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Interactions of Barbiturates of Various Pharmacological Categories with Benzodiazepine Receptors

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SUMMARY

Numerous barbiturates, such as (±)-pentobarbital, reversibly enhance the affinity for equilibrium binding of [^{3}H]diazepam to well-washed rat cortical membranes in a chloride-dependent and picrotoxinin-sensitive manner [Leeb-Lundberg *et al.*, *Proc. Natl. Acad. Sci. U. S. A.* 77:7468–7472 (1980)]. The chemical specificity and stereospecificity of this barbiturate effect *in vitro* has been examined in detail for comparison with pharmacological actions of barbiturates as central nervous system depressants and modulators of inhibitory transmission mediated by γ-aminobutyric acid. One group of barbiturates, chemically and pharmacologically related to (±)-pentobarbital, such as (±)-secobarbital, (±)-dimethylbutyl barbituric acid, (−)-dimethylbutyl barbituric acid, amobarbital, and (+)-and (−)-pentobarbital, behaved similarly in this system. These compounds enhanced [^{3}H]diazepam binding to the same maximal level (about 125% above baseline), with EC_{50} values ranging from 30 to 300 μM ("full agonists"). Although (−)-isomers of pentobarbital and dimethylbutyl barbituric acid were more potent than the racemic pair, the (+)-isomer of pentobarbital was still equally efficacious. All of the active compounds in this series are nervous system depressants with anesthetic/hypnotic activity, but some of them additionally show excitatory activity *in vivo*. Another group of barbiturates, all with N^1-methyl substitutions, showed a stereospecific enhancement of [^{3}H]diazepam binding, but with a maximal effect lower than that of the (±)-pentobarbital series. In three cases, the isomer having more central nervous system depressant activity, (+)-hexobarbital, (−) N^1-methyl, 5-phenyl, 5-propyl barbituric acid, and (−)-mephobarbital, enhanced [^{3}H]diazepam binding, but to a maximal level varying from 35% to 75%. In addition, these agents reduced the (±)-pentobarbital enhancement down to their own maximal level, suggestive of a "partial agonist/antagonist" action. The pharmacologically weak (−)-hexobarbital had no effect on [^{3}H]diazepam in the absence or presence of pentobarbital, a lack of activity shared by the compound barbital. (+)-Mephobarbital and the excitatory barbiturate (+)-methyl, phenyl, propyl barbituric acid did not enhance equilibrium [^{3}H] diazepam binding on their own, but they reversed the enhancement by (±)-pentobarbital completely and competitively. This "antagonist" property was shared by another group of barbiturates typified by phenobarbital and (±)-metharbital. The decrease in the K_D for the [^{3}H]diazepam binding induced by (±)-pentobarbital was due to a saturable decrease both in the dissociation rate constant (k_{-1}) and in the association rate constant (k_1), with the effect on k_{-1} predominating. The K_D values calculated from the kinetic constants measured in the absence or presence of (±)-pentobarbital were consistent with equilibrium binding parameters. The EC_{50} for (±)-pentobarbital to decrease k_{-1} was 100 μM. Phenobarbital, although inducing no apparent effect on equilibrium [^{3}H]diazepam binding, also caused a saturable decrease in both k_{-1} and k_1, with an EC_{50} on k_{-1} of 260 μM. However, the changes in the two rates were equal, therefore not altering the net value of K_D for [^{3}H]diazepam binding. Thus phenobarbital acts at the same receptor sites as pentobarbital in this *in vitro* system, but in a somewhat different manner. These results suggest that barbiturates can be divided into different groups depending on the type of interaction with benzodiazepine receptor binding. The qualitatively different types of interaction with [^{3}H]diazepam binding *in vitro* suggest that the categories so defined might correspond to different categories of pharmacological actions of barbiturates as anesthetics,

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hypnotics, convulsants, anticonvulsants, or inactive. Testing this theory will require more quantitative information.

INTRODUCTION

Barbiturate depressants have had significant impact on the therapy of several convulsive disorders (1). They also possess properties that make them useful as drugs for relieving anxiety and for inducing sedation, sleep, and general anesthesia. Their precise molecular mechanism of action is not yet understood, although the general theory has been that barbiturate depressants potentiate inhibitory synaptic transmission (2, 3), attenuate excitatory synaptic transmission (3, 4), or block electrically excitable membranes (5). Even if some barbiturates at some doses affect all three mechanisms, recent electrophysiological results suggest that one of the major targets for these drugs in the central nervous system is inhibitory synaptic transmission, specifically that involving the neurotransmitter GABA[1] (2, 3, 6, 7). Several observations have shown that barbiturates might potentiate GABA transmission by either facilitating the release or blocking the uptake of GABA. However, these effects occur only at supratherapeutic concentrations (8). Barbiturates cause increased membrane chloride ion conductance responses very similar to those caused by GABA (2), as well as potentiating the postsynaptic response to GABA (3, 6, 7, 9) and specifically reversing the antagonism of GABA responses by picrotoxin (3, 7, 10), picrotoxin-like cage convulsants, and bicuculline (10).

Several central nervous system depressants, including barbiturates and pyrazolopyridines, inhibit the binding of the convulsant GABA synaptic antagonist dihydropicrotoxinin in a competitive manner and with potencies that roughly agree with pharmacological activity (11–13), suggesting that these drugs bind to a picrotoxin-sensitive receptor site that is closely associated with the GABA receptor-ionophore complex. The same depressant drugs also were able to perturb the binding of benzodiazepines (12–15) to receptor sites in mammalian brain membranes. High-affinity benzodiazepine binding sites show a specificity which correlates well with pharmacological potencies of the benzodiazepines (16), and the affinity of at least a portion of these sites is enhanced by GABA, suggesting a close coupling of GABA and benzodiazepine receptors (17). The picrotoxinin-sensitive enhancement by pyrazolopyridines (13, 15) and barbiturates (12–14) of benzodiazepine receptor binding shows that a receptor site for these drugs is also coupled to the GABA-benzodiazepine receptor complex. The effects on benzodiazepine receptors of pyrazolopyridines (15) and barbiturates (14) were specifically dependent on anions that can penetrate GABA-activated ion channels, and the barbiturates showed a chemical specificity and stereospecificity which correlated with anesthetic activity *in vivo* and the ability to enhance GABAergic transmission (14). Both barbiturates (18, 19) and pyrazolopyridines (20) also enhance specific Na^+ -independent [^{3}H]GABA binding in a manner resembling the interaction with benzodiazepine binding, further supporting the existence of a three-drug receptor-ionophore complex. Two of the receptors are for GABA and benzodiazepines, whereas the third drug receptor site binds barbiturates and related depressants and picrotoxinin and related convulsants.

