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Characterization of Barbiturate-stimulated Chloride Efflux from Rat Brain Synaptoneurosomes

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Abstract

Membrane chloride (Cl^-) permeability was studied in a novel subcellular brain preparation, the synaptoneurosome. Using a radioactive tracer exchange technique, Cl^- transport was determined by measuring $^{36}Cl^-$ efflux from rat cerebral cortical synaptoneurosomes. Barbiturates increased $^{36}Cl^-$ efflux in a dose-dependent manner with the following relative order of potency: 5-(1,3-dimethylbutyl)-5-ethyl barbituric acid ((−)-DMBB) > pentobarbital > secobarbital > (+)-DMBB > hexobarbital > amobarbital > mephobarbital. Phenobarbital and barbital were virtually inactive. A good correlation was observed between the potencies of these barbiturates in stimulating $^{36}Cl^-$ efflux and their anesthetic potencies in mice ($r = 0.90$, $p < 0.01$) and their abilities to enhance [^{3}H] diazepam binding to brain membranes ($r = 0.77$, $p < 0.05$). The effect of pentobarbital in enhancing $^{36}Cl^-$ efflux was reversed by the γ-aminobutyric acid (GABA) antagonists picrotoxin and bicuculline. Picrotoxin and bicuculline both decreased $^{36}Cl^-$ efflux in the absence of pentobarbital, suggesting the presence of endogenous GABA. Incubation of synaptoneurosomes with 4,4′-di-isothiocyano- or dinitro-2,2′-disulfonic acid stilbene, inhibitors of anion transport, also decreased both basal and pentobarbital-induced $^{36}Cl^-$ efflux. Pentobarbital (500 μM) was most effective in inducing $^{36}Cl^-$ efflux in the cerebellum, hippocampus, and cortex (23.7, 23.6, and 22.5%, respectively), and was less effective in stimulating $^{36}Cl^-$ efflux in the striatum (15.1%) and pons-medulla (6.2%). The relative efficacy of pentobarbital in enhancing $^{36}Cl^-$ efflux among these various brain regions was highly correlated ($r = 0.96$, $p = 0.01$) with the relative densities of [^{35}S]-t-butylbicyclophosphorothionate-binding sites, a measure of GABA-gated Cl^- channel density. These data suggest that pharmacologically relevant Cl^- transport associated with the benzodiazepine/GABA/Cl^- ionophore receptor complex can be measured in a subcellular preparation from brain, the synaptoneurosome.

Barbiturates are among the most extensively studied central nervous system depressants and are commonly used as anesthetics, sedative/hypnotics, anxiolytics, and anticonvulsants (Harvey, 1975). The pharmacological actions of barbiturates appear to be due to their ability to potentiate γ-aminobutyric acid (GABA)-mediated inhibition of synaptic neurotransmission (Nicoll et al., 1975; Ransom and Barker, 1975; Haefely et al., 1979) and/or inhibit excitatory synaptic neurotransmission (Barker and Ransom, 1978a, b; Nicoll and Wojtowicz, 1980). Electrophysiologic studies have shown that barbiturates inhibit neuronal excitability by enhancing GABA-mediated chloride (Cl^-) conductance as well as by directly increasing Cl^- conductance (Barker and Ransom, 1978a, b; Mathers and Barker, 1980; Nicoll and Wojtowicz, 1980). Furthermore, barbiturates reverse the antagonism of GABA-activated Cl^- conductance produced by convulsants such as picrotoxin and bicuculline (Barker and Ransom, 1978; Bowery and Dray, 1978).

The molecular mechanisms mediating the actions of barbiturates on GABAergic synaptic activity have also been investigated. Barbiturates have been shown to enhance GABA receptor binding in a Cl^--dependent fashion (Olsen and Snowman, 1982). However, barbiturates do not bind to the GABA recognition site itself but rather to a site which is closely associated with the Cl^- ionophore (Olsen, 1982). Receptor-binding studies have indicated that barbiturates inhibit the binding of [^{3}H]dihydropicrotoxinin and [^{35}S]-t-butylbicyclophosphorothionate (TBPS) to sites that have also been linked to the Cl^- ionophore (Ticku and Olsen, 1978; Olsen, 1982; Squires et al., 1983; Ramanjaneyulu and Ticku, 1984). Although electrophysiologic and receptor-binding studies have provided evidence for the functional coupling of barbiturate/picrotoxin-binding sites and the GABA receptor/Cl^- ionophore complex, attempts to directly study these interactions using biochemical techniques have generally been unsuccessful due to methodologic limitations in measuring Cl^- transport in cell-free systems. Recently, GABA-mediated Cl^- transport has been studied in intact cultured chick embryo cerebral neurons (Thampy and Barnes, 1984) and in rat hippocampal slices (Wong et al., 1984). In the latter study, barbiturates were shown to induce $^{36}Cl^-$ flux, as well as to enhance the effect of GABA on $^{36}Cl^-$ flux. We have recently reported the use of a novel subcellular preparation (the "synaptoneurosome") from rat brain to study $^{36}Cl^-$ efflux (Schwartz et al., 1984). Ultrastructural and biochemical studies have shown that this preparation contains both pre- and postsynaptic membranes which form closed vesicles that retain a number of receptor-mediated properties and maintain an electrochemical gradient (Hollingsworth et al., 1984, 1985). In a preliminary report, we observed that pentobarbital enhanced $^{36}Cl^-$ efflux from synaptoneurosomes prepared from rat cerebral cortex in a picrotoxin-sensitive fashion (Schwartz et al., 1984). In the present study, we report in detail the characterization of barbiturate-induced $^{36}Cl^-$ efflux from

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rat brain synaptoneurosomes. Our results suggest that the effect of barbiturates in stimulating $^{36}Cl^-$ efflux from synaptoneurosomes is mediated via barbiturate/picrotoxin recognition sites that are coupled to the benzodiazepine/GABA receptor.

Materials and Methods

Preparation of synaptoneurosomes. Synaptoneurosomes were prepared from cerebral cortices of adult, male Sprague-Dawley rats (200 to 250 gm) as previously described (Hollingsworth et al., 1985). Cerebral cortices were dissected free from white matter, and approximately 1 gm of tissue was homogenized in 7 ml of buffer containing 20 mM HEPES-Tris, 118 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, and 2.5 mM $CaCl_2$ (pH 7.4) using a glass-glass homogenizer (five strokes). The homogenate was transferred to a 40-ml tube, diluted with 30 ml of ice-cold (0 to 4°C) buffer, and then filtered by gravity through three layers of nylon mesh (160 μm, TETKO Inc., Elmsford, NY) placed in a Millipore Swinex filter holder. The filtrate was then gently pushed through a 10-μm Millipore filter (LCWP 047) using a 10-ml syringe. Care was taken to prevent the development of hydrostatic pressure in the filter which would result in large shear forces on the tissue. The filtered preparation was centrifuged at 1000 × *g* for 15 min. After discarding the supernatant the pellet was gently resuspended in buffer to a final protein concentration of 10 mg/ml unless otherwise specified. Protein was determined by the method of Lowry et al. (1951).

