It was suggested that the mechanism responsible for this effect of barbiturates on the Glc carriers was a decrease in the number of functional hexose transporters and/or a decrease in the intrinsic activity of the carriers. The major Glc carrier of the BBB is GLUT-1 (Pardridge, 1991; Carruthers, 1990; Bell *et al.*, 1993; Baldwin, 1993). This suggests that the function of GLUT-1 may in general be sensitive to barbiturates.

Although studied in detail for more than a century, the molecular mechanisms responsible for general anesthesia remain obscure (Akeson & Deamer, 1991; Franks & Lieb, 1987, 1990; Jørgenson *et al.*, 1991; Trudell, 1991). Recently, the notion that general anesthetics are "nonspecific" agents that have as their primary target the lipid portions of cell membranes has been questioned (Franks & Lieb, 1987, 1990). This is due in large part to the lack of correlation of "lipid bilayer stabilization" concepts (Gruner & Shyamsunder, 1991; Trudell, 1991) and the observed pharmacology of general anesthesia (Richter & Holtman, 1982; Willow &

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* Address correspondence to this author at: Anesthesiology, CFP-1, Henry Ford Hospital, 2799 W. Grand Blvd., Detroit, MI 48202-2689, (tel) 313/876-3030, (fax) 313/876-2018.

‡ The Department of Physiology & Biophysics, SUNY.

§ The Department of Neurological Surgery, SUNY.

|| The Department of Anesthesiology, Henry Ford Hospital.

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[1] Abbreviations: 2AS, 2-(anthroyloxy)stearic acid; 12AS, 12-(anthroyloxy)stearic acid; BBB, blood−brain barrier; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; FB, fluorescence buffer; GLUT-1, type 1 isoform of the facilitative hexose transporters; IC_{50}, 50% inhibitory concentration, K_{app}, apparent rate constants of simple first-order exponentials; NRK, normal rat kidney; Q_{max}, maximal fluorescence quenching; SB, sulfate buffer; TB, transport buffer; T_{max}, maximum velocity for Glc transfer.

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Johnston, 1983). Franks and Lieb (1987, 1990) have discussed an alternative view that general anesthetics act directly on "amphiphilic pockets of circumscribed dimensions on particularly sensitive proteins". Based on this idea, the most likely targets for general anesthetics were proposed to be intrinsic membrane proteins that have multiple amphipathic bilayer spanning helices. Ion channels, ion pumps, and facilitative transporters constitute a large class of proteins that have this property. Different classes of general anesthetics affect different classes of these membrane proteins selectively [cf. Pancrazio *et al.* (1993), Asano and Ogasawara (1981, 1982), and Rooney *et al.* (1993)].

Our focus on the *in vivo* effects of barbiturates on Glc transfer across the BBB leads us to review the effects of barbiturates on membrane permeability [see Willow and Johnston (1983) and Richter and Holtman (1982) for reviews]. In contrast to the many studies on the membrane effects of local anesthetics [cf. Dahl-Hansen and Clausen (1973), Kutchai *et al.* (1980a,b), Cooper and Kohn (1980), Clausen *et al.* (1973), Abu-Salah (1991), and Abu-Salah *et al.* (1982)], the action of barbiturates on hexose transport has not been extensively examined. A very early study by Lowry *et al.* (1964) suggested that barbiturates alter glycolytic rate in brain tissue. Later work, by Clausen and collaborators (1973), on "membrane stabilizers" suggested that barbiturates can suppress insulin-stimulated, but not basal, hexose transport in rat adipocytes. Similarly, Salah *et al.* (1982) reported modest inhibition by barbiturates of Glc uptake in human erythrocytes. Krieglstein and Mwasekaga (1987) and Okazaki *et al.* (1992) have recently reported that barbiturates inhibit Glc utilization in neuroblastoma cells.

In an attempt to elucidate the molecular mechanisms for the decreases in hexose transport that we have observed *in vivo* for the BBB (Otsuka *et al.*, 1991a), the hypothesis that barbiturates diminish GLUT-1-mediated hexose transport was tested in cultured mammalian cell lines and human erythrocytes. The interaction of barbiturates with purified and reconstituted GLUT-1 was also studied. Our findings suggest that barbiturates can interact directly with GLUT-1 and inhibit the intrinsic activity of the carrier.

EXPERIMENTAL PROCEDURES

Materials. Sodium pentobarbital was obtained from Anthony Products (Arcadia, CA) as a 260 mM stock dissolved in 10% (v/v) EtOH. Barbital was obtained from Fischer Scientific Co. (Pittsburgh, PA) and was prepared as a 40 mM stock solution in transport buffer (TB) consisting of 140 mM NaCl, 5 mM KCl, 2.5 mM $MgSO_4$, 1.0 mM $CaCl_2$, and 20 mM HEPES, pH 7.4. These solutions were kept at <30 °C and used within 1 month. Thiobutabarbital was obtained from Byk Gulden Konstanz (Germany) and was prepared daily as a 250 mM stock in H_2O. Halothane was obtained from Halocarbon Laboratories (North Augusta, SC) and contained 0.01% (v/v) thymol as a preservative. Stock halothane solutions were prepared immediately before use as 15 mM stocks in TB and were sealed to avoid evaporation. EtOH was obtained from Quantum Chemical Corp., USI Division (Tuscoloa, IL), and was diluted to a 10% (v/v) stock. [^{3}H]-2-dGlc was obtained from New England Nuclear Research Products (Boston, MA). Glc-6-phosphate dehydrogenase, dGlc, Glc, Fru, Trp, fatty acid-free fraction V bovine serum albumin (BSA), sequence grade trypsin, cytochalasin B, and $NADP^+$ were obtained from Sigma Chemical Co. (St. Louis, MO). High temperature silicone oil (*d* 1.05) was obtained from Aldrich Chemical Co. (Milwaukee, WI). 2- and 12-(anthroyloxy)stearic acid (2AS and 12AS, respectively) were obtained from Molecular Probes, Inc. (Eugene, OR). Concentrated stocks of 2AS and 12AS dissolved in EtOH were stored protected from light at −20 °C.