We have examined further the effects of different barbiturates and their stereoisomers on equilibrium and kinetics of [^{3}H]diazepam binding. On the basis of their differential effects on [^{3}H]diazepam binding, barbiturates can be divided into different groups, possibly correlating with their different major pharmacological actions.

MATERIALS AND METHODS

N-[*methyl*-^{3}H]Diazepam (83.5 Ci/mmole), [21, 22-^{3}H] dihydrostrychnine (51.4 Ci/mmole), and [^{3}H]naloxone (45 Ci/mmole) were purchased from New England Nuclear Corporation (Boston, Mass). [*G*-^{3}H]Kainic acid (4 Ci/mmole) was purchased from Amersham Corporation (Arlington Heights, Ill.) *N*-(2′,3′-[^{3}H]Propyl)-*N*,*N*-dimethyl(-2-aminoethyl)benzilate (propylbenzilylcholine) was prepared as described by Hulme *et al.* (21). Nonradioactive diazepam was donated by H. Möhler, Hoffman-La Roche (Basel, Switzerland). Nonradioactive (±)-3-quinuclidinyl benzilate was donated by N. J. M. Birdsall, National Institute for Medical Research, London, England. The (+)- and (−)-hexobarbital, (+)- and (−)-MPPB and (+)- and (−)-mephobarbital were gifts of J. Knabe, University of Saarlandes (Saarlandes, Germany). The (+)- and (−)-isomers of pentobarbital were gifts of J. Barker, National Institutes of Health (Bethesda, Md.). (±)-DMBB, amobarbital, and (±)-secobarbital were gifts of Eli Lilly (Indianapolis, Ind.). CHEB was the gift of H. Downes, University of Oregon (Portland, Ore.). (−)-DMBB was a gift of R. Young, New England Nuclear Corporation. Barbital was a gift of Merck (Rahway, N. J.), (±)-mephobarbital and phenobarbital were gifts of Sterling-Winthrop (New York, N. Y.), and (±)-metharbital was a gift of Abbott Laboratories (New York, N. Y.). All other drugs and materials were obtained from commercial sources.

White male Sprague-Dawley rats (200–300 g) were decapitated and their brains were rapidly removed and bathed in ice-cold 0.32 M sucrose. Cerebral cortex was then removed and homogenized in 20 volumes of 0.32 M sucrose in a glass homogenizer fitted with a Teflon pestle (12 passes, 400 rpm). The homogenate was centrifuged at 2000 rpm (1000 × *g*) for 10 min at 0–4° (Beckman JA 17 rotor). The pellet (P_1) was discarded, and the supernatant fraction was centrifuged at 45,000 rpm (140,000 × *g*) for 45 min (Spinco rotor 60 Ti). The pellet ($P_2 + P_3$) was resuspended [Ultra-Turrax (Cincinnati, Ohio), 25 sec at setting 40%] in 25 volumes of ice-cold double-distilled water (osmotic shock) and centrifuged at 45,000 rpm for 45 min. The $P_2 + P_3$ pellet was washed again in buffer (0.2 M NaCl-10 mM sodium phosphate, pH 7.0 ± 0.1). The pellet was resuspended to a final protein concentration of 0.2–1.0 mg/ml in buffer.

[1] The abbreviations used are: GABA, γ-aminobutyric acid; MPPB, *N*′-methyl, 5-phenyl,5-propylbarbituric acid; DMBB, 5-ethyl,5-[1,3-dimethylbutyl]barbituric acid; CHEB, 5-ethyl,5-[2-cyclohexylideneethyl]barbituric acid.

Aliquots of the membrane suspension were incubated in triplicate for 60 min at 0° with 0.5 nM [^{3}H]diazepam with and without drugs in a total volume of 1 ml. At the end of this incubation, the membranes were trapped on Whatman GF/B filters. Two 2-ml additions of 0.2 M NaCl were added to the incubation vial and poured onto the filter. The filters were placed into plastic vials and counted in 5 ml of CytoScint-toluene, 2:1 v/v (WestChem Products) in a Beckman 3155T scintillation counter. Efficiency (44%) was routinely determined with [^{3}H]toluene. Background was determined in the presence of 10 μM unlabeled diazepam and usually accounted for 10% of the total radioactivity.

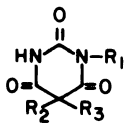
The binding of all other ligands was measured by centrifugation, using the same membrane fractions and assay buffer as described for [^{3}H]diazepam. Approximately 1 mg of membrane protein was incubated in a volume of 1.0 ml, without and with nonradioactive ligands (to estimate background) and 0.5 mM pentobarbital, for 15 min at 0°. Reactions were terminated by centrifugation for 10 min at 50,000 × *g* (20,000 rpm, Beckman rotor JA 20.1), followed by twice rinsing the pellets superficially with 2 ml of 0.2 M NaCl at 0°. The pellets were solubilized overnight in 0.2 ml of Soluene 350 (Packard), followed by radioactivity measurement in 3 ml of toluene containing 0.5% 2,5-diphenyloxazole, efficiency 35%.

