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MECHANISM OF ACTION OF BARBITURATES

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INTRODUCTION

Despite the widespread use of barbiturates and their clear potential for abuse, little is known of their neurochemical mechanisms of action. Our purpose is to review the neurochemical effects of acute and chronic barbiturate administration, with particular emphasis on the relationship between neurochemical changes and the development of tolerance and dependence. The electrophysiological effects of barbiturates have been reviewed recently (1–3), and Okamoto (4) has compared the neurophysiological and neurochemical effects of barbiturates to the effects of alcohol. The clinical characteristics of barbiturate abuse have been reported by Wesson & Smith (5), and the membrane actions of barbiturates and other anesthetics have been reviewed by Seeman (6) and Roth (7).

ACUTE EFFECTS OF BARBITURATES

Experimental Approaches

The structure-activity relationship (SAR) of barbiturates is an important pharmacological tool that has sometimes been neglected in neurochemical studies. A number of barbiturates are used therapeutically and are readily available for laboratory study. The most potent of the clinically useful

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barbiturates is thiopental; the least potent is barbital. In vivo, the difference in anesthetic potency is about tenfold (8). Barbiturate effects result from the action of the un-ionized species, and the barbiturate potencies generally are correlated with the lipid solubility of the un-ionized species (6, 9, 10). Comparison of potencies of these drugs in vivo, however, is greatly complicated by differences in absorption, distribution, metabolism, and degree of ionization (11). For example, pentobarbital is 14 times more lipid soluble than barbital, but is only 5 to 7 times more potent in producing anesthesia or death (6, 8). Pentobarbital, however, is 16 times more potent than barbital in blocking nerve conduction in vitro. Presumably, the better correlation obtained in vitro is due to elimination of complicating factors such as absorption. There is an excellent correlation between many diverse in vitro actions of barbiturates and their lipid solubilities (10).

There are differences among barbiturates, however, that cannot be explained by differences in lipid solubility. These include the anticonvulsant effects, convulsant effects, and isomeric differences. The anticonvulsant effects of the barbiturates are to some extent independent of the sedative effects, resulting in therapeutically useful drugs (such as phenobarbital) which, at dosages that produce minimal sedation, are effective anticonvulsants. This is not true of most other barbiturates (12). A striking difference among barbiturates is the convulsant effects found in certain derivatives with bulky alkyl groups (13, 14). For two of these compounds, N-methyl-5-propyl-5-phenylbarbiturate and 5-(1,3-dimethylbutyl)-5-ethyl barbituric acid (DMBB), the (+) isomers are convulsants, whereas the (–) isomers are anesthetics (13, 15). The (–) isomer of DMBB, as well as other anesthetic barbiturates, is a potent antagonist of the convulsant effects of the (+) isomer of DMBB (13). It has been suggested that the stereoselective effects of DMBB are due to differences in the formation of hydrogen bonds at receptor sites (16). The stereoisomers of a number of other barbiturates also have been shown to differ in their effects, with the S(–) isomer generally being more potent than the R(+) isomer in producing anesthesia or death (15, 17–22). The R(+) isomer of several barbiturates, including pentobarbital, has been observed to produce more excitation than the S(–) isomer (13, 15, 18). Thus, for many barbiturates, but not all, the excitatory effects predominate for the R(+) isomer whereas the depressant effects predominate with the S(–) isomer. Unfortunately, most comparisons of barbiturate isomers have been restricted to anesthetic and lethal effects. One exception is the observation (20) that the isomers of secobarbital are equipotent as anticonvulsants, even though the S(–) isomer is a more potent anesthetic and is more toxic than the R(+) isomer. It would be interesting to compare the effects of various barbiturate stereoisomers on other behavioral and physiological parameters. When comparing the in vivo potencies of barbitu-

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rate isomers, it should be noted that stereoselective metabolism or distribution may occur, resulting in different isomer tissue levels. For example, three minutes after intravenous administration of equal dosages of pentobarbital isomers, the brain concentration of (+) pentobarbital is 40% higher than the concentration of (–) pentobarbital (22). The (–) isomer, however, is the more potent anesthetic, indicating that this potency difference cannot be explained by brain level differences.