Measurement of chloride-36 efflux. $^{36}Cl^-$ efflux was studied using a modification of the methods described by Kasai and Changeux (1971) for measurement of cation flux in *Torpedo* microsacs and by White and Miller (1981) for measurement of $^{36}Cl^-$ efflux in *Torpedo* membrane vesicles. Synaptoneurosomes (10 mg of protein/ml) were incubated with $^{36}Cl^-$ (5 μCi/ml, specific activity, 12.5 mCi/gm; New England Nuclear, Boston, MA) for 60 min at 0 to 4°C. Aliquots of synaptoneurosomes (60 μl) equivalent to approximately 0.6 mg of protein were then diluted 50-fold with buffer of the same composition as described above at 25°C, unless otherwise specified, in the presence or absence of various drugs. After rapid mixing, the diluted suspension was filtered at various time intervals under vacuum through Whatman GF/C filters which were immediately washed with 5 ml of ice-cold buffer. The filters were placed in 7 ml of Ready-Solv (Beckman Instruments, Fullerton, CA) scintillation fluid and counted at an efficiency of >75%. The amount of $^{36}Cl^-$ retained by the synaptoneurosomes at time zero was obtained by filtering an undiluted aliquot of preincubated synaptoneurosomes followed by washing as described above. The loss or efflux of $^{36}Cl^-$ from the preincubated synaptoneurosomes was determined by measuring the amount of radioactivity retained by the filters. Typically the amount of $^{36}Cl^-$ retained at time zero represented approximately 3% of the $^{36}Cl^-$ added to the synaptoneurosomes (20,000 cpm). In certain experiments, rubidium-86 ($^{86}Rb^+$) efflux was also measured (3 μCi/ml, specific activity, ~1.9 mCi/mg; New England Nuclear), in a manner similar to that previously described using a synaptosomal preparation (Albuquerque et al., 1981). (±)-Bicuculline methiodide (Pierce Chemical Co., Rockford, IL) was dissolved in buffer immediately before assay. (−)- and (+)-5-(1,3-dimethylbutyl)-5-ethyl barbituric acid, (DMBB) were the gift of Dr. Kenner Rice; 4,4′-dinitro-2,2′-disulfonic acid stilbene was the generous gift of Dr. Iaov Cabantchik. All other drugs were obtained from commercial sources.

Results

The dilution (50-fold) of synaptoneurosomes preincubated with $^{36}Cl^-$ resulted in a rapid loss of $^{36}Cl^-$ retained in the filtered membranes (Fig. 1). As previously observed (White and Miller, 1981), such dilution results in an exchange of $^{36}Cl^-$ for nonradioactive Cl^- via a time-dependent diffusion process. The exchange or "loss" of $^{36}Cl^-$ from the synaptoneurosomes is referred to as $^{36}Cl^-$ efflux. As early as 5 sec after dilution, approximately 40%, 66%, and 73% of the $^{36}Cl^-$ initially retained by the synaptoneurosomes was lost when dilution was carried out at 0°C, 25°C, and 37°C, respectively (Fig. 1). The initial rapid phase(s) of $^{36}Cl^-$ efflux[2] and the slower phase which followed were both dependent on the temperature of the dilution buffer (Fig. 1). The efflux of $^{36}Cl^-$ at 25°C reached equilibrium by 40 min (see Fig. 3) and, although increasing or decreasing the temperature of the dilution buffer resulted in a corresponding change in the equilibrium time, the amount of $^{36}Cl^-$ retained at equilibrium was the same (data not shown), indicating that equilibrium represents a temperature-dependent rate of exchange of $^{36}Cl^-$.

In order to determine whether $^{36}Cl^-$ might be nonspecifically sequestered or bound to the vesicular membranes, various conditions that would disrupt the integrity of the formed vesicles were examined. As early as 10 sec after dilution in hypotonic buffer (5 mM Tris, pH 7.4) or in the detergent sodium dodecyl sulfate (SDS, 0.5%) only 35% and 9%, respectively, of the $^{36}Cl^-$ was retained compared to synaptoneurosomes diluted in isotonic buffer (Table I). In addition, preincubation of the preloaded synaptoneurosomes at 60°C (for 3 min) resulted in an 86% loss of $^{36}Cl^-$ when measured 10 sec after dilution (Table I).

The amount of $^{36}Cl^-$ retained at 2 min after dilution was also dependent on the concentration of protein; a linear relationship between the amount of $^{36}Cl^-$ retained and protein concentration (prior to dilution) was observed between 2 and 17 mg of protein/ml

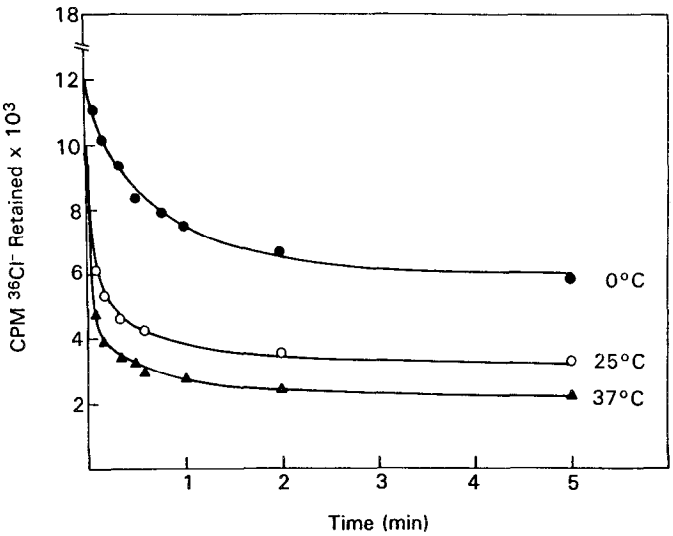

Figure 1. Effect of temperature on the time course of $^{36}Cl^-$ efflux from rat cerebral cortical synaptoneurosomes. Synaptoneurosomes preincubated with $^{36}Cl^-$ (at 0°C) were subsequently diluted with buffer at 0°C, 25°C, or 37°C and filtered at various times as described under "Materials and Methods." Data are from a single experiment performed in triplicate and are representative of three experiments.