Binding to muscarinic acetylcholine receptors was measured using [^{3}H]propylbenzilylcholine (10 nM) and 2 μM (±)-3 quinuclidinyl benzilate for background (21). The specific binding to background ratio was 5:1. Binding to opiate receptors (22) was measured with 1 nM [^{3}H] naloxone, estimating background, which was about 5%, with 10 μM nonradioactive morphine sulfate. [^{3}H]Kainic acid binding (23) was assayed with 5 nM radioactive ligand and estimating background with 0.1 mM kainic acid; the signal to noise ratio was 2.5. This ligand was incubated for 60 min rather than 15 min. [^{3}H]Dihydrostrychnine (1 nM) binding was assayed as described for spinal cord of rat (24) and various regions of pigeon nervous system (25). Using 0.1 mM nonradioactive strychnine for estimating background, the signal to noise

TABLE 1

Enhancement of [^{3}H]diazepam binding by various barbiturates

Rat cortex membranes were prepared and assayed for [^{3}H]diazepam binding (at 0.5 nM) with and without 200 μM of the various barbiturates shown. Percentage enhancement refers to the increase in [^{3}H]diazepam specifically bound in the presence of 200 μM barbiturate compared with that bound in the absence of barbiturate, divided by this control level of binding, × 100. The results are the average of at least three experiments that varied ≤10%. For a discussion of the experimental error and statistical significance, see legend to Fig. 1.



BARBITURATE	RING SUBSTITUTION			ENHANCEMENT
	R_1	R_2	R_3	(%)
(-)DMBB	H	$-CH_2CH_3$	$-CH(CH_3)CH_2CH(CH_3)_2$	110
(±)DMBB				102
(±)SECOBARBITAL	H	$-CH_2-CH=CH_2$	$-CH(CH_3)CH_2CH_2CH_3$	80
(-)PENTOBARBITAL	H	$-CH_2CH_3$	$-CH(CH_3)CH_2CH_2CH_3$	65
(±)PENTOBARBITAL				64
(-)MEPHOBARBITAL	CH_3	$-CH_2CH_3$		62
(+)HEXOBARBITAL	CH_3	$-CH_3$		46
(+)PENTOBARBITAL				38
AMOBARBITAL	H	$-CH_2CH_3$	$-CH_2CH_2CH(CH_3)_2$	32
(-)MPPB	CH_3	$-CH_2CH_2CH_3$		22
CHEB	H	$-CH_2CH_3$	$-CH_2-CH=$	18
(±)MEPHOBARBITAL				15
(-)HEXOBARBITAL				12
PHENOBARBITAL	H	$-CH_2CH_3$		0
(±)METHARBITAL	CH_3	$-CH_2CH_3$	$-CH_2CH_3$	0
BARBITAL	H	$-CH_2CH_3$	$-CH_2CH_3$	0
(+)MPPB				0
(+)MEPHOBARBITAL				0

ratio under our assay conditions was 0.75 in rat brain stem and 0.5 in rat cortex, with 85% of the specific binding displaceable by 1 mM glycine.

RESULTS

Numerous barbiturates increased the specific binding of [^{3}H]diazepam to well-washed fresh rat cortical membranes. Table 1 shows the degree of enhancement by 200 μM barbiturates of [^{3}H]diazepam binding measured at one subsaturating concentration. As previously reported (14), the enhancement was due to a change in affinity for [^{3}H]diazepam, without a change in the maximal number of binding sites. This effect was observed with numerous commonly employed hypnotic barbiturates such as pentobarbital, but also by some barbiturates with excitatory activity such as CHEB.

The maximal enhancement of equilibrium [^{3}H]diazepam binding by (±)-pentobarbital under the conditions employed was approximately 125% over baseline (225% of control). This maximal level of enhancement was also reached by several other barbiturates, all having similar structures. Figure 1 shows a concentration-dependence curve of (±)-secobarbital in the absence and presence of a constant nonsaturating amount of (±)-pentobarbital (a dose-response curve for (±)-pentobarbital is included for comparison). These drugs reached a common maximal level of enhancement of [^{3}H]diazepam binding, indicating that the effects of the two drugs are probably mediated through a common, mutually exclusive, receptor site mechanism. Amobarbital, (±)-DMBB, (−)-DMBB, and the (+)- and (−)-isomer of pentobarbital showed the same characteristics as (±)-secobarbital when combined with a constant, nonsaturating amount of (±)-pentobarbital. The only property that differed between these drugs was the respective EC_{50} value; these values were 30 μM [(−)-DMBB], 100 μM [(±)-secobarbital], 130 μM [(±)-pentobarbital], and 300 μM (amobarbital). (−)-Pentobarbital (120 μM) was more potent than the (+)-isomer (300 μM).

Not all barbiturates that stimulated [^{3}H]diazepam binding gave the same maximal response as (±)-pentobarbital. Three examples of barbiturates which are methylated at the N^1 nitrogen atom showed a stereospecific modulation of benzodiazepine receptor binding. Whereas their stereoisomers showed little or no effect, (−)-mephobarbital, (+)-hexobarbital, and (−)-MPPB showed enhancement of [^{3}H]diazepam binding, although to a lower maximal degree than (±)-pentobarbital. Figure 2A, B, and C shows the effects of (−)-MPPB, (−)-mephobarbital, and (+)-hexobarbital, respectively, in the presence of a constant nonsaturating amount of (±)-pentobarbital. These three compounds had EC_{50} values of 100 μM, 50 μM, and 150 μM, respectively, for their own effects. When an excess of one of these drugs was added concurrently with pentobarbital, the maximal enhancement was equal to that obtained with the drug which gave a lower max-

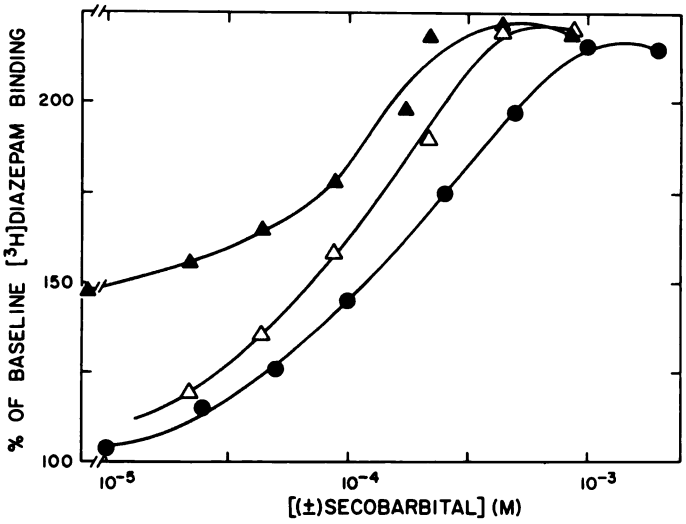


FIG. 1. *Concentration dependence for barbiturate "full agonists" interacting with benzodiazepine binding*