Cell Culture. 3T3-C2 murine fibroblasts were originally provided by Dr. H. Green (Harvard Medical School, Boston, MA). C6 rat glioblastoma cells were obtained from American Type Culture Collection (Rockville, MD). N2A rat neuroblastoma cells were provided by Dr. E. Beyer (Washington University, St. Louis, MO). Clone 52E normal rat kidney (NRK) cells were originally provided by Dr. M. Rosenfeld (New York University Medical School, New York, NY). FTO rat hepatocarcinoma cells were provided by Dr. Y. Hod (State University of New York, Stony Brook, NY). All cell lines were cultured as previously described for 3T3-C2 cells (Haspel *et al.*, 1986, 1985a). Unless otherwise indicated, all cells were grown to confluence in high Glc (4.5 g/L), Dulbecco's modified Eagle's medium (DMEM) containing nonessential amino acids, 2 mM Gln, 100 units/mL penicillin, 100 μg/mL streptomycin, and 9% (v/v) heat-inactivated calf serum in a 5% humidified CO_2 atmosphere at 37 °C on 35 mM six-well dishes for hexose transport determinations or single 100 mM dishes for Glc-6-phosphate content determinations. Murine 3T3-C2 fibroblasts were plated at a density of ~8000 cells/cm^2 and fed at least every third day. Quiescent cells were used in all experiments, and confluent monolayers were refed 12−24 h before use. Fresh thaws of frozen stocks of the different cell lines were utilized for ≤15 passages. Other cell lines were treated similarly, but plated at a slightly lower initial density. Confluent monolayers of 3T3-C2 cells were Glc-deprived by feeding cells media containing no Glc, *dialyzed* calf serum, and Fru (1 g/L) (Haspel *et al.*, 1986, 1991).

Assay of Hexose Uptake by Cultured Cells. [^{3}H]-2-dGlc uptake was assayed as previously described (Haspel *et al.*, 1991; Ortiz *et al.*, 1992). Cells were rapidly washed once with TB and preincubated for the times indicated (generally, 15 min at 23 °C) in TB containing the indicated agents. This solution was rapidly removed, and uptake was initiated by addition of fresh TB containing the indicated agents and [^{3}H]-2-dGlc (generally, 100 μM, 0.5 μCi/mL) with or without 20 mM Glc as a competitor. After the indicated times (generally, 15 min at 23 °C) this solution was rapidly removed and uptake was terminated by rapid washing with iced phosphate-buffered saline [0.9% (w/v) NaCl, 10 mM NaH_2PO_4, pH 7.4]. Cells were solubilized in 1% (w/v) sodium dodecyl sulfate and cell-associated radioactivity and cellular protein determined by scintillation counting and bicinchonate assay, respectively. Specific uptake of [^{3}H]-2-dGlc was calculated by subtracting cell-associated radioactivity values observed in the presence of 20 mM Glc from the respective noncompeted values and was normalized to cellular protein content. Generally, transport results were normalized to control values, set to 100%, for each experiment, and the percentages of control for each condition are reported.

Assays of Hexose Uptake by Human Erythrocytes. (A) Isolation of Human Erythrocytes. Blood was drawn daily by venipuncture into heparinized tubes and centrifuged at 750*g* for 5 min at 4 °C. The plasma, buffy coat, and ~10%

of the packed red blood cells were removed. The erythrocytes were then washed three times by centrifugation and resuspension in iced TB (≤20% hematocrit).

(B) Unidirectional Influx. Packed erythrocytes were resuspended at ~20% hematocrit (~2 × 10^9 cells/mL) in TB and the cells counted with an hemocytometer. Aliquots (100 μL) of cell suspension were preincubated at 4 °C for 15 min in TB (final volume of 900 μL) containing the indicated agents. After preincubation, 100 μL of TB containing the indicated agents and [^{3}H]-2-dGlc (1 mM, 10 μCi/mL) with or without Glc (250 mM) was added at 4 °C with mixing to initiate uptake. Triplicate aliquots (200 μL) were rapidly layered over ~100 μL aliquots of silicone oil in narrow 400 μL microcentrifuge tubes, and uptake was terminated at 30 s by centrifugation (10000*g* for 10 s at 4 °C) of the erythrocytes through the oil. The tips of the microcentrifuge tubes containing the cell pellets were removed. The cells were lysed with H_2O (100 μL), the endogenous catalase was inactivated by heating (~15 min at 85 °C), and the hemoglobin was bleached with 200 μL of 30% (v/v) H_2O_2. Cell-associated radioactivity was determined by scintillation counting. Specific uptake of [^{3}H]-2-dGlc was calculated by subtracting cell-associated radioactivity values observed in the presence of 25 mM Glc from the respective noncompeted values and normalizing to cell number.

(C) Equilibrium Exchange Influx. Glc influx under equilibrium exchange conditions was assayed essentially as described previously for equilibrium exchange efflux (Haspel *et al.*, 1985b). Aliquots of packed erythrocytes were incubated (1 h at 37 °C) at ≤5% hematocrit in TB containing 130 mM Glc. The Glc-loaded cells were centrifuged at 750*g* for 5 min at 4 °C and resuspended at ~50% hematocrit in TB containing 130 mM Glc. Aliquots (500 μL) of this suspension were preincubated with the indicated agents and samples (30 μL) transferred to the bottom of 15 mL conical centrifuge tubes. Exchange was initiated by the rapid addition with mixing of TB (1 mL) containing Glc (130 mM), and [^{3}H]-2-dGlc (5 μCi/mL) to the cells at 23 °C. Exchange was terminated at 5, 10, 15, 20, 30, and 600 s by the rapid addition with mixing of 8 mL of iced stop solution (174 mM NaCl, 1.25 mM KI, 1 μM $HgCl_2$, 0.1 mM phloretin, 25 μM cytochalasin B), and the solution was kept on ice for <1 h. A zero time value was obtained by premixing the initiation and stop solutions and adding the mixture to the cells simultaneously. Samples were centrifuged at 1000*g* at 4 °C for 15 min, the supernatants carefully removed, and the cell pellets solubilized in 0.5 mL of 2% (v/v) Triton X-100. Duplicate 200 μL aliquots were heated (~5 min at 85 °C) to inactivate endogenous catalase, and the hemoglobin was bleached with 100 μL of 30% (v/v) H_2O_2. Cell-associated radioactivity and total cellular protein were then determined by scintillation counting and bicinchonate assay, respectively. Cell-associated radioactivities for each time and condition were normalized to protein and the duplicates averaged. The value for 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 (Haspel *et al.*, 1985b), fractional equilibrium exchange influx was calculated and plotted versus time. A simple first-order exponential rate equation was used to obtain the half-time ($\tau_{1/2}$) for equilibrium exchange influx for each condition.