Several other experimental approaches have produced clues to the mechanism of action of barbiturates. The partitioning of barbiturates into model membranes is influenced by the cholesterol content of the membrane (23). Other aspects of membrane composition may also influence barbiturate distribution, resulting in membrane areas with relatively high drug concentrations. This is potentially important because lipids and proteins in these areas would be perturbed more strongly than those in the surrounding membrane, leading to selective effects on certain membrane functions. Other potentially important observations concern the effects of barbiturates on the squid giant axon. The blockade of nerve conduction produced by these drugs appears to be due to an effect on the inner surface of the axon (24). Both lipids and proteins are asymmetrically distributed across the nerve membrane (25, 26), and barbiturates may selectively perturb membrane components localized on the cytoplasmic half of the membrane bilayer. The physicochemical basis for this selectivity remains to be explored.

In summary, the SAR of the barbiturates may prove to be useful in determining which neurochemical actions lead to physiological or behavioral alterations. Although lipid solubility is an important determinant of barbiturate potency, it cannot completely explain the quantitative and qualitative differences among some of the barbiturates. It is likely that a high degree of lipid solubility allows the drug access to its hydrophobic sites of action, whereas other factors, such as hydrogen bonding, determine the molecular interactions that result in neurochemical perturbations.

Perturbation of Membrane Physical Properties by Barbiturates

There is evidence that barbiturates penetrate into membrane lipid and alter the physical state of the lipid. Resultant changes in ion channels and membrane-bound enzymes have been hypothesized as a mechanism of action of barbiturates (6, 27). In support of the membrane hypothesis, phenobarbital and pentobarbital have been shown to form hydrogen bonds with phosphatidylcholine, a major lipid component of brain membranes (28). Barbiturates also have been shown to decrease the temperature of the gel-to-liquid crystalline phase transition of phosphatidylcholine and phos-

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phatidylethanolamine vesicles (27), which indicates that barbiturates facilitate the "melting" or "fluidization" of membrane lipids. Studies with ESR probes indicate phospholipid-cholesterol vesicle fluidization by barbiturates, although pure phospholipids or phospholipids mixed with small amounts of cholesterol appear to be made more rigid by barbiturates (29–32). Nerve membranes contain large amounts of cholesterol, and studies with model systems would predict brain membrane fluidization by barbiturates. Harris & Schroeder (33), using fluorescent probe techniques, demonstrated that pentobarbital and secobarbital increase the fluidity of the hydrophobic core of synaptic membranes prepared from mouse brain. These effects are similar to those observed with ethanol (34, 35) and are consistent with the pharmacologic similarities of alcohols and barbiturates. In contrast, one ESR study (29) indicates that thiopental increases synaptic membrane surface rigidity without affecting membrane core fluidity. This discrepancy apparently is due to differences in the type of probe molecules used in ESR and florescence studies and has been noted in a study with model membranes (36) in which fluorescence techniques indicate that pentobarbital fluidizes lipid membranes. This change is correlated with an increase in cation permeability. In contrast, changes in ESR parameters suggested an increase in rigidity and could not be correlated with changes in ion fluxes. Interestingly, relatively small changes in membrane fluidity (as measured by a fluorescent probe) resulted in substantial changes in ion permeability, suggesting the existence of a mechanism for amplifying the weak membrane perturbations produced by low concentrations of barbiturates (36).

In addition to altering membrane fluidity, barbiturates may affect the surface charge of brain membranes. Matthews & Nordmann (37) found that amobarbital decreases the surface charge of synaptic vesicles from cerebral cortex. This effect is independent of calcium availability and may lead to vesicle fusion and enhanced release of neurotransmitters without neuronal depolarization. There is evidence (presented below) that barbiturates enhance spontaneous neurotransmitter release.

In summary, the interaction of barbiturates with membrane lipids suggests a plausible mechanism for alteration of neuronal function, but it has not been determined that these interactions are consistent with the SAR of the barbiturates. For example, demonstration of a different interaction of convulsant and anesthetic barbiturates with membrane lipids would strengthen the membrane hypothesis.