Rat cortex membranes were prepared and assayed for [^{3}H]diazepam binding (at 0.5 nM) as described under Materials and Methods. Varying concentrations of (±)-secobarbital, with (▲) and without (△) 100 μM (±)-pentobarbital. A dose-response curve for (±)-pentobarbital (●) is included for comparison.

In a typical example, the specific binding in the absence of barbiturates was 3810 ± 41 (1% error) cpm (4125 ± 31 cpm with radioactive ligand alone, 315 ± 27 cpm in the presence of 10 μM nonradioactive diazepam). The specific binding in the presence of 20 μM secobarbital was 4635 ± 32 (1% error) cpm, which corresponds to an enhancement over control of 22 ± 1%, and is statistically significantly different from the control according to Student's *t*-test ($p < 0.005$). The value in the presence of 20 μM secobarbital *plus* 100 μM pentobarbital was 6076 ± 105 (1.5% error) cpm, corresponding to a 59% enhancement. In general, with typical binding and error values similar to these, a difference in binding of 10% is significant according to the *t*-test ($p < 0.01$). Furthermore, all observations were typical of at least three experiments which varied by ≤ 10%.

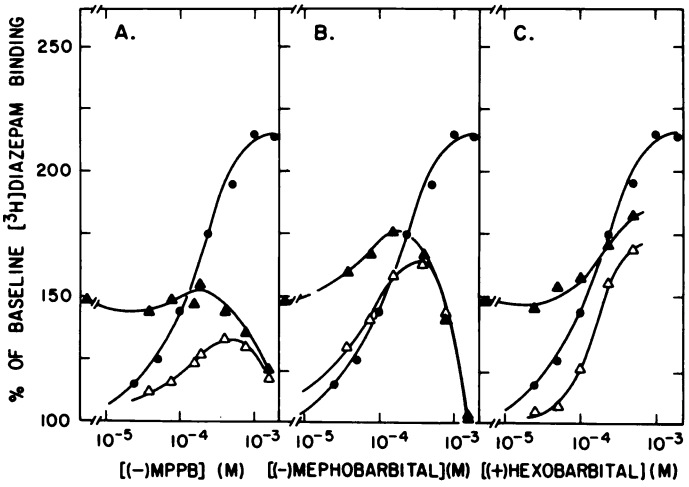


FIG. 2. *Concentration dependence for barbiturate "partial agonists/antagonists" interacting with benzodiazepine binding*

Rat cortex membranes were prepared and assayed for [^{3}H]diazepam binding (at 0.5 nM) as described under Materials and Methods. Varying concentrations of (A) (−)-MPPB, (B) (−)-mephobarbital, and (C) (+)-hexobarbital were included in the assays without (△) or with (▲) 100 μM (±)-pentobarbital. A dose-response curve for (±)-pentobarbital (●, full agonist) is included for comparison. The results are typical of three experiments in which individual points, EC_{50} values, and maximal effects varied ≤ 10%.

imal effect; i.e., high concentrations of these agents could actually lower the effect of pentobarbital in the manner of a partial agonist. This competitive effect was further evident when the concentration of (±)-pentobarbital was varied in the presence of a constant concentration of either (−)-MPPB, (−)-mephobarbital, or (+)-hexobarbital (data not shown). At higher concentrations of these drugs (1 mM), the [^{3}H]diazepam binding was not enhanced by (±)-pentobarbital above the level induced by the other agent alone.

The (+)-isomers of mephobarbital (pharmacology unknown) and MPPB [a convulsant, (26)], did not alter baseline [^{3}H]diazepam binding to any significant extent, but they definitely were able to compete with (±)-pentobarbital for its binding site. As can be seen in Fig. 3, both compounds reversed the enhancement caused by 100 μM pentobarbital with IC_{50} values of 200 μM and 100 μM, respectively. The inhibition curves were shallow, with some effects in the submicromolar range.

The (−)-isomer of hexobarbital was a very weak enhancer of [^{3}H]diazepam binding. This weak depressant drug, and also another non-enhancing compound, barbital, did not compete with (±)-pentobarbital enhancement. However, several other barbiturates which did not enhance [^{3}H]diazepam binding on their own also were able to reverse the effect of those which did enhance. Included in this group were compounds generally used for their anticonvulsant actions such as phenobarbital and (±)-metharbital. Figure 3 shows that these two barbiturates competed with (±)-pentobarbital in a strictly mass-action manner. The inhibition by phenobarbital was apparently competitive (K_i 200 μM) and affected only the affinity of benzodiazepine binding, not the number of binding sites (27).

As mentioned previously, enhancing barbiturates lower the K_D for [^{3}H]diazepam binding. This effect could be brought about by either decreasing the dissociation rate k_1 or increasing the association rate k_1 of the ligand-receptor complex. As Fig. 4A shows, 500 μM (±)-pentobarbital (a near-maximal concentration) decreased k_{-1} of [^{3}H]diazepam binding from 0.431 min^{-1} to 0.132 min^{-1}. However, Fig. 4B shows that k_1 was likewise decreased from 4.19×10^8 to 2.98×10^8 M^{-1} min^{-1}. If the decrease had occurred only in k_1, the equilibrium affinity constant would have been altered in the opposite direction. However, the additional change in k_{-1} not only neutralizes the effect in k_1, but also changes the net effect on K_d. The K_d values calculated from the kinetic rate constants, both with and without (±)-pentobarbital, were reasonably close to the experimental values obtained from Scatchard plots (Table 2). The effect on k_{-1} (Fig. 5) was saturable and showed an EC_{50} of 100 μM. This is approximately equal in potency to the effect on equilibrium [^{3}H]diazepam binding, although the threshold for the former effect might be slightly lower.