Fluorescence Studies of Proteoliposomes Containing Purified GLUT-1. Purified GLUT-1 protein reconstituted into endogenous phospholipids was prepared from clarified octyl glucoside extracts of alkali-stripped human erythrocyte membranes by anion exchange chromatography followed by exhaustive dialysis (Baldwin & Lienhard, 1989). The GLUT-1-containing proteoliposomes were dialyzed against a fluorescence buffer (FB) consisting of 100 mM NaCl, 1 mM EDTA, and 75 mM HEPES, pH 7.5. The purity and identity of GLUT-1 were determined after electrophoresis on SDS−polyacrylamide gels (11%) by staining with Coomassie blue and immunoblotting before and after mild trypsinization (Haspel *et al.*, 1988). As expected, native GLUT-1 migrated broadly at M_r ~55 000 and was immunoreactive, while mildly trypsinized GLUT-1 showed a broadly migrating band at M_r ~30 000 and a sharp immunoreactive band at M_r ~19 000.

Intrinsic protein fluorescence of native or mildly trypsinized (Haspel *et al.*, 1988) GLUT-1 (~20 μg/mL), BSA (20 μg/mL), or Trp (200 ng/mL) in FB (~1.5 mL) was measured at 23 °C with stirring on an ISS-K2 spectrofluorometer using software from ISS (Champaign, IL). To reduce scattered light, stocks of GLUT-1 containing proteoliposomes (~100 μg/mL) were sonicated (~100 W for 30 s at 23 °C) before use and a 310 nm band-pass filter was employed. Pentobarbital, barbital, or Glc was added in small aliquots (2−10 μL) from concentrated stocks and emission spectra (300−420 nm) were recorded at an excitation wavelength of 280 nm after ~5 min of incubation at 23 °C. The fluorescence emission intensities were calculated from the area of the emission spectra and corrected for any dilutions. Fractional quenching was calculated by dividing the intensities obtained after additions by the intensity before any additions. Plots of fractional quenching versus concentration were computer fit to simple hyperbolic functions of saturable binding to a single class of binding sites. Apparent affinities (K_d) and maximal quenching (Q_{max}) were calculated, and the reliability of the fits was assessed from the coefficients of variation of the parameters.

The effect of pentobarbital on the physical state of the membrane was monitored with 2AS or 12AS as fluorescent probes of two "depths" of the hydrocarbon interior of the lipid bilayer (Scarlata, 1991). 2AS (6 μM) or 12AS (2.5 μM) was added from ethanolic stocks and incubated with GLUT-1-containing proteoliposomes (~20 μg/mL) for 10 min at 23 °C with stirring. This allows these fatty acid probes to insert into the lipid bilayer. Fluorescence emission at 430 nm was recorded before and ~5 min after addition of pentobarbital (10 mM) with an excitation wavelength of 381 nm. These intensity values were corrected for any dilutions.

RESULTS

Pentobarbital Inhibits Hexose Transport in 3T3-C2 Murine Fibroblasts. The effect of pentobarbital on hexose transport by 3T3-C2 murine fibroblasts was examined (Figure 1). 3T3-C2 cells were preincubated for 15 min with pentobarbital (0, 0.5, or 10 mM) or vehicle [0.4% (v/v) EtOH], and [^{3}H]-2-dGlc uptake was determined in the continued presence of anesthetic or vehicle for 1, 5, or 15 min. Pentobarbital inhibited hexose transport by ~95% and ~50% at 10 and 0.5 mM, respectively. The uptake of [^{3}H]-2-dGlc was linear

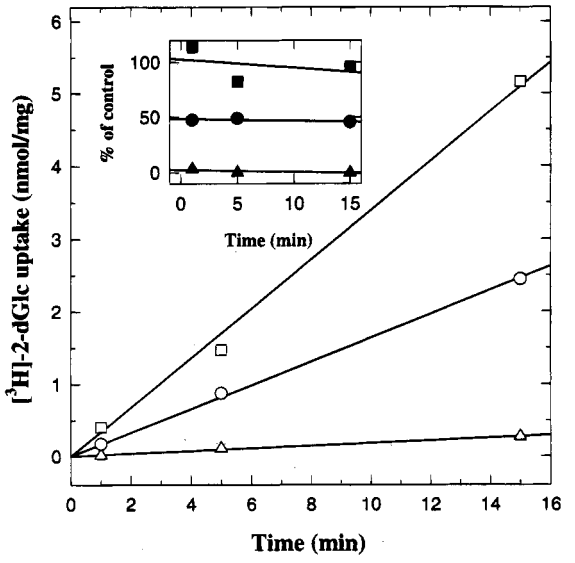

FIGURE 1: Pentobarbital inhibits hexose transport in 3T3-C2 murine fibroblasts. 3T3-C2 cells were grown to confluence in 35-mm six-well dishes and refed 12−24 h before use. Cells were then preincubated at 23 °C in TB containing 10 mM pentobarbital (△, ▲, 0.5 mM pentobarbital (○, ●), vehicle [0.4% (v/v) EtOH] (□, ■) or with no additions (i.e., control) (not shown) for 15 min. [^{3}H]-2-dGlc uptake was then assayed at 23 °C as described in the Experimental Procedures, in triplicate, in the continued presence of the indicated agents for the indicated times. The open symbols represent hexose transport as nmol of [^{3}H]-2-dGlc/mg of cellular protein. The closed symbols represent hexose transport as % of control uptake normalized for each time of uptake (*inset*). The lines drawn are based on least-squares linear regressions. The results are representative of three similar experiments.