TABLE I

Effects of various conditions on $^{36}Cl^-$ efflux from cerebral cortical synaptoneurosomes

Synaptoneurosomes preincubated with $^{36}Cl^-$ were diluted in either buffer, 5 mM Tris-HCl, pH 7.4, or 0.5% SDS and filtered 10 sec after dilution as described under "Materials and Methods." Prior to dilution an aliquot of preloaded $^{36}Cl^-$ synaptosomes was heated as indicated and placed back on ice. Dilution and filtration were carried out as described under "Materials and Methods." Data are the means ± SEM from three experiments performed in quadruplicate.

Condition	$^{36}Cl^-$ Efflux at 10 sec (% decrease from basal)
Hypotonic lysis (5 mM Tris-HCl)	64.4 ± 10.9
SDS	90.3 ± 0.7
Tissue heated at 65°C × 3 min	86.4 ± 3.8

[2] It is possible that the initial rapid loss of $^{36}Cl^-$ (within 5 sec) consists of multiple phases. In fact, in similar experiments where $^{22}Na^+$ efflux was measured in *Torpedo* microsacs, the dilution-induced loss of $^{22}Na^+$ was characterized by a complex decay curve consisting of several exponentials (Kasai and Changeux, 1971). In the present study the efflux of $^{36}Cl^-$ measured between 5 and 300 sec can be best fit to a single exponential decay curve, using the equation: $f(t) = \Delta y\ (-kt) + y_e$ (adapted from Grover, 1984) where t = time, Δy = cpm at 5 sec minus cpm at equilibrium time, and y_e = cpm at equilibrium time, and using a nonlinear curve fitting program (Knott, 1979).

(data not shown). A concentration of 10 mg of protein/ml was routinely used in subsequent experiments.

In a previous report we observed that dilution of cerebral cortical synaptoneurosomes in buffer containing pentobarbital results in an enhancement of $^{36}Cl^-$ efflux with an EC_{50} value of approximately 200 μM (Schwartz et al., 1984). The effect of pentobarbital (500 μM) on the time course of $^{36}Cl^-$ efflux is shown in Figure 2. Within the first 5 sec, pentobarbital stimulated $^{36}Cl^-$ efflux by approximately 22% compared to synaptoneurosomes diluted in the absence of drug (basal efflux). As equilibrium was approached, the amount of $^{36}Cl^-$ retained in the presence or absence of pentobarbital approached the same value (Fig. 2). In separate experiments in which the synaptoneurosomes were hypotonically lysed either during preincubation with $^{36}Cl^-$ or during dilution, pentobarbital had no effect on $^{36}Cl^-$ efflux (data not shown). In order to determine whether the pentobarbital-enhanced $^{36}Cl^-$ efflux occurred via a nonspecific perturbation of the membrane, the effects of pentobarbital on $^{86}Rb^+$ efflux were also measured. Pentobarbital (0.3 to 3 mM) had no significant effect on $^{86}Rb^+$ efflux from nondepolarized synaptoneurosomes (Table II). However, in veratrine-depolarized synaptoneurosomes, pentobarbital, at concentrations greater than 300 μM, decreased $^{86}Rb^+$ efflux in a dose-dependent manner (Table II). Phencyclidine (100 μM) also decreased veratrine-stimulated $^{86}Rb^+$ efflux (not shown) in a manner similar to that reported by Albuquerque et al. (1981) using synaptosomes.

Pentobarbital-enhanced membrane permeability to chloride could also be studied by measuring $^{36}Cl^-$ uptake in synaptoneurosomes (Schwartz et al., 1985b). In these experiments larger signals were obtained with similar doses of pentobarbital; however 3 to 5 times the amount of $^{36}Cl^-$ was needed to perform these assays, making them expensive.

The effects of a series of anesthetic and/or sedative barbiturates in altering $^{36}Cl^-$ efflux were also examined. In each case the pharmacologically active anesthetic/sedative barbiturates produced a dose-dependent enhancement of $^{36}Cl^-$ efflux (Fig. 3). (−)-DMBB, pentobarbital, secobarbital, and (+)-DMBB were among the most potent barbiturates studied, whereas phenobarbital and barbital were virtually inactive. The effect of the barbiturates in enhancing $^{36}Cl^-$ efflux is stereospecific since (−)-DMBB was more potent than (+)-DMBB (Fig. 3) (at 300 μM, $p < 0.05$). Significantly, previous studies have shown that barbiturates enhance the binding of benzodiazepine receptor agonists to the benzodiazepine receptor in proportion to their anesthetic potencies (Skolnick et al., 1981, 1982; Leeb-Lundberg and Olsen, 1982). The potencies of the various barbiturates tested in enhancing $^{36}Cl^-$ efflux were correlated with their anesthetic potencies in mice (Butler, 1942) ($r = 0.90$, $p < 0.01$, Fig. 4*A*) and with their abilities in enhancing [^{3}H]diazepam binding to benzodiazepine receptors in rat cerebral cortical membranes (Leeb-Lundberg and Olsen, 1982) ($r = 0.77$, $p < 0.05$, Fig. 4*B*).

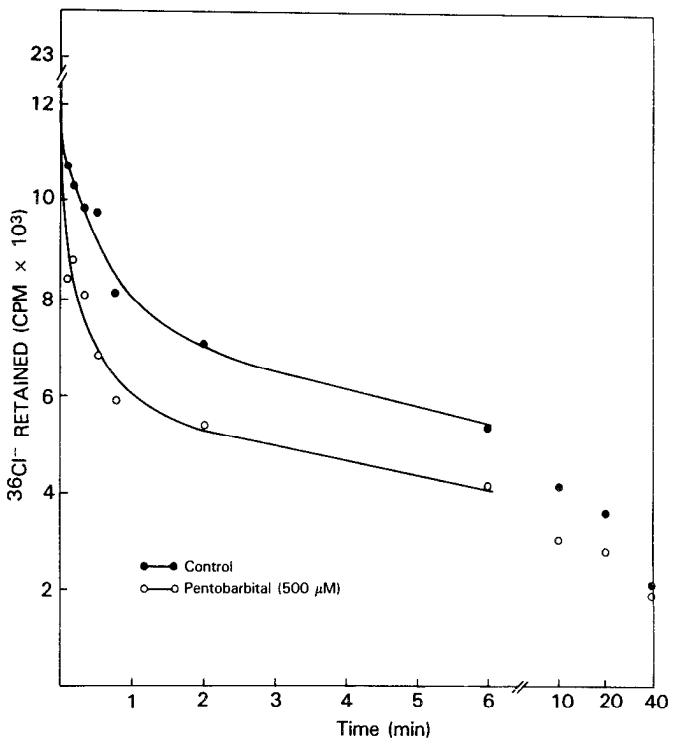

Figure 2. Effect of pentobarbital on $^{36}Cl^-$ efflux from cerebral cortical synaptoneurosomes. Synaptoneurosomes were preincubated with $^{36}Cl^-$, diluted in the presence or absence of pentobarbital (500 μM), and filtered at various times after dilution as described under "Materials and Methods." Data are from a single experiment performed in triplicate and are representative of four to seven experiments.