Phenobarbital (500 μM), just like (±)-pentobarbital, significantly decreased both k_{-1} and k_1 for [^{3}H]diazepam binding (Fig. 4A and B). However, the change in both rate constants was approximately equal, thus not altering the equilibrium affinity constant. This agrees with the fact that phenobarbital did not alter equilibrium [^{3}H]-diazepam binding (Table 2). Figure 5 shows that the effect of phenobarbital on k_{-1} was saturable with an EC_{50} of 260 μM. This value agreed well with the K_i for reversing (±)-pentobarbital-enhanced [^{3}H]diazepam binding.

Finally, the specificity of the barbiturate effect on [^{3}H]diazepam was tested by examining the effect of 0.5 mM (±)-pentobarbital on the binding of some other receptor ligands. No significant effect was observed on the binding of the muscarinic ligand [^{3}H]propylbenzylilcho-

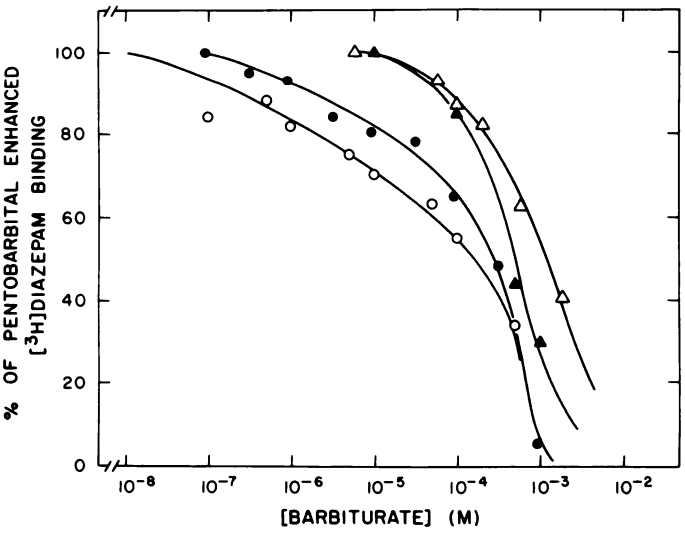


FIG. 3. *Concentration dependence for weakly hypnotic barbiturates interacting with (±)-pentobarbital-enhanced benzodiazepine binding*

Rat cortex membranes were prepared and assayed for [^{3}H]diazepam binding (at 0.5 nM). Varying concentrations of (+)-MPPB (○), (+)-mephobarbital (●), phenobarbital (▲), and (±)-metharbital (△) were included with 100 μM (±)-pentobarbital in the assay. Control (±-pentobarbital enhancement of [^{3}H]diazepam binding was 45% above baseline, and is normalized to 100% in the figure. The results are the average of three experiments in which individual points and IC_{50} values varied ≤ 10%.

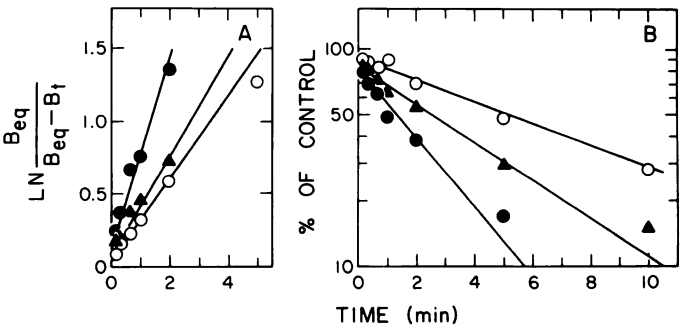


FIG. 4. *Kinetics of diazepam receptor binding in the presence and absence of (±)-pentobarbital and phenobarbital*

A. Association rate. Rat cortex membranes were prepared and assayed for [^{3}H]diazepam binding (at 0.5 nM). At the times indicated, the assays were rapidly terminated. The results are typical of at least three determinations. B. Dissociation rate. Rat cortex membranes were prepared and assayed for [^{3}H]diazepam binding (at 0.5 nM). Following equilibration, the samples were made 10 μM in nonradioactive diazepam (without significantly altering the incubation volume), and the assays were rapidly terminated at the times indicated. The results are typical of at least three determinations. ●, Control; ○, plus 500 μM (±)-pentobarbital; ▲, plus 500 μM phenobarbital.

TABLE 2

Rate constants of [^{3}H]diazepam binding in the presence and absence of anesthetic/hypnotic and anticonvulsant barbiturates

Rat cortex membranes were prepared and assayed for [^{3}H]diazepam binding (at 0.5 nM) with and without 500 μM barbiturates. The kinetic parameters were obtained from association and dissociation rate experiments described under Fig. 4A and B. The values for k_1 and k_{-1} are the mean ± standard error of the mean of six (control) and three (plus barbiturate) experiments. The experimental K_D values for [^{3}H]diazepam binding are typical under the conditions used.

Drug	k_1[a]	k_{-1}[b]	K_D	
			Calculated[c]	Experimental[d]
	$M^{-1}\ min^{-1} \times 10^{-8}$	min^{-1}	nM	nM
None	4.19 ± 0.43	0.431 ± 0.032	1.03	2.23
(±)-Pentobarbital	2.98 ± 0.28	0.132 ± 0.012	0.44	0.86
Phenobarbital	2.08 ± 0.33	0.263 ± 0.018	1.15	2.5

[a] Association rate constant k_1 was estimated from the equation $k_{app} = k_1[S] + k_{-1}$, where k_{app} is the slope of the logarithmic plot of ligand binding association with time and $[S]$ is the [^{3}H]diazepam concentration (28).

[b] Dissociation rate constant k_{-1} was calculated from the equation $k_{-1} = 0.693/t_{1/2}$, where $t_{1/2}$ is the time when 50% of the initial specifically bound [^{3}H]diazepam remains, following addition of excess nonradioactive ligand.