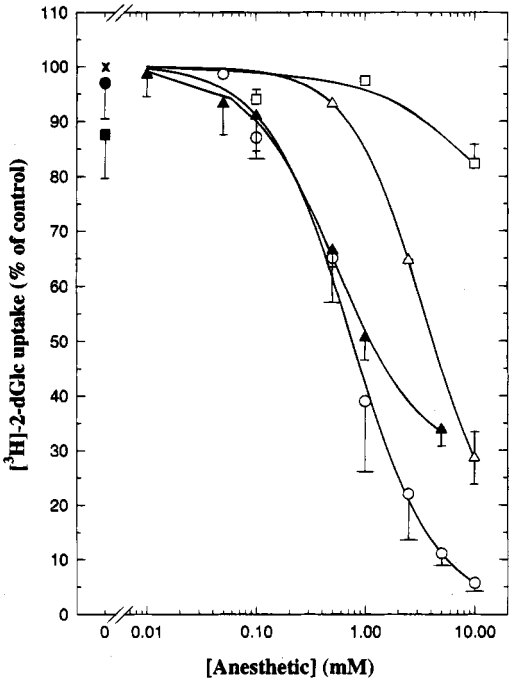

FIGURE 2: Concentration dependence for the inhibition of hexose transport by barbiturates in 3T3-C2 cells. 3T3-C2 cells were grown and refed as in Figure 1. Cells were then preincubated at 23 °C for 15 min in TB containing different concentrations of pentobarbital (○), thiobutabarbital (▲), barbital (△), halothane (□), 0.4% (v/v) EtOH (■, vehicle), 0.2% (v/v) EtOH (●, vehicle), or with no additions (×, control). [^{3}H]-2-dGlc uptake was then assayed at 23 °C as described in the Experimental Procedures, in triplicate, for 15 min in the continued presence of the indicated agents. Results are expressed as % of control. The means of individual experiments (N = 2 for barbital, N = 4 for pentobarbital, thiobutabarbital, halothane, and vehicle) are presented. Each data set is normalized to its respective control. Error bars are ±SD.

with time in the presence or absence of anesthetic, and the percent inhibition was constant at all times examined. This is consistent with hexose transport, not phosphorylation of dGlc, being the rate-limiting step for dGlc uptake (Carruthers, 1990; Bell *et al.*, 1993) and suggests that pentobarbital inhibits hexose transport rather than Glc metabolism.[2] This inhibition by pentobarbital of hexose transport in 3T3-C2 cells was (i) not altered by decreasing the preincubation time with anesthetic to 0, 1, or 5 min (not shown); (ii) not reversed by washing and incubation for 0, 5, or 15 min at 23 °C with TB or TB containing 1% (w/v) BSA (not shown); (iii) not prevented by inclusion of 1% (w/v) BSA in the preincubation and uptake solutions (not shown); and (iv) not significantly altered at 4 or 37 °C (not shown). Furthermore, when hexose transport is upregulated ~3-fold by Glc deprivation (Haspel *et al.*, 1986; Ortiz & Haspel, 1993), the percent inhibition caused by pentobarbital is essentially unchanged (not shown). Taken in concert, these observations suggest that inhibition of hexose transport by pentobarbital is relatively rapid, not readily reversible, not prevented by binding of the anesthetic to plasma proteins, not temperature-dependent, and not dependent on the level of cell surface expression of hexose transporter.

Concentration Dependence for the Inhibition of Hexose Transport by Barbiturates in 3T3 Cells. The effects of three barbiturates, halothane, and EtOH on hexose transport in 3T3-C2 cells were examined at different concentrations (Figure 2). The IC_{50}'s for inhibition by pentobarbital, thiobutabarbital, and barbital were 0.8, 1.0, and 4 mM, respectively. The shape of the dose−response curve for thiobutabarbital is not ideal because of limited solubility of this anesthetic in aqueous buffers at ≥1 mM. This order is loosely consistent with the Meyer and Overton rule (Franks & Lieb, 1990) in which the solubility of general anesthetics in nonpolar solvent correlates with their relative potency. These concentrations also compare well with the doses used both clinically in humans and *in vivo* for animal studies (Richter & Holtman, 1982; Willow & Johnston, 1983). These concentrations are reasonably consistent with the content (~200 nmol/g) of pentobarbital found in the brains of anesthetized rats (Richter & Waller, 1975). In contrast, halothane or EtOH (i.e., vehicle), both at anesthetic concentrations (Akeson & Deamer, 1991), inhibits hexose transport only slightly (Figure 2). This suggests that, under these conditions, barbiturates, as a class of general anesthetics, can selectively inhibit hexose transport.

Effect of Pentobarbital on the Kinetics of Hexose Transport in 3T3-C2 Cells. The effect of pentobarbital on the kinetics of hexose transport in 3T3-C2 cells was examined (Figure 3). Cells were preincubated for 15 min with 0.5 mM pentobarbital or vehicle [0.4% (v/v) EtOH], and the uptake of different concentrations of [^{3}H]-2-dGlc was determined in the continued presence of the indicated agents. Estimates of the apparent K_m's and V_{max}'s were made by fitting the

[2] Glc-6-phosphate content, measured as in Ortiz and Haspel (1993), of confluent and refed 3T3-C2 cells is decreased (~50%) by 10 mM pentobarbital (not shown). This agrees qualitatively with the observed inhibition of hexose transport (Figures 1 and 2) and confirms the earlier observations of Krieglstein and Mwasekaga (1987).

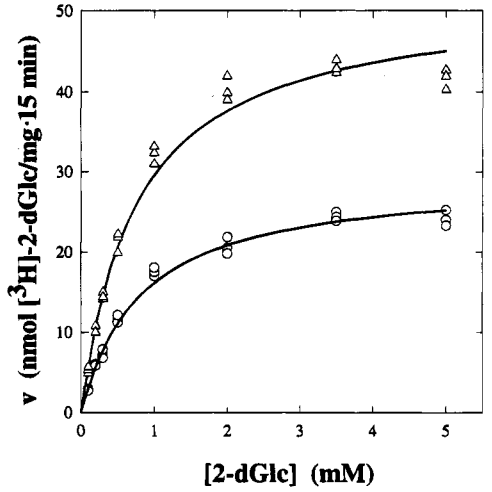

FIGURE 3: Effect of pentobarbital on the kinetics of hexose transport in 3T3-C2 cells. 3T3-C2 cells were grown and refed as in Figure 1. Cells were then preincubated at 23 °C for 15 min in TB containing vehicle [0.2% (v/v) EtOH] (△) or 0.5 mM pentobarbital (○). The concentrations of dGlc indicated and 2 μCi/mL [^{3}H]-2-dGlc were employed. [^{3}H]-2-dGlc uptake was then assayed at 23 °C as described in the Experimental Procedures, in triplicate, for 15 min in the continued presence of the indicated agents. The results are presented as hexose transport velocity (v) in nmol of [^{3}H]-2-dGlc/(mg of cellular protein·15 min) and are corrected for the different specific activities. The curves for vehicle- and 0.5 mM pentobarbital-treated cells were made by computer fitting the data to a simple saturable carrier model. The results are representative of three similar experiments, and the individual determinations from one of these are depicted.

data to a simple saturable carrier model (Carruthers, 1990). The reliability of the fits was assessed from the coefficients of variation of the parameters. The apparent K_m's determined in pentobarbital-treated and untreated cells are not significantly different [i.e., 0.52 ± 0.15 mM and 0.52 ± 0.13 mM, respectively, (mean ± SD, N = 3)]. The apparent V_{max} determined in pentobarbital-treated cells is ~50% of that observed in untreated cells [i.e., 47.6 ± 12.0 and 25.2 ± 2.7 nmol/(mg·15 min) for control and pentobarbital-treated cells, respectively (mean ± SD, N = 3)]. These results suggest that barbiturates do not alter the affinity of the hexose transporter for Glc.[3] In addition, the change in apparent V_{max} correlates well with the percent inhibition observed in both Figures 1 and 2.