TABLE II

The effects of pentobarbital on $^{86}Rb^+$ efflux from cerebral cortical synaptoneurosomes

Synaptoneurosomes were incubated with $^{86}Rb^+$ (3 μCi/ml) for 1 hr at 0°C. Aliquots (0.3 mg of protein) were diluted in buffer (50-fold) containing pentobarbital in the absence or presence of veratrine (50 μM). The suspensions were filtered 2 min after dilution as described under "Materials and Methods." Data are the means ± SEM for a single experiment performed in quadruplicate and are representative of two to four experiments. The cpm $^{86}Rb^+$ retained during the basal and veratrine-depolarized conditions were 13,819 ± 421 and 7,358 ± 121, respectively.

Pentobarbital (mM)	$^{86}Rb^+$ Retained (% of basal)	$^{86}Rb^+$ Retained (% of veratrine-depolarized)
0	100.0 ± 3.0	100.0 ± 1.6
0.3	91.8 ± 3.7	98.3 ± 0.9
0.5	99.0 ± 0.7	110.1 ± 1.7[a]
1.0	90.9 ± 2.8	113.2 ± 1.8[a]
3.0	89.0 ± 3.4	150.6 ± 4.5[a]

[a] $p < 0.01$ compared to the veratrine control group (Student's *t* test).

Studies with the GABA receptor/Cl^- ionophore antagonists picrotoxin and bicuculline demonstrated that the pentobarbital-sensitive $^{36}Cl^-$ efflux was reversed by both picrotoxin and bicuculline (Fig. 5, *A* and *B*). In addition, both picrotoxin and bicuculline decreased basal $^{36}Cl^-$ efflux in the absence of pentobarbital (Fig. 5, *A* and *B*). The EC_{50} values for picrotoxin and bicuculline in inhibiting basal $^{36}Cl^-$ efflux were approximately 70 μM and 150 μM, respectively (data not shown). Occasionally, the antagonists had no effect on basal $^{36}Cl^-$ efflux, yet they still decreased the pentobarbital-induced $^{36}Cl^-$ efflux (data not shown). In related experiments, the glycine antagonist strychnine (1 mM) had no effect on either basal or pentobarbital-induced $^{36}Cl^-$ efflux (data not shown).

The ability of pentobarbital to enhance $^{36}Cl^-$ efflux from synaptoneurosomes prepared from various brain regions was also examined. Stimulation of $^{36}Cl^-$ efflux by pentobarbital (500 μM) was greatest in the cerebellum, hippocampus, and cortex, moderate in the striatum, and least in the pons-medulla (Table III). The effects of pentobarbital in stimulating $^{36}Cl^-$ efflux in these brain regions is highly correlated with the regional distribution of [^{35}S]TBPS-binding sites as reported by Squires et al (1983) ($r = 0.96$, $p = 0.01$, Fig. 6).

The effect of 4,4′-di-isothiocyano-2,2′-disulfonic acid stilbene (DIDS), which has been reported to irreversibly inhibit anion transport in red blood cells (Cabantchik et al., 1978), was also examined. DIDS (200 μM) completely prevented the pentobarbital-induced efflux of $^{36}Cl^-$ and, like the GABA antagonists, DIDS decreased $^{36}Cl^-$ efflux in the absence of pentobarbital (Fig. 7). The EC_{50} value for DIDS in inhibiting basal $^{36}Cl^-$ efflux was approximately 75 μM (not shown). However, in some experiments in which the disulfonyl stilbene had no effect on basal $^{36}Cl^-$ efflux, an inhibition of pentobarbital-induced $^{36}Cl^-$ efflux was still observed (Table IV). Similar results were obtained with a more specific and reversible anion transport inhibitor, 4,4′-dinitro-2,2′-disulfonic acid stilbene (DNDS) (30 μM to 1 mM) (Barzilay and Cabantchik, 1979) (Table IV).

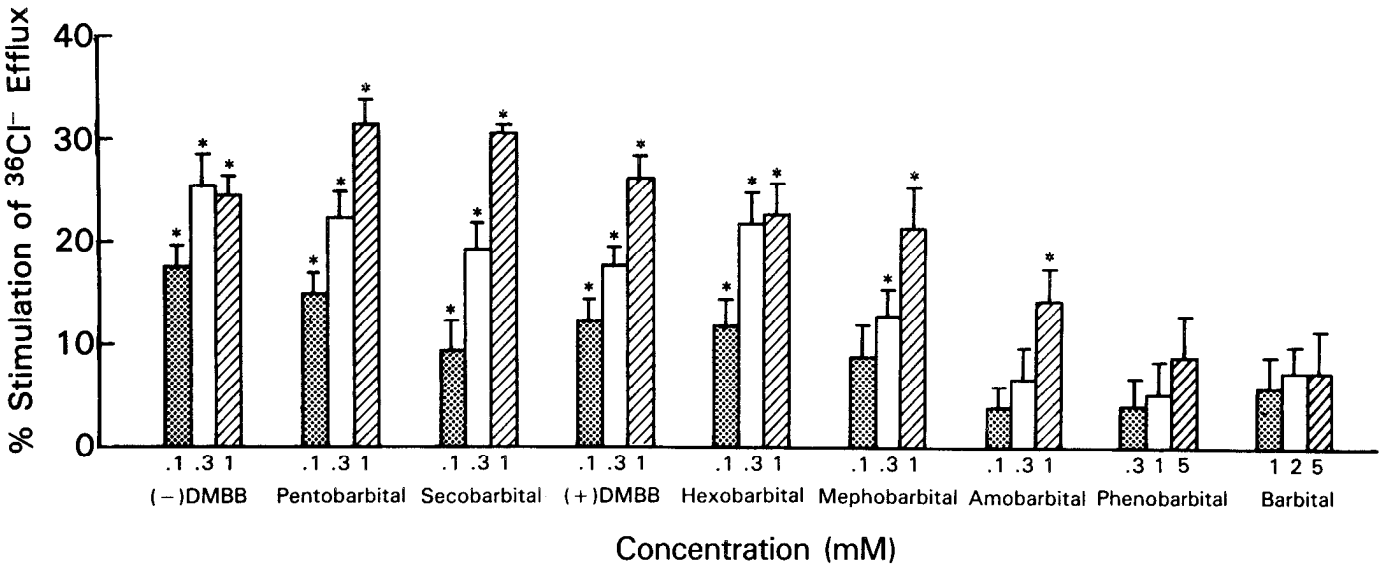

Figure 3. Effect of various barbiturates on $^{36}Cl^-$ efflux from rat cerebral cortical synaptoneurosomes. Synaptoneurosomes were preincubated with $^{36}Cl^-$, diluted in the presence or absence of various barbiturates, and filtered 2 min later as described under "Materials and Methods." Data are the means ± SEM for three to nine experiments performed in quadruplicate. *, $p < 0.01$ compared to basal $^{36}Cl^-$ efflux for each experiment (Student's t test).