[c] The calculated dissociation affinity constant for [^{3}H]diazepam binding, based on the relationship $K_D = k_{-1}/k_1$.

[d] The affinity constant obtained from Scatchard plots,using exactly the same conditions as used for the association and dissociation experiments.

line, the opiate ligand [^{3}H]naloxone, or [^{3}H]dihydrostrychnine to fresh washed rat cortex membranes (assayed in the same buffer employed for [^{3}H]diazepam binding). The binding of [^{3}H]kainic acid also was not enhanced but was weakly inhibited (20%) by 0.5 mM (±)-pentobarbital.

DISCUSSION

The majority of barbiturates have been tested so far only in living animals. Comparisons of depressant activity *in vivo* with structure-function studies at the cellular and molecular levels may obviously be complicated by pharmacokinetic variables. Nevertheless, depressant barbiturates have been shown to potentiate postsynaptic responses to GABA (2, 3, 6, 7, 9), and the sleep-inducing activity of a series of barbiturates was found to correlate well with their ability to potentiate GABA-mediated inhibition (10). Several barbiturates exist as stereoisomers. Interestingly, these stereoisomers not only differ in potency, but in some cases appear to have different actions, such as hypnotic activity versus no activity, or hypnotic activity versus excitatory activity. Such stereoisomers should be useful for *in vitro* structure-function studies.

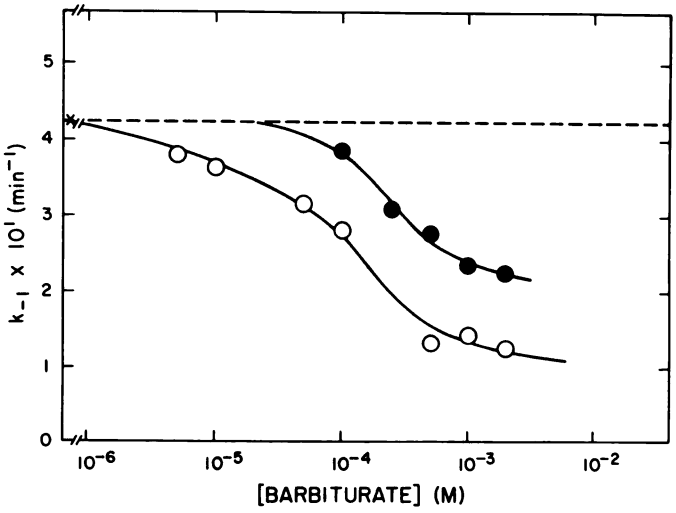


FIG. 5. *Dissociation rate constant (k_{-1}) of specific [^{3}H] diazepam binding at varying concentrations of (±) pentobarbital and phenobarbital*

The membranes were prepared and the assay was carried out as described in Fig. 4B. ×, Control; ○, plus varying concentrations of (±)-pentobarbital, and ●, phenobarbital. The results are typical of at least three experiments that varied ≤ 10%.

Barbiturate depressants generally have anticonvulsant activity (1). Those clinically useful as antiepileptics have high anticonvulsant to sedative potency ratios, and the structure-activity relationships for anesthetic/hypnotic action and useful anticonvulsant action are different (1). Whether these differences in activity of anesthetic and anticonvulsant barbiturates reflect different mechanisms or sites of action or are the result of different potencies on a single cellular mechanism is not yet clear. Previous reports have indicated that barbiturates in both categories have effects on GABAergic postsynaptic events, but have slight differences in their mechanism of action. Thus phenobarbital, but not pentobarbital, was reported to block repetitive nerve firing at doses equivalent to or lower than those enhancing GABA action in cultured neurons (29). Pentobarbital, but not phenobarbital, was found to mimic GABA action at opening chloride channels in the pharmacologically relevant dose range [phenobarbital was effective only at very high concentrations (30)]; both could enhance GABA responses at relevant concentrations, with pentobarbital more potent (2, 9, 29, 30). Likewise, although pentobarbital was found to be more potent than phenobarbital in potentiating GABA in brain slices, the two were roughly equal in reversing picrotoxin antagonism (7), leading that investigator to speculate that these two effects might correlate with anesthetic/hypnotic and anticonvulsant activity, respectively.

The *in vitro* modulation by barbiturates of [^{3}H]diazepam binding to mammalian brain membranes appears to be related to pharmacological actions of these drugs *in vivo* and GABA postsynaptic responses (14, 31). These *in vitro* barbiturate effects now have been observed by at least two other laboratories (32, 33). The GABA chlo-

ride channel-blocking drug picrotoxin competitively inhibits the enhancement of [^{3}H]diazepam binding by barbiturates (14, 31–33) and pyrazolopyridines (13, 15), consistent with observations that these drugs inhibit the binding of [^{3}H]dihydropicrotoxinin (11–13). An association with the GABA receptor is implied by the observations that GABA agonists also enhance [^{3}H]diazepam binding (17) and that this GABA effect is potentiated by pyrazolopyridines (13, 15) and barbiturates (32). Furthermore, the GABA receptor antagonist bicuculline indirectly inhibits (at least partially) the enhancement of [^{3}H]diazepam binding by pyrazolopyridines and barbiturates (13, 15, 31–33). Direct evidence for coupling of pyrazolopyridine/barbiturate-binding sites to GABA receptors is provided by observations that a similar spectrum of pyrazolopyridines (2) and barbiturates (18, 19, 31) enhances GABA receptor binding *in vitro*. Furthermore, the enhancement of [^{3}H]diazepam by barbiturates (14) and pyrazolopyridines (15) is dependent upon the presence of certain anions that are the same anions shown to permeate GABA-regulated inhibitory postsynaptic chloride ion channels in brain (34) which are enhanced by barbiturates (35).