Role of Calcium in Barbiturate Actions

Barbiturates affect the transport of calcium in a variety of excitable tissues. The depolarization-stimulated accumulation of calcium in autonomic ganglia (38) and in brain synaptosomes (39–41) is blocked in vitro by

barbiturates. Various nerve endings may differ in sensitivity to this effect, since synaptosomes from cerebellum are more inhibited than synaptosomes from other brain regions (42). Calcium currents are reduced by barbiturates in the squid giant synapse (43), and indirect evidence indicates that calcium availability is reduced in the leech Retzius cell (44). The intrasynaptosomal, ATP-dependent sequestration of calcium is inhibited by pentobarbital (45). Pentobarbital concentrations in the range of 0.1 to 1.0 mM are required for inhibition of calcium transport in these systems. Brain concentrations of 0.1 mM pentobarbital produce anesthesia (46). Anesthetic, but not convulsant, barbiturates have been shown to stimulate synaptosomal calcium-ATPase in vitro (47). Willow & Johnston (47) suggested that this effect could enhance the efflux of calcium from synaptosomes. Barbiturate effects are also seen in vivo, since an acute injection of pentobarbital reduces the calcium content of synaptic membranes (48). This may be related to the observation that pentobarbital displaces calcium from the cytoplasmic surface of the erythrocyte membrane (49). Although barbiturates have been shown to increase the binding of calcium to phospholipids (50, 51), their predominant effect on biological membranes appears to be a decrease in calcium binding (48, 49). A functional role for the calcium antagonist effects of the barbiturates is indicated by the observation that an elevation of external calcium reverses the nerve-blocking action of barbital and pentobarbital (52); however, analogous results were not obtained in the CNS, since an increase in brain calcium potentiated pentobarbital anesthesia and did not alter barbiturate hypothermia (53). The relevance of calcium transport inhibition to the sedative or anesthetic actions of barbiturates is also questionable because of the observation that convulsant barbiturates inhibit calcium uptake by synaptosomes (39). The effects on calcium, however, may be related to the anticonvulsant actions of barbiturates. This is supported by observations that nonbarbiturate anticonvulsants inhibit calcium uptake (40) and that a convulsive barbiturate can possess anticonvulsant activity at subconvulsive dosages (14).

In summary, barbiturate concentrations in the anesthetic range clearly inhibit calcium accumulation by neural tissue. The pharmacological importance of this effect and the mechanism responsible remain obscure. The influx process is generally assumed to be inhibited, but an enhancement of efflux, which could probably account for the observations, may also occur. The role of calcium in barbiturate effects on neurotransmitter release is discussed in the following section.

Acute Effects of Barbiturates on Synaptic Transmission

ELECTROPHYSIOLOGICAL STUDIES A number of excellent electrophysiological studies have contributed greatly to knowledge of the barbitu-

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rate effects at various synapses. These studies have been reviewed elsewhere (1–3, 54), and a detailed presentation is beyond the scope of this discussion. The essence of the studies is that sedative barbiturates inhibit excitatory transmission and enhance inhibitory transmission. The inhibition of excitatory transmission involves a decrease in the postsynaptic effects of excitatory neurotransmitters (55–57) and perhaps a presynaptic inhibition of transmitter release (see below). Excitatory transmission is decreased not only by anesthetic barbiturates, but by anticonvulsant and convulsant barbiturates as well (1, 56–58). This process is moderately sensitive to barbiturates, with 0.1 to 0.5 mM pentobarbital inhibiting a variety of excitatory synapses. The augmentation of inhibitory transmission has been studied in detail at a variety of synapses which use GABA as a neurotransmitter. At these synapses, barbiturates have been shown to enhance the effects of GABA, to reverse the effects of its antagonists, and, in some cases, to mimic its effects (56, 59, 60). The anesthetic barbiturates produce all three of these effects, whereas the anticonvulsant barbiturates have minimal GABA-mimetic activity (3, 58). In contrast, a convulsant barbiturate produced only a minor enhancement of the effects of GABA and did not appear to produce any GABA-mimetic effects (1, 61). The enhancement of GABA effects is much greater for the (–) isomer of pentobarbital than for the (+) isomer (62). This observation gives further evidence of a role for GABA in the depressant, but not excitatory, effects of the barbiturates. Thus, anesthetic, anticonvulsant, and convulsant barbiturates produce distinct effects at GABAergic synapses. Inhibitory synapses are extremely sensitive to effects of anesthetic barbiturates. Concentrations of pentobarbital as low as 1 μM produce GABA-mimetic effects and enhanced the action of GABA (59), whereas 5 μM reduce the effects of GABA antagonists (60). The sensitivity and selectivity of the GABA synapse to barbiturates suggests that it is an important site for the anesthetic and anticonvulsant actions of these drugs.