Barbiturates Inhibit Hexose Transport in a Variety of Cultured Cell Lines. The effect of pentobarbital on hexose transport in a variety of cultured cell lines was examined (Figure 4). These cell lines included 3T3-C2 murine fibroblasts, FTO rat hepatocarcinoma cells, NRK epithelial cells, C6 rat glioblastoma cells, and N2A rat neuroblastoma cells. The different cell lines were preincubated for 15 min with pentobarbital (0, 1, or 10 mM) or vehicle [0.4% (v/v) EtOH], and [^{3}H]-2-dGlc uptake was determined in the continued presence of anesthetic or vehicle for 15 min. Pentobarbital significantly inhibits hexose transport in all of

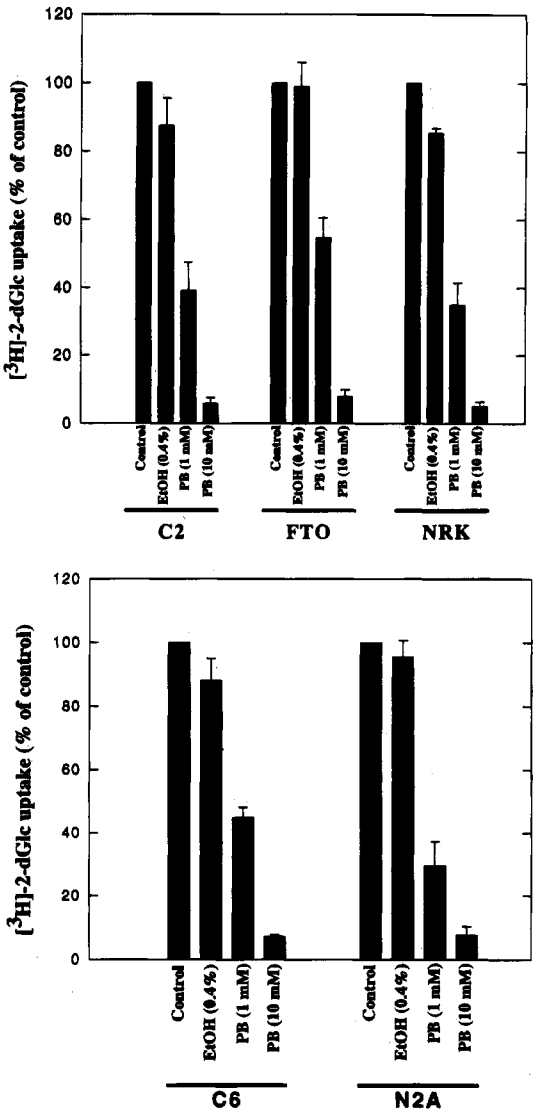

FIGURE 4: Pentobarbital inhibits hexose transport in a variety of cultured cell lines. 3T3-C2 (C2) murine fibroblasts, FTO rat hepatocarcinoma, NRK epithelial cells, C6 rat glioblastoma cells, or N2A rat neuroblastoma cells were grown and refed as for 3T3-C2 cells in Figure 1. Cells were then preincubated at 23 °C for 15 min in TB containing no additions (control), 0.4% (v/v) EtOH (vehicle), 1 mM pentobarbital (PB), or 10 mM pentobarbital. [^{3}H]-2-dGlc uptake was then assayed at 23 °C as described in the Experimental Procedures, in triplicate, for 15 min in the continued presence of the indicated agents. The top and bottom panels show results of cell lines of "non-neural" and "neural" origins, respectively. Results are expressed as % of control. The means of the individual experiments are presented. Each data set is normalized to its respective control. Error bars are ±SD and N = 3 for all cell lines except C6 cells, for which N = 2.

these cell lines (i.e., >90% inhibition for 10 mM and ~60% inhibition for 1 mM). In contrast, 0.4% (v/v) EtOH does not significantly alter hexose transport in any of these cell lines (i.e., <10% inhibition). This cell line independence, and the fact that all of these immortalized cell lines predominately express hexose transporter protein GLUT-1 (Bell *et al.*, 1993), suggests that barbiturates can inhibit GLUT-1 in a manner which is independent of cellular environment.[4]

[3] In this kinetic analysis we are determining *apparent* K_m's and V_{max}'s because dGlc is phosphorylated by hexokinase to dGlc-6-P. It should be emphasized that these *composite* kinetic constants are clearly a function of the "enzymatic" properties of both the hexose transporter and hexokinase (Carruthers, 1990). With this analysis the *lack* of any change in apparent K_m allows us to reasonably conclude that Glc affinity is relatively unaltered. Reciprocal and offsetting change in the K_m's of the carrier and hexokinase are an unlikely possibility.

[4] GLUT-1 overexpressed in *Xenopus* oocytes is inhibited (≥50%) by 10 mM pentobarbital (Kushmerick and Haspel, unpublished observations).