The effect of pyrazolopyridines and barbiturates *in vitro* is specific for the GABA-benzodiazepine receptor complex, because pyrazolopyridines (1 μM) did not perturb the binding of a dopamine receptor ligand, [^{3}H]-spiroperidol, a muscarinic receptor ligand, [^{3}H]quinuclidinyl benzilate, or an alpha-adrenergic receptor ligand, [^{3}H]clonidine (36). Pentobarbital (up to 0.5 mM) did not have any effect on the binding of the muscarinic receptor ligand [^{3}H]propylbenzylilcholine, the opiate receptor ligand [^{3}H]naloxone, the excitatory amino acid receptor ligand [^{3}H]kainic acid, or the putative glycine receptor ligand [^{3}H]dihydrostrychnine (this study). All of these studies were carried out in 10 mM sodium phosphate buffer (pH 7.0) plus 0.2 M NaCl. The absence of effects of barbiturates on [^{3}H]kainic acid binding assayed in Tris-citrate buffer has also been reported (23), but there are likewise no effects of barbiturates on benzodiazepine or GABA receptor binding in this chloride-free buffer.

Additionally, and equally important, the barbiturate enhancement of [^{3}H]diazepam binding is both chemically specific (e.g., secobarbital > pentobarbital = hexobarbital > amobarbital > phenobarbital) and stereospecific (e.g., (+)-hexobarbital > (−)-hexobarbital). This specificity would not be expected for nonspecific hydrophobic interactions with biological macromolecules known to occur at high concentrations (≥1 mM) of barbiturates (1). Although all active barbiturates are weak acids (1), there is no correlation between pK_a values and either pharmacological potency or activity *in vitro* on [^{3}H]diazepam binding. As the case of anesthetic/hypnotic potency (1), there is a correlation between lipid solubility and the enhancement of [^{3}H]diazepam binding by barbiturates. Whether this correlation implies that both effects involve somewhat indirect actions via membrane lipids or, rather, hyprophobic interactions with a specific membrane protein receptor remains to be seen, although the stereospecificity supports the latter possibility. The current study examines in more detail the interactions with benzodiazepines by barbiturates of varying pharmacological potencies and activities.

First, despite the fact that all of the active barbiturates (ref. 14 and Table 1) and pyrazolopyridines (13) inhibit the binding of [α-^{3}H]dihydropicrotoxinin to mammalian brain membranes (11), and micromolar picrotoxinin competitively blocks the enhancement of [^{3}H]diazepam binding by these agents (13–15, 31–33), there is not a good correlation in barbiturate potency between these two *in vitro* assays. Apparently [^{3}H]dihydropicrotoxinin is not specific for the barbiturate/pyrazolopyridine binding sites involved in perturbation of GABA and benzodiazepine receptors; in addition, this ligand has a rather low binding affinity with a resulting low ratio of specific binding to background (11, 12). Therefore the subpopulation of picrotoxinin/barbiturate receptors which appears to be coupled to the GABA-benzodiazepine receptor complex can be studied more appropriately with radioactive benzodiazepine or GABA receptor binding.

Second, dose-response curves reveal that barbiturates can be placed into several categories which show different types of interaction with [^{3}H]diazepam binding. The enhancement by one group of barbiturates reached the same maximal, nonadditive, and mutually exclusive degree of enhancement (≃125%). Included in this group are the two stereoisomers of pentobarbital as well as (−)-DMBB, (±)-DMBB, (±)-secobarbital, and amobarbital. These barbiturates therefore can be designated full agonists. All of the full agonists found so far are unmethylated at N^1, with any assymetrical carbon atom situated in their hydrophobic aliphatic side chains.

Whereas the (−)-isomers of pentobarbital and DMBB are strictly depressant agents, the (+)-isomers have both depressant and excitatory actions; that is, they are preanesthetic convulsants (2, 37). (+)-Pentobarbital and (+)-DMBB do not cause either a time-dependent or a concentration-dependent antagonism of (±)-pentobarbital enhancement of [^{3}H]diazepam binding at concentrations lower than those leading to, or time periods prior to, enhancment of [^{3}H]diazepam binding (data not shown). Rather, they they show an enhancement which reaches the same maximal level but with lower potency than their (−)-isomers. One possible explanation for this observation is that the excitatory and depressant actions of this group are not mediated through the same receptors (2, 37), and that enhancement of [^{3}H]diazepam binding by these drugs reflects their depressant activity (discussed below). A less satisfactory explanation would be that our conditions are not sufficient to distinguish depressant from excitatory effects of these drugs on this system. The excitatory agent CHEB (37), also an N^1-unmethylated compound, weakly but significantly enhanced [^{3}H]diazepam binding. Since analogues of CHEB produce postexcitatory depression much like (+)-DMBB and (+)-pentobarbital, CHEB itself is likely to be a preanesthetic convulsant (37), and therefore also to have (±)-pentobarbital-like actions at the tissue or cellular level. A barbiturate with strictly excitatory activity, (+)-MPPB, did not enhance [^{3}H]diazepam binding but inhibited the enhancement by other barbiturates as described below.

Not all active barbiturates enhanced [^{3}H]diazepam binding to the same maximal extent. The depressant drugs, (+)-hexobarbital, (−)-mephobarbital, and (−)-MPPB, had maximal enhancement lower than the full

agonists, defining a second category of interaction. These drugs all competed with (±)-pentobarbital enhancement by reversing it down to their own maximal level. Furthermore, the effect of (−)-mephobarbital and (−)-MPPB reached a peak but not actually a plateau, and declined back to baseline at high concentrations; they also reversed (±)-pentobarbital enhancement of [^{3}H]diazepam binding down to baseline. Because of lack of solubility of (+)-hexobarbital at high concentrations, it is not yet clear whether it shares this property. These "partial agonists/antagonists" have structures different from those of full agonists in that they all are N^1-methylated and assymetrical at carbon atom C^5. In all cases the stereoisomer with little or no depressant activity was correspondingly weak or inactive in enhancing [^{3}H]diazepam binding. There is another type of drug which enhances [^{3}H]diazepam binding in a partial agonist manner, namely the anxiolytic pyrazolopyridines such as etazolate. This drug also enhanced [^{3}H]diazepam binding to a lower maximal level (90%) than did pentobarbital (13), with reversal of the enhancement back to baseline at high concentrations (15).