BIOCHEMICAL STUDIES Many of these studies have focused on the inhibitory effects of acute barbiturate exposure on the uptake and release of neurotransmitters. These and other neurochemical effects of barbiturates on synaptic transmission are summarized in the following sections.

Inhibition of neurotransmitter release Since barbiturates inhibit calcium accumulation by nerve endings, they would be expected to inhibit the release of neurotransmitters. Indeed, barbiturates have been shown to inhibit the release of ACh (63–66), NE (64, 67), GABA (67–70), and glutamate (68–70). The inhibition of ACh release has been studied in detail. It has been found that the cortex is more sensitive than the striatum to the

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inhibitory effects of pentobarbital on ACh release (65), and a similar regional difference has been found with synaptosomal calcium uptake (42). A 0.1 mM concentration of pentobarbital inhibited cortical ACh release (65). The potencies of six anesthetic barbiturates were correlated with their lipid solubilities (66). Other studies have shown that phenobarbital and pentobarbital are more potent inhibitors of ACh release than of NE release (64). Thus, the antirelease effects of pentobarbital display some specificity with regard to different brain areas and different neurotransmitters. Other than the correlation with lipid solubility, the structural specificity of barbiturates for inhibition of neurotransmitter release is unclear. There is some evidence that convulsant barbiturates inhibit release of ACh (38, 71) and that the R(+) isomer of secobarbital is more potent than the S(–) isomers for the inhibition of ACh release from midbrain (71). This SAR suggests that inhibition of ACh release is not responsible for the sedative or anesthetic effects of barbiturates. In addition, the pentobarbital concentrations (0.2 to 1.0 mM) required to affect noncholinergic systems are seldom achieved in vivo.

In summary, although the inhibitory barbiturate effects on neurotransmitter release may contribute to reduction of excitatory transmission at certain synapses, there is no compelling evidence for involvement of these effects in barbiturate sedation or anesthesia.

Stimulation of neurotransmitter release Electrophysiological evidence suggests that barbiturates enhance the resting release of ACh at the neuromuscular junction (72–74). This enhancement may be responsible for the muscle twitches seen during the induction of barbiturate anesthesia. Also, there is evidence that low concentrations (0.01 and 0.02 mM) of pentobarbital enhance the resting and stimulated release of ACh from brain tissue (65, 68, 75). In these same studies, higher concentrations of pentobarbital were shown to inhibit ACh release. The increased resting release of neurotransmitter produced by these drugs may be related to the observations (discussed above) that barbiturates reduce the surface charge on synaptic vesicles (37) and inhibit intrasynaptosomal sequestration of calcium (45). These alterations would be expected to enhance vesicle fusion and spontaneous release of neurotransmitters. Even though barbiturates inhibit the depolarization-stimulated release of several neurotransmitters, they also enhance the resting and stimulated release of at least one neurotransmitter, ACh. This enhancement occurs with lower concentrations of barbiturates than those required to inhibit transmitter release. Electrophysiological studies suggest that the (–) isomer of pentobarbital is much more effective than the (+) isomer in stimulating neurotransmitter release (62). Most experiments have tested racemic mixtures of barbiturates. The mechanisms responsible

for the biphasic effects on neurotransmitter release are not known, but may be due to distinct effects of the two isomers.