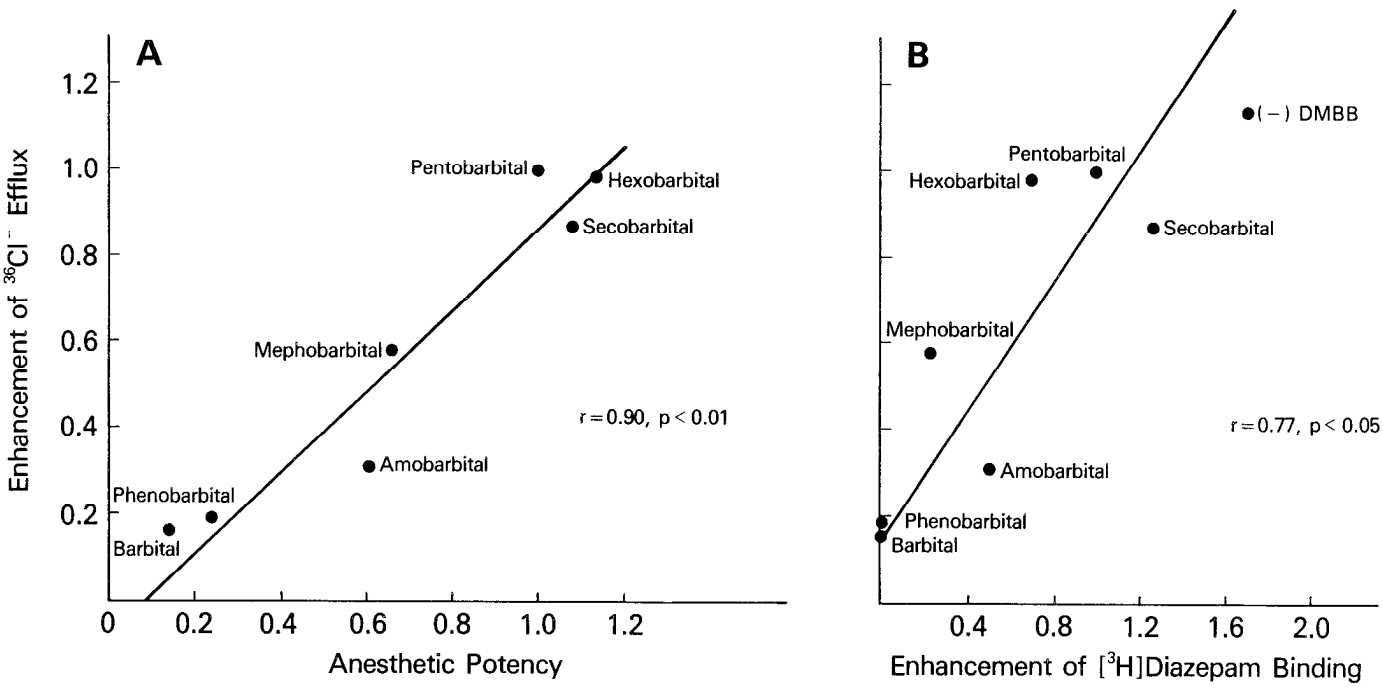

Figure 4. Correlation of barbiturate-enhanced $^{36}Cl^-$ efflux with anesthetic potency in mice and barbiturate-enhanced [^{3}H]diazepam binding in rat brain. The percentage of stimulation of $^{36}Cl^-$ efflux from cerebral cortical synaptoneurosomes by various barbiturates (300 μM) relative to pentobarbital was correlated in *A* with the anesthetic dose (AD_{50}) in mice relative to pentobarbital (Butler, 1942) ($r = 0.90$, $p < 0.01$) and in *B* with the percentage of enhancement of [^{3}H]diazepam binding in cerebral cortical membranes by barbiturates (200 μM) relative to pentobarbital (Leeb-Lundberg and Olsen, 1982) ($p < 0.077$, $p < 0.05$). Significance was determined using Pearson's product moment.

Discussion

To date, there have been few biochemical studies of neurotransmitter-gated Cl^- transport in brain. Although $^{36}Cl^-$ uptake and efflux have recently been reported in cultured chick embryonic cerebral neurons (Thampy and Barnes, 1984) and rat hippocampal slices (Wong et al., 1984), respectively, the development of a suitable technique for studying Cl^- transport in a subcellular brain preparation has been more difficult. Methodologic limitations associated with these studies include highly permeable or "leaky" membrane preparations as well as a lack of intact postsynaptic vesicular structures. Recently, we reported the successful use of a synaptoneurosomal preparation from cerebral cortex to study pentobarbital-induced $^{36}Cl^-$ efflux (Schwartz et al., 1984). This preparation, which has been defined and characterized using morphologic and biochemical techniques (Hollingsworth et al., 1984, 1985), is a modification of the vesicular preparation originally described by Chasin et al. (1974) to study adenylate cyclase activity, cyclic AMP accumulation (Daly et al., 1980), and the effects of depolarizing agents on membrane potential (Creveling et al., 1980). Synaptoneurosomes represent resealed membrane fragments and, on the basis of ultrastructural analysis, consist of synaptosomes and synaptosomes attached to