The stereoisomers of the N^1-methyl barbiturates just discussed demonstrate other categories of interaction with [^{3}H]diazepam binding sites. In one group was (−)-hexobarbital, an extremely weak depressant drug (26), which neither enhanced the binding of [^{3}H]diazepam nor modified the enhancement by (±)-pentobarbital. This over-all weak activity *in vivo* and *in vitro* was shared by the compound barbital.

On the other hand, the excitatory (+)-MPPB (26) and also (+)-mephobarbital (inactive as hypnotic; ref. 26) did not enhance [^{3}H]diazepam binding, but they were not inactive in this system. Both reversed the enhancement by those barbiturates which were active; the concentration-dependence curves deviated significantly from the law of mass action, and involved some high-affinity interactions (Fig. 3). The effect of these compounds resembled that of the convulsant drug picrotoxinin. This indicates that at least one convulsant barbiturate has an *in vitro* effect at the GABA-benzodiazepine-barbiturate receptor sites which is different from the enhancement observed with depressant barbiturates. The similar *in vitro* effect of (+)-mephobarbital suggests that it might have some drug actions like (+)-MPPB, although *in vivo* studies are complicated by rapid metabolism of mephobarbital to phenobarbital (1, 26). It is also interesting that the modest enhancement by (±)-mephobarbital (Table 1) appears to reflect the summation of relatively potent enhancement by the (−)-isomer and reversal by the (+)-isomer. This could have implications for clinical use, and suggests that the pharmacological spectrum for the two pure isomers should be investigated.

The final category of barbiturates interacting with [^{3}H]diazepam binding includes compounds such as phenobarbital and (±)-metharbital, used therapeutically as anticonvulsants. These compounds, like (+)-MPPB and (+)-mephobarbital, did not enhance equilibrium [^{3}H]diazepam binding but were able to compete with (±)-pentobarbital and reverse its enhancement. This effect required high but still relevant concentrations, and the dose-response curves for phenobarbital and metharbital, unlike those for (+)-MPPB and (+)-mephobarbital, followed the law of mass action. The nonbarbiturate depressant chlormethiazole, useful in treating status epilepticus, resembles phenobarbital in inhibiting both the pentobarbital enhancement of [^{3}H]diazepam binding and the binding of [^{3}H]dihydropicrotoxinin (27).

Thus barbiturates can be divided into several groups on the basis of these *in vitro* effects. Two groups, the pentobarbital series and some isomers of the N^1-methyl barbiturates, enhance [^{3}H]diazepam binding, although to different maximal extents. It would be interesting to examine these two classes for possible differences in pharmacological actions. With the possible exception of CHEB (discussed above), all of these compounds are active depressants with anesthetic/hypnotic activity, and the potency in these studies correlates with pharmacological activity (14). However, some of these compounds have potent excitatory activity in addition to depressant actions, and the relationship of their enhancing activity *in vitro* to actions *in vivo* requires further study. In the N^1-methyl series, there is a marked agreement between the stereospecificity for hypnotic activity (26) and the enhancement of [^{3}H]diazepam binding *in vitro*. The anxiolytic pyrazolopyridines which enhance [^{3}H]diazepam binding are reputedly not very active as sedatives (15, 36). We have reported that the interactions of etazolate and pentobarbital with [^{3}H]diazepam binding are not equal with respect to sensitivity to bicuculline inhibition (13). However, etazolate has activity like (−)-pentobarbital on chloride channels of cultured mouse spinal neurons.[2]

Therefore we might conclude that all barbiturates and related depressants which affect GABA postsynaptic receptor-ionophores as studied electrophysiologically (2, 3, 7, 9, 10, 25, 30, 35) have effects on [^{3}H]diazepam binding. These interactions, which are picrotoxinin- and chloride-sensitive, define a barbiturate-benzodiazepine-GABA receptor-chloride ion channel complex (31, 38) which appears to mediate some of the depressant actions of barbiturates. The different categories of interaction with this complex by barbiturates may be related to their pharmacological actions as anesthetic/hypnotics, convulsants, anticonvulsants, or inactive compounds.

The third and last conclusion is that all of the enhancing barbiturates specifically decrease the K_D for [^{3}H]diazepam binding. Interestingly, this effect is apparently due to a decrease in both the on-rate and the off-rate for [^{3}H]diazepam. With pentobarbital, the off-rate was affected approximately twice as much as the on-rate, thus causing a decrease in the over-all calculated K_D. Barbiturates have been shown to alter the kinetics of GABA-stimulated membrane conductance increases, delaying the time to peak and also prolonging the decay kinetics of GABA responses, the net effect being an enhancement of the over-all response (2, 3, 9, 29, 35). However, the main effect would be on the time constant of the conductance response, rather than on the amplitude. Since the benzodiazepine receptor appears to be part of the GABA receptor-ionophore complex, one might speculate that the effects on [^{3}H]diazepam on- and off-rate correlate with the (±)-pentobarbital effects on the response of the cell to GABA. Phenobarbital also altered the kinetics

[2] R. E. Study and J. L. Barker, personal communication.

of [^{3}H]diazepam binding. This effect might be related to the prolongation by phenobarbital of GABA-membrane conductance responses (9). Just like (±)-pentobarbital, phenobarbital decreased both the on- and the off- rate of [^{3}H]diazepam binding in a saturable and dose-dependent manner. However, the effect was slightly smaller than that of (±)-pentobarbital, and the ratio of the rate constants was not different from control, thus not altering the calculated K_D. This agrees with the fact that phenobarbital did not change equilibrium [^{3}H]diazepam binding. It is interesting that the action *in vitro* of barbiturates used as anticonvulsants should differ somewhat from those of barbiturates used clinically as hypnotics/anesthetics. These different effects of pentobarbital and phenobarbital *in vitro* might be related to their different actions on the membrane chloride channels (7, 29, 30), a possibility worthy of more investigation.

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