Other effects on neurotransmitters The electrophysiological evidence that GABA has a leading role in barbiturate actions makes it important to consider neurochemical interactions between these drugs and GABA. Evidence suggests that acute pentobarbital administration enhances the synthesis of GABA in mouse brain and that the increased GABA availability may be involved in barbiturate narcosis (76). Recent biochemical studies have shown that barbiturates enhance the effects of muscimol (a GABA agonist) on DA turnover in the rat retina (77). A convulsant barbiturate did not enhance the effects of muscimol. Thus, these studies support the electrophysiological observations that suggest a role for enhanced GABAergic transmission in the anesthetic, but not convulsant, effects of barbiturates. Receptor binding studies, however, have failed to detect any interactions between GABA and barbiturates (78). Therefore, barbiturates do not affect the postsynaptic binding of GABA, even though GABA-mimetic actions have been observed electrophysiologically (see above). The barbiturates probably affect other components of the GABA receptor system, one component being the chloride ionophore that appears to be coupled to the GABA receptor. A report (78) suggests that dihydropicrotoxinin produces its antagonism of GABA by affecting this chloride ionophore. Ticku & Olsen (79) demonstrated that a variety of barbiturates inhibit the binding of radioactive dihydropicrotoxinin to rat brain membranes. The anesthetic and anticonvulsant barbiturates competitively inhibit binding (Hill coefficients of about 1.0) and the IC_{50} for pentobarbital was 0.05 mM. Convulsant barbiturates are also potent inhibitors of binding, but the interaction is not of a simple competitive nature, since the Hill coefficients were about 0.4 (79). These experiments demonstrate a novel approach to the neurochemical actions of barbiturates, but are technically difficult because of the unfavorable ratio (10:1) of nonspecific to specific binding.

In other experiments, in vitro exposure to pentobarbital has been shown to inhibit the uptake of GABA and catecholamines by brain synaptosomes (80, 81). The concentrations required to produce these effects ranged from 1 to 5 mM. Acute administration of pentobarbital to mice also inhibited synaptosomal uptake of NE and, to a lesser extent, DA (81). Pentobarbital also has been shown to decrease the turnover of catecholamines in brain (82).

In summary, acute exposure to barbiturates affects many aspects of synaptic function. The effects on the GABA synapse appear to be involved in barbiturate sedation and anesthesia, but the neurochemical basis

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of these effects is obscure. Recent studies of the complexity of the GABA synapse (83) should prove useful in understanding the actions of barbiturates.

Macromolecules in Barbiturate Action on the Central Nervous System

The barbiturate-type sedative-hypnotics contain a heterocyclic, six-membered ring having a structure resembling that of uracil and thymine. The structure of barbituric acid (the basic structure of all barbiturates) is actually 2,4,6-trioxohexahydropyrimidine or 6-hydroxyuracil (84). Several studies suggest that natural pyrimidines or structurally similar compounds may depress arousal levels (85–89). In terms of biochemical studies, earlier investigations show that the barbiturates feature a highly selective affinity for forming hydrogen-bonded complexes with molecules containing adenine (90). Selectivity in hydrogen bonding is an important aspect of biologic organization, especially in the nucleic acids. Derivatives of adenine selectively form hydrogen bonds with thymine or uracil derivatives. This complementarity has two components. One component is geometric and enables nucleotide polymers to become organized into regular double-stranded helical molecules; the other component is electronic and is a consequence of electronic distribution with the individual purines and pyrimidines. The association constants for hydrogen bonding between barbiturates and adenine derivatives are about 1000, which is an order of magnitude greater than that found between uracil and adenine derivatives (90). It is not clear, however, whether this affinity is sufficient to allow association in vivo. The association constants are similar for the intermolecular crystalline complexes formed between a variety of anesthetic barbiturates and several different adenine derivatives. Although results thus far are from aggregate solutions with chemical derivatives of adenine, the phenomenon of hydrogen bonding may be a key to the way barbiturates act in living systems. As noted above, the barbiturates also form hydrogen bonds with membrane lipids (28).

An in vivo study (91) demonstrated that pentobarbital directly inhibited RNA synthesis, as measured by incorporation of [^{14}C]-formate and [^{32}P]-phosphate into RNA. Pentobarbital inhibits growth and synthesis of nucleic acids and proteins in murine and mastocytoma cells grown in culture (92). Recent evidence suggests that acute phenobarbital administration decreases protein synthesis in rat cerebral cortex (93); however, this inhibition of protein synthesis may be due to the hypothermia produced by barbiturates (94).

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BARBITURATE TOLERANCE AND PHYSICAL DEPENDENCE

Methods Available for Induction of Barbiturate Tolerance and Dependence

In 1903, Fischer and von Mering introduced the first hypnotic barbiturate, barbital (diethylbarbituric acid), under the trade name of Veronal ®. Veronal was clinically tested for two years, and in 1905 Kress (95) reported toxic symptoms in a large number of subjects using