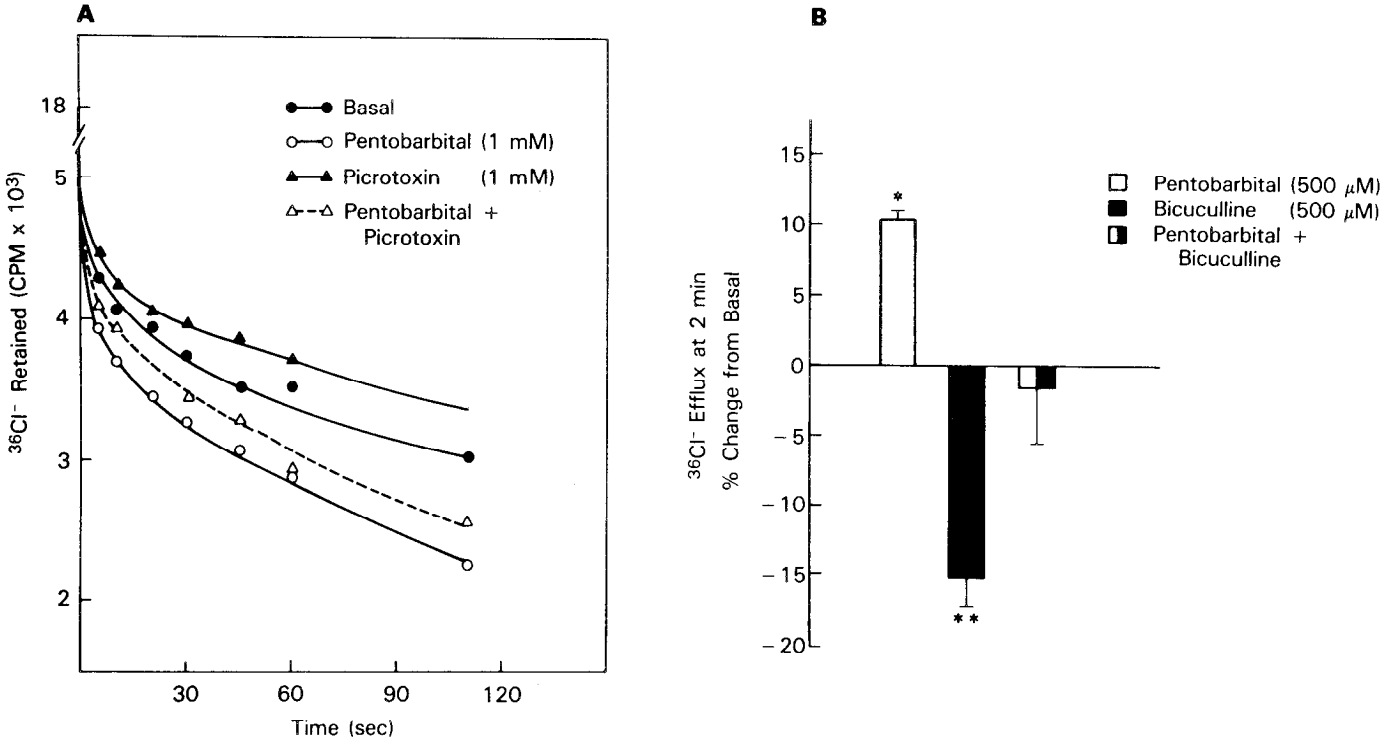

Figure 5. Effect of pentobarbital and picrotoxin (*A*) and bicuculline (*B*) on $^{36}Cl^-$ efflux from cerebral cortical synaptoneurosomes. Synaptoneurosomes preincubated with $^{36}Cl^-$ were diluted with the drugs indicated. The suspensions were filtered at various times after dilution (*A*) or at 2 min after dilution (*B*). Data in *A* are the means of five to eight replications from a single experiment and are representative of three experiments. Data in *B* are the means ± SEM of three to five experiments performed in quadruplicate. *, $p < 0.02$; **, $p < 0.001$ compared to basal (Student's *t* test).

TABLE III

Pentobarbital-enhanced $^{36}Cl^-$ efflux in various brain regions

Synaptoneurosomes from different brain areas preincubated with $^{36}Cl^-$ were diluted in the absence or presence of pentobarbital (500 μM). Two minutes later the suspensions were filtered as described under "Materials and Methods." Data are the means ± SEM for a single experiment performed in quadruplicate, and are representative of three experiments.

Brain Region	Pentobarbital-enhanced $^{36}Cl^-$ Efflux (% stimulation at 2 min)
Cerebellum	23.7 ± 0.7[a]
Hippocampus	23.6 ± 0.9[a]
Cortex	22.5 ± 0.1[a]
Striatum	15.1 ± 0.5[a]
Pons-medulla	6.2 ± 0.9[b]

[a] $p < 0.005$ (Student's *t* test) compared to basal $^{36}Cl^-$ efflux from each brain region.

[b] $p < 0.05$ (Student's *t* test) compared to basal $^{36}Cl^-$ efflux from each brain region.

postsynaptic membrane fragments which have resealed in a vesicular fashion ("neurosome," Hollingsworth et al., 1984). In these preparations two distinct populations of vesicles are observed by electron microscopy, one with a fairly uniform diameter of approximately 0.6 μm (synaptosome) and a larger vesicle with a diameter of 1.0 μm (neurosome) (Hollingsworth et al., 1985). It is possible that the mild homogenization conditions used in preparing synaptoneurosomes and/or the presence of intact postsynaptic vesicular structures are two factors that make this preparation useful for studying receptor-mediated Cl^- transport.

Membrane permeability to ions such as Na^+, Cl^-, and Rb^+ has been studied in a variety of vesicular preparations using the dilution method described here (Kasai and Changeux, 1971; Albuquerque et al., 1981; White and Miller, 1981). The dilution initiates the exchange of radioactive ion with nonradioactive ion and, thus, reflects the membrane permeability to the ion in question. The effects of drugs and neurotransmitters on the membrane permeability of ions is superimposed on the dilution-induced efflux. Using this technique, White and Miller (1981) have studied Cl^- permeability of membrane vesicles prepared from the electric organ of *Torpedo californica*. The time course for $^{36}Cl^-$ exchange in their study is similar to the time course of $^{36}Cl^-$ efflux observed in the present study using synaptoneurosomes. In our experiments, the earliest time point at which $^{36}Cl^-$ efflux could be reliably measured was 5 sec, and it is apparent from Figure 2 that pentobarbital has already increased the rate of $^{36}Cl^-$ efflux by the earliest time point. In fact, the parallel nature of the efflux curves (cf. Fig. 2) suggests that the major portion of the $^{36}Cl^-$ efflux induced by pentobarbital occurs within 5 sec. These data are consistent with the rapid increase in membrane Cl^- conductance induced by barbiturates observed electrophysiologically (Barker and Ransom, 1978b). It is unlikely that pentobarbital is acting by displacing $^{36}Cl^-$ from membrane-bound anion-binding sites since pentobarbital had no effect in lysed synaptoneurosomes. The effect of pentobarbital appears to be relatively selective for Cl^- transport as evidenced by the lack of effect on $^{86}Rb^+$ efflux under resting conditions. These data, coupled with data that pentobarbital enhances $^{36}Cl^-$ efflux with varying efficacy in various brain regions and data that the pentobarbital-induced efflux is reversed by picrotoxin and bicuculline (see below), suggest that pentobarbital is not causing a nonspecific membrane perturbation which would result in an increase in membrane Cl^- permeability. However, the ability of pentobarbital to decrease $^{86}Rb^+$ efflux in depolarized synaptoneurosomes suggests that barbiturates may also act at K^+ channels to decrease K^+ conductance. It should be pointed out that phencyclidine (PCP), which also has anesthetic properties, decresed $^{86}Rb^+$ efflux from depolarized synaptoneurosomes in a manner similar to that observed in synaptosomes (Albuquerque et al., 1981). It has been suggested that PCP may act at K^+ channels in brain (Albuquerque et al., 1981).