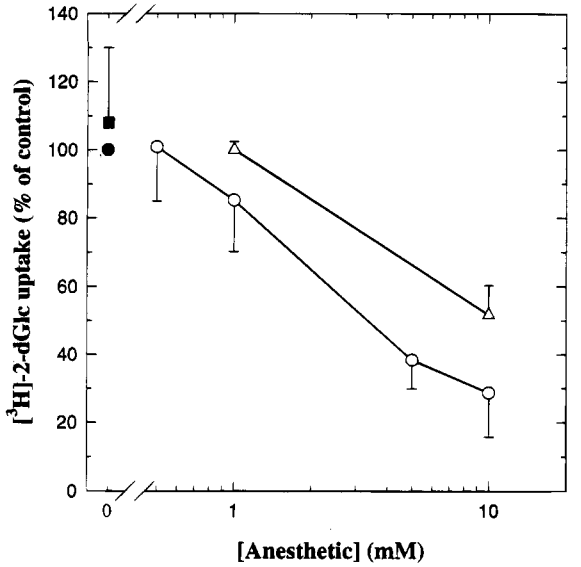

FIGURE 5: Barbiturates inhibit hexose transport in human erythrocytes. Isolated human erythrocytes were preincubated at 4 °C in TB containing the indicated concentrations of pentobarbital (○), barbital (△), 0.4% (v/v) EtOH (■, vehicle), or no additions (●, control). Unidirectional influx of [^{3}H]-2-dGlc was then assayed at 4 °C as described in the Experimental Procedures, in triplicate, for 30 s in the continued presence of the indicated agents. Results are expressed as % of control. The means of individual experiments ($N = 3$ for barbital, and $N = 5$ for pentobarbital and vehicle) are presented. Each data set is normalized to its respective control. Error bars are ±SD.

Barbiturates Inhibit Hexose Transport in Human Erythrocytes. (A) Unidirectional Influx. Hexose transport in human erythrocytes is mediated predominately by GLUT-1 (Carruthers, 1990). The effect of barbiturates on hexose transport in human erythrocytes was examined in two ways. Unidirectional influx of [^{3}H]-2-dGlc into human erythrocytes was assayed at 4 °C. This was done to slow uptake of dGlc and enabled us to more accurately determine the extent of influx. However, even under these experimental conditions untreated erythrocytes are at ~80% of equilibrium at 30 s of uptake (not shown), and these deviations from linearity with time should be considered. Uptake of [^{3}H]-2-dGlc by erythrocytes treated with different concentrations of pentobarbital or barbital was examined (Figure 5). Both pentobarbital and barbital significantly inhibit unidirectional influx of dGlc. The IC_{50}'s for inhibition of hexose transport in human erythrocytes are ~3 and ≥10 mM for pentobarbital and barbital, respectively. Furthermore, an anesthetic concentration of EtOH [i.e., 0.4% (v/v)] does not significantly inhibit unidirectional influx of dGlc.

(B) Equilibrium Exchange Influx. Glc influx into human erythrocytes under equilibrium exchange conditions was also examined (Table 1). This assay employed [^{3}H]-2-dGlc as a radioactive tracer of hexose flux and can be used to more accurately, relative to unidirectional influx (Figure 5), determine the *absolute* half-times ($\tau_{1/2}$) for Glc equilibration at 23 °C. If a simple carrier model is assumed, then the data fit a first-order exponential (Carruthers, 1990). The $\tau_{1/2}$ for equilibrium exchange influx of Glc in control erythrocytes is ~10 s. This value agrees with previous determinations (Haspel *et al.*, 1985b). In contrast, the $\tau_{1/2}$ determined for erythrocytes treated with cytochalasin B (25 μM), a potent inhibitor of hexose transport (Baldwin, 1993), is ~150 s and, as expected, is equivalent to ~95% inhibition. The $\tau_{1/2}$'s determined for erythrocytes treated with 10 mM pentobarbital, 1 mM pentobarbital, or 0.4% (v/v) EtOH are ~28, 14, and 13 s, respectively. These results demonstrate that barbiturates can inhibit hexose transport in human erythrocytes by ~60% and indicate that hexose transporter function is affected by this class of general anesthetic. The results also suggest that barbiturates can interact with GLUT-1.[4,5]

Table 1: Pentobarbital Inhibits Equilibrium Exchange Influx of Glc in Human Erythrocytes[a]

agents	concn (mM)	$\tau_{1/2}$ (s) (expt A, B)	Glc influx (% of control)[b] (expt A, B)
none	nd	10, 11	100, 100
EtOH	68[c]	nd, 13	nd, 85
pentobarbital	1	14, nd	71, nd
pentobarbital	10	25, 32	40, 34
cytochalasin B	0.025[d]	138, 171	7, 6

[a] Isolated human erythrocytes were loaded (1 h, 37 °C) with 130 mM Glc in TB as described in the Experimental Procedures and preincubated at 23 °C in TB containing 130 mM Glc and the indicated agents. Equilibrium exchange influx of Glc was than assayed at 23 °C as described in detail in the Experimental Procedures in the continued presence of the indicated agents. Results are expressed as half-times of equilibrium exchange influx ($\tau_{1/2}$) for each experiment, A or B, and results of individual experiments are presented (nd, indicates not determined). [b] Calculated as follows: $100 \times [K_{app}(agent)/K_{app}(none)]$; K_{app}'s are the apparent rate constants of the simple first-order exponentials. [c] Equivalent to 0.4% (v/v) EtOH. [d] Added from a 50 mM stock in Me_2SO.

Barbiturates Interact with Purified GLUT-1. The fluorescence intensity of GLUT-1 is known to decrease upon D-Glc binding (Gorga & Lienhard, 1982; Baldwin, 1993). We observed similar concentration-dependent quenching of the intrinsic fluorescence of GLUT-1 upon titration with D-Glc (0−200 mM) (Figure 6A). L-Glc (200 mM) was unable to quench the intrinsic fluorescence of GLUT-1 (not shown). This demonstrates the expected stereospecificity of GLUT-1 for Glc (Carruthers, 1990). A small red shift in the emission spectra (not shown) was also observed upon Glc binding. The K_d and Q_{max} for this saturable process are 30 mM and 0.41, respectively, and these values agree qualitatively with those previously reported (Gorga & Lienhard, 1982; Carruthers, 1990). In the two site model, this K_d has generally been attributed to the apparent affinity of the inner Glc binding site (Carruthers, 1990). The Q_{max} we report is somewhat larger than that previously observed (Gorga & Lienhard, 1982), but this may be attributable to the greater sensitivity of our instrument, the excitation wavelength employed, and the procedures used to reduce scattered light. These results indicate that our preparation of GLUT-1 is functionally intact.

The effects of pentobarbital (0−15 mM) and EtOH (0.4%) on the intrinsic fluorescence of GLUT-1 were examined in the presence and absence of 200 mM D-Glc (Figure 6B). Pentobarbital quenches the intrinsic fluorescence of GLUT-1 in a concentration-dependent fashion, and Glc did not alter the quenching. No significant shift in emission maximum is observed (not shown). The K_d and Q_{max} for this saturable

[5] Band 3-mediated anion exchange by human erythrocytes was assayed as $^{35}SO_4^{2-}$ uptake (Haspel *et al.*, 1985b). Preliminary results indicate that 10 and 1 mM pentobarbital inhibit anion exchange by ~60% and ~20% [38.7 ± 2.6% and 79.3 ± 2.3% of control at 5 min of uptake (mean ± SD, $N = 4$)], respectively (not shown).