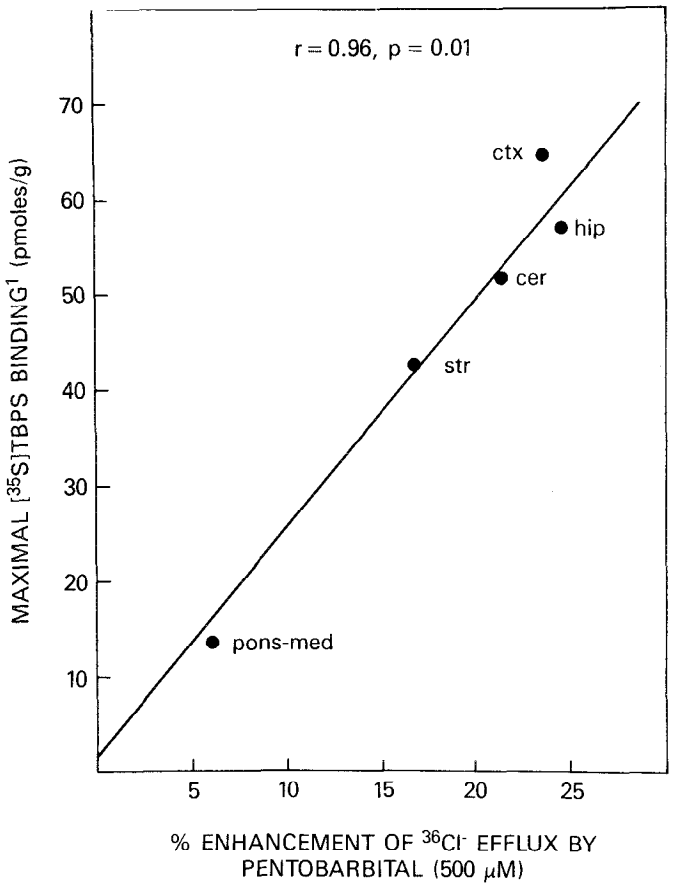

Figure 6. Correlation of pentobarbital-enhanced $^{36}Cl^-$ with maximal [^{35}S]TBPS binding (Squires et al., 1983) in various brain regions. $^{36}Cl^-$ efflux data are the mean of three experiments performed in quadruplicate ($r = 0.96$, $p = 0.01$, Pearson's product moment). *cer*, cerebellum; *ctx*, cortex; *hip*, hippocampus; *pons-med*, pons-medulla, *str*, striatum.

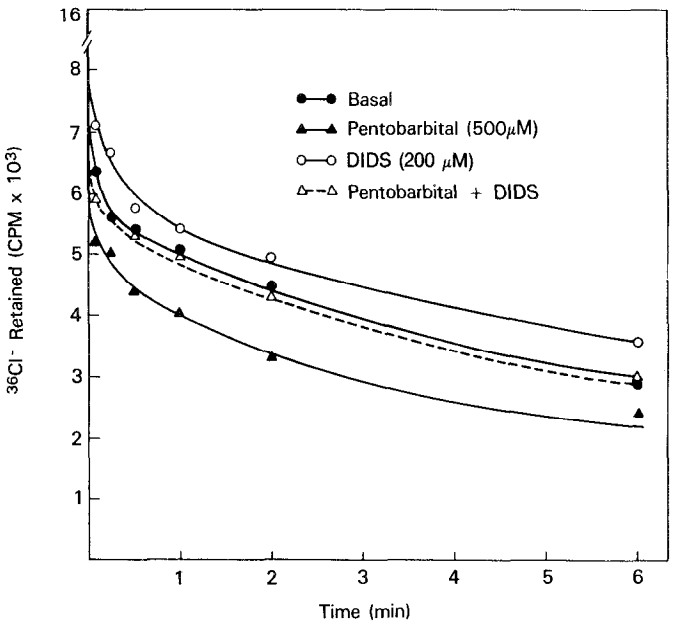

Figure 7. Effect of pentobarbital and DIDS on $^{36}Cl^-$ efflux from cerebral cortical synaptoneurosomes. Synaptoneurosomes preincubated with $^{36}Cl^-$ were diluted in buffer containing pentobarbital (500 μM), DIDS (200 μM), or both and filtered at various times after dilution as described under "Materials and Methods." Data are from a single experiment performed in quadruplicate and are representative of four experiments.

TABLE IV

Effect of disulfonyl stilbenes on pentobarbital-enhanced $^{36}Cl^-$ efflux

Synaptoneurosomes preincubated with $^{36}Cl^-$ were diluted with the drugs indicated and filtered 2 min later as described under "Materials and Methods." Data are from two separate experiments performed in quadruplicate.

Drug	Stimulation of $^{36}Cl^-$ Efflux (%)
Pentobarbital (500 μM)	16.5 ± 2.5
DIDS (200 μM)	2.5 ± 4.8
DIDS + pentobarbital	5.2 ± 2.7[a]
Pentobarbital (500 μM)	26.4 ± 1.3
DNDS (100 μM)	2.3 ± 2.8
DNDS + pentobarbital	19.5 ± 3.1[a]

[a] $p < 0.05$ compared to pentobarbital (Student's t test).

The effects of pentobarbital to increase $^{36}Cl^-$ efflux especially at concentrations > 500 μM could also be due to a leakage of GABA from the synaptosomal vesicles. However, this is unlikely, since most evidence suggests that barbiturates in high concentrations decrease the release of GABA (for review, see Richter and Holtman, 1982).

Several other barbiturates were also shown to enhance $^{36}Cl^-$ efflux, and these effects were correlated with their potencies as anesthetics (see Fig. 4*A*) (Butler, 1942). In addition, these barbiturates stimulate $^{36}Cl^-$ efflux with a relative potency that is similar to their abilities to enhance GABA-mediated inhibition of hippocampal neuronal activity (Dunwiddie and Worth, 1984; Olsen et al., 1985) and GABA and benzodiazepine receptor binding in brain membranes (see Fig. 4*B*) (Leeb-Lundberg and Olsen, 1982; Olsen and Snowman, 1982; Skolnick et al., 1982). These correlations further support the concept of a functional coupling between benzodiazepine/GABA receptors and the barbiturate recognition sites associated with the Cl^- ionophore in the regulation of membrane Cl^- permeability (Paul et al., 1981; Olsen, 1982; Skolnick and Paul, 1982).