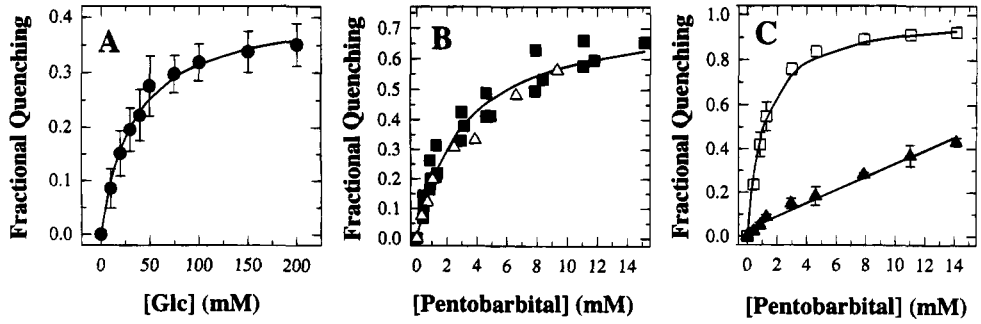

FIGURE 6: Pentobarbital interacts with purified GLUT-1. GLUT-1-containing proteoliposomes (~20 μg/mL) (panels A and B), BSA (20 μg/mL) (panel C), or Trp (200 ng/mL) (panel C) in FB were incubated with Glc (0−200 mM) or pentobarbital (0−15 mM) as indicated. Fluorescence emission spectra were recorded as described in the Experimental Procedures, and fractional quenching was calculated. The mean of five experiments is presented in panel A (●), and the error bars are ±SD. Individual determinations from four similar experiments are presented in panel B, and the △ and ■ represent measurements in the presence and absence of 200 mM Glc, respectively. The mean of two experiments is presented in panel C, the error bars are ±SD, and the □ and ▲ represent results for BSA and Trp, respectively. The curves were computer fit to simple hyperbolic functions of saturable binding except for the Trp curve which was fit by linear regression. The calculated Q_{max}'s derived from these fits are 0.41, 0.75, and 1.0 for panels A, B, and C (BSA results), respectively. The calculated K_d's derived from these fits are 30 mM, 3 mM, and 1 mM for panels A, B, and C (BSA results), respectively.

process are 3 mM and 0.75, respectively. This affinity and lack of dependence on Glc binding is in reasonable agreement with the hexose transport results (Figures 2, 3, and 5). Barbital (10 mM) also quenched (~25%) the intrinsic fluorescence of GLUT-1 (not shown). Mild trypsinization of intact GLUT-1 inactivates the carrier functionally (Baldwin, 1993). Mild trypsinization of GLUT-1 did not alter the K_d and Q_{max} for pentobarbital (not shown). Ethanol (0.4%) did not greatly alter the fluorescent intensity or emission maxima (not shown).

In a similar manner, pentobarbital was able to quench the intrinsic fluorescence of BSA (Figure 6C). The K_d and Q_{max} for this saturable process are 1 mM and 1.0, respectively. As expected, this result is consistent with the binding of barbiturates to albumin measured by equilibrium dialysis (Nillson *et al.*, 1976). This supports, to some extent, this experimental approach. In contrast, the quenching of Trp fluorescence by pentobarbital (Figure 6C) exhibits a linear concentration dependence consistent with collisional quenching.

Using 2AS and 12AS as fluorescent probes, the effect of pentobarbital on the packing of the acyl chains of the lipid bilayer (Scarlata, 1991) of the GLUT-1 containing proteoliposomes was also examined (not shown). Pentobarbital (10 mM) did not alter the emission spectra of either of these bilayer embedded probes. This suggests that the quenching by barbiturates of the intrinsic fluorescence of GLUT-1 is not mediated simply by a change in the physical state of the membrane lipid. Taken in concert, these results suggest that barbiturates are able to interact directly with GLUT-1 protein and this interaction is not altered by Glc occupancy or mild proteolytic inactivation.

DISCUSSION

We have demonstrated that barbiturates can inhibit hexose transport in cultured cells (Figures 1−4) and human erythrocytes (Figure 5 and Table 1). Under our experimental conditions, this inhibition is relatively rapid, not readily reversible, not prevented by the presence of albumin (1% w/v), not temperature-dependent, and not dependent on the level of cell surface expression of hexose transporters. Inhibition of hexose transport by barbiturates follows to a first approximation the Meyer-Overton rule (Franks & Lieb, 1990, 1987) correlating lipid solubility with potency. The concentrations at which *partial inhibition* is observed are also consistent with the estimated brain levels of barbiturates in both animal and clinical studies (Richter & Waller, 1975; Richter & Holtman; 1982). This inhibition is also distinct in that two other anesthetics, namely, halothane and ethanol, do not greatly inhibit hexose transport (Figures 2 and 5). Furthermore, barbiturates decrease the apparent V_{max} but not the apparent K_m of hexose transport (Figure 3), and this inhibition is similar in multiple cell types (Figures 4 and 5 and Table 1).[4] This suggests that barbiturates are altering either the number of functional hexose transporters or the intrinsic activity of the carriers. A change in the number of functional hexose transporters might require membrane trafficking events as demonstrated for insulin-stimulated hexose transport or protein synthesis and/or degradation as demonstrated for growth/nutritional regulation of hexose transport (Bell *et al.*, 1993; Baldwin, 1993). Given the demonstrated rapidity of the inhibition of hexose transport by barbiturates, its lack of temperature dependence, its independence of the cell surface expression of the hexose transporter, and the observed effects in human erythrocytes, these processes seem unlikely to be involved. An alteration in the intrinsic activity of GLUT-1 is more consistent with the characteristics of this inhibition. In this regard, we have demonstrated that barbiturates quench the intrinsic fluorescence of purified GLUT-1 protein and that this quenching is independent of Glc occupancy and mild proteolytic inactivation (Figure 6). The properties of this interaction fail to correlate with a change in the physical state of the membrane lipids. This suggests that barbiturates interact directly with GLUT-1 and that this interaction somehow alters hexose transport. Taken in concert, these results suggest that it is the ability of GLUT-1 to translocate Glc across the membrane, not Glc binding *per se*, which is sensitive to barbiturates and that the site of barbiturate binding is distinct from the substrate binding sites of the carrier. On the basis of thermodynamic, kinetic, mutational, and structural considerations both Baldwin (1993) and Carruthers (1990) have expressed the notion that the translocation step itself may be quite sensitive to subtle conformational changes in the carrier protein.