[^{35}S]TBPS, a GABA antagonist cage convulsant, has been reported to label a site on the Cl^- ionophore (Squires et al., 1983), and the labeling of this site by [^{35}S]TBPS is allosterically modulated by barbiturates, benzodiazepines, and GABA (Supavilai and Karobath, 1984; Ramanjaneyulu and Ticku, 1984). The good correlation observed between the magnitude of the pentobarbital-enhanced $^{36}Cl^-$ efflux and the density of [^{35}S]TBPS-binding sites (Squires et al., 1983) in various brain regions ($r = 0.96$, Fig. 7) supports the notion that the barbiturate-induced efflux of $^{36}Cl^-$ is due to activation of the GABA-associated Cl^- channel. The regional distribution of the pentobarbital-induced $^{36}Cl^-$ efflux is also similar to the regional brain distribution of binding sites for benzodiazepines, GABA, and picrotoxinin in brain (Mohler and Okada, 1978; Ticku et al., 1978).

Wong et al. (1984) have suggested that in hippocampal slices barbiturates may also activate a GABA-insensitive Cl^- ionophore since the maximal effect of barbiturate on $^{36}Cl^-$ efflux exceeded that induced by GABA. Since high concentrations of barbiturates were used, it is also possible that a direct effect of barbiturates on Cl^- permeability was measured. We have observed a similar phenomenon with pentobarbital versus muscimol- or GABA-induced $^{36}Cl^-$ efflux using the synaptoneurosome preparation (Schwartz et al., 1985b).

Since basal $^{36}Cl^-$ efflux is inhibited by bicuculline and picrotoxin, it is likely that endogenous GABA is present in sufficient concentration to contribute to basal $^{36}Cl^-$ efflux. The concentrations of picrotoxin and bicuculline (10 to 1000 μM) that decrease basal $^{36}Cl^-$ efflux in synaptoneurosomes are similar to those that directly increase the excitability of cultured mouse spinal neurons (Barker et al., 1984; Barker and Ransom, 1978b). The convulsants picrotoxin and bicuculline also decrease the pentobarbital-induced inhibition of cultured spinal neurons (Barker and Ransom, 1978b; Bowery and Dray, 1978). Similarly, both of these drugs antagonize the pentobarbital-induced $^{36}Cl^-$ efflux in synaptoneurosomes.

The presence of endogenous GABA in the vesicular preparation may be a limitation to this technique, as it has been in many studies relating to the GABA/chloride ionophore. The variation in the amount of endogenous GABA between experiments could account for the variation in the ability of the antagonists to decrease basal $^{36}Cl^-$ efflux. Efforts to remove endogenous GABA, either enzymatically or by repeated washing of the synaptoneurosomes, have met with limited success (unpublished observations). Another limitation of this flux assay is the relatively small absolute signal produced by the drugs. Thus, near-maximal concentrations of drugs such as pentobarbital, bicuculline, and picrotoxin are often necessary to attain statistically significant changes in efflux. Evidence from our laboratory indicates that better signals may be obtained using a $^{36}Cl^-$ uptake assay (Schwartz et al., 1985b) although the latter is considerably more expensive due to higher concentrations of $^{36}Cl^-$ employed. Nevertheless, the use of the synaptoneurosome preparation to study receptor-mediated chloride transport appears to be promising. For example we have recent evidence to show that picrotoxin inhibition of pentobarbital-induced $^{36}Cl^-$ efflux differs in two strains of mice (Schwartz et al., 1985a, b) that have different seizure sensitivity to picrotoxinin (Seale et al., 1985).

The increase in Cl^- permeability induced by pentobarbital was also shown to be sensitive to the disulfonyl stilbenes, DIDS and DNDS, which are both inhibitors of the anion transport protein in red blood cells (Cabantchik et al., 1978; Barzilay and Cabantchik, 1979). There is some evidence that the disulfonyl stilbenes might also act on the Cl^- ionophore associated with the benzodiazepine/GABA receptor complex. Costa et al. (1981) have reported that anion-dependent [^{3}H]diazepam binding in rat brain membranes is inhibited by DIDS. Although these findings do not prove that the effect of disulfonyl stilbenes is acting directly on the GABA-associated Cl^- ionophore, the evidence presented here suggests that a DIDS-sensitive ion transporter may be associated with the GABA/Cl^- ionophore. However, in our experiments, DIDS decreased Cl^- permeability in the absence of pentobarbital, and, thus, we may simply be observing a phrmacological antagonism of the pentobarbital-induced $^{36}Cl^-$ efflux by DIDS rather than a true biochemical interaction. Nevertheless, in several experiments, DIDS and DNDS, in concentrations which had no effect on basal $^{36}Cl^-$ efflux, reversed the pentobarbital-induced $^{36}Cl^-$ efflux. It should be noted that another disulfonyl stilbene, 4-acetamido-4′-isothiocyano-2,2′-disulfonic acid stilbene (SITS), has been shown to decrease $^{36}Cl^-$ uptake in cultured chick cerebral neurons, without effecting GABA-induced $^{36}Cl^-$ uptake (Thampy and Barnes, 1984). The reasons for the discrepancy in these results is unclear but could be related to differences in the tissue preparations used. Since the disulfonyl stilbenes have become useful tools for studying anion transport in a variety of systems (Thomas, 1976; Pollard et al., 1977; Cabantchik et al., 1978; White and Miller, 1981), our data indicate that these compounds might also be used to study the molecular properties of the Cl^- ionophore associated with the GABA receptor. However, much more work will be necessary to ascertain whether a DIDS-sensitive anion transporter is associated with the GABA/Cl^- ionophore.

The technique described here for measuring barbiturate-induced $^{36}Cl^-$ efflux has enabled us to study for the first time in a cell-free system the functional interactions between activation of barbiturate/picrotoxin-binding sites and the resultant changes in Cl^- permeability. The functional properties of other components of the benzodiazepine/GABA/Cl^- ionophore receptor complex are currently being studied using this technique. Furthermore, the possibility that the benzodiazepine/GABA/Cl^- ionophore receptor complex can be regulated by well known post-translational modifiers (phosphorylation, glycosylation) like the nicotinic cholinergic receptor (Teichberg et al., 1977; Vandlen et al., 1979) can now be examined.

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