Our conclusion that hexose transporter activity is altered by barbiturates *contrasts* with that of Okazaki *et al.* (1992), who suggested that Glc phosphorylation by hexokinase is involved in the effect of barbiturates on dGlc uptake. We observe linear uptake of dGlc with time in the presence or absence of barbiturate (Figure 1), while Okazaki *et al.* (1992) observed some minor nonlinearity. Importantly, the barbiturate and radiolabeled sugar were added simultaneously in the Okazaki *et al.* (1992) study, while in our experiment (Figure 1) the cells were preincubated for 15 min with anesthetic before dGlc uptake was assayed. We cannot exclude that in their system a slight change in the inhibition with time of exposure might explain this difference. More significantly, Okazaki *et al.* (1992) report that the uptake of 3-O-MeGlc, a relatively nonmetabolizable Glc analog (Ortiz & Haspel, 1993), is not altered by barbiturates and used this observation to give further support to the notion of phosphorylation, not transport, being altered by barbiturates. We attempted to confirm these observations but found that 3-O-MeGlc equilibrates rapidly (<30 s) when assayed at 4 °C (not shown) and believe that this may explain the inability of Okazaki *et al.* (1992) to observe inhibition of 3-O-MeGlc uptake at 22 °C. Assays of 3-O-MeGlc uptake in confluent monolayers of cultured cells require careful corrections for extracellular trapping and are hampered by technical problems associated with both the rapid equilibration times of 3-O-MeGlc and the low levels of uptake [cf. Whitesell *et al.* (1990)]. The observation of inhibition by barbiturates of hexose transport by both human erythrocytes (Figure 5 and Table 1) and *Xenopus* oocytes overexpressing GLUT-1[4] also supports our conclusion that barbiturates act on the hexose transport process itself and not at a distal step. Further support for this conclusion can be found in our demonstration of a direct interaction of barbiturates with purified GLUT-1 protein (Figure 6). Additional *in vivo* support for this concept can be found in studies demonstrating that the transfer of 3-O-MeGlc across the BBB is decreased by barbiturates (Otsuka *et al.*, 1991a).

The implications of our findings to the physicochemical mechanisms involved in anesthetic action need to be addressed. As discussed by Franks and Lieb (1987, 1990), anesthetics will in general obey the Meyer-Overton rule, but this correlation with lipophilicity is often restricted to a series of analogs of a particular class of anesthetics. In other words, the correlation with hydrophobicity is limited and suggests some specificity with respect to the targets of different classes of anesthetics. For instance, both barbiturates and halothane have been shown to modulate K^+ channels in a variety of preparations (Pancrazio, 1993). The types of K^+ channels affected by these anesthetics are, however, clearly distinct. Several other examples of such specificity with regard to anesthetic class have also been reported [cf. Asano and Ogasawara (1981, 1982) and Rooney *et al.* (1993)]. The octanol/water partition coefficients of halothane and most barbiturates are not sufficiently different (Firestone *et al.*, 1986) to explain this unusual specificity. This suggests that the barbiturate moiety may itself impart some specificity with respect to the multiple targets[5] of this class of anesthetic.

Barbiturates, as opposed to volatile hydrocarbons such as halothane, are amphipathic in nature (Richter & Holtman, 1982). Because of this property, barbiturates probably partition into membranes at or near the head groups of the phospholipids comprising the membrane bilayer. In contrast, halothane may partition into the hydrocarbon core of the bilayer and not the membrane surface. The differential affects of these two classes of anesthetics could then be explained by differential sensitivity of their targets. Some membrane proteins could be more sensitive to anesthetics which adsorb to the membrane interface, while others could be more sensitive to anesthetics which dissolve into the membrane interior. We suggest that the former would be more sensitive to barbiturates, while the latter would be more sensitive to volatile hydrocarbons. Conceptually, this effect of barbiturates on membrane permeability is reminiscent of early studies by Andersen *et al.* (1976) on the effects of small dipolar organic molecules such as phloretin on membrane permeability. In these studies a role for interfacial dipole potentials having differential effects on diverse permeability pathways was indirectly implicated as part of the physicochemical mechanism. In our case, more specific interactions with putative barbiturate binding sites on the affected membrane proteins should also be considered. It is, however, important to reconsider all of these concepts in the continued pursuit of the underlying mechanisms involved in barbiturate action.

Finally, it should be stated that our observations are most likely not the fundamental basis of barbiturate-induced anesthesia. The findings do, however, have broad mechanistic implications for the wide utility of barbiturates in neurosurgery. It has been suggested that postoperative problems associated with elevated intracranial pressures could, at least in part, be curtailed by a reduction in cerebral metabolism [Michenfelder, 1978; Michenfelder *et al.*, 1976; and see Todd and Warner (1992) for a critical discussion]. With respect to this, a barbiturate-induced reduction in cerebral blood flow and metabolism has been observed *in vivo* (Gjedde & Rasmussen, 1980; Ingvar *et al.*, 1980; Nilsson & Seisjo, 1975). Conventional wisdom holds that barbiturates lower brain neuroactivity (i.e., action potentials, neurotransmitter release, etc.) and in this way decrease cerebral blood flow. Recently, this has also been attributed, at least in part, to a reduction in Glc transfer across the BBB caused by direct inhibition of the Glc carriers of the endothelial cells (Otsuka *et al.*, 1991a). Since the major Glc carrier of these endothelial cells is GLUT-1 (Pardridge, 1991), our observations hint that the inhibition of GLUT-1 hexose transporters by barbiturates may be quite relevant to the clinical pharmacology of neurosurgical anesthesia. It will be interesting to determine if GLUT-1 exhibits greater sensitivity to barbiturates than the other hexose transporter isoforms (Bell *et al.*, 1993).[4,5] These observations also suggest that screening of barbiturates for their effects on hexose transport both *in vivo* and *in vitro* would be quite informative and could lead to improvements in the protocols employed in obtaining the "cerebral protection" required during neurosurgery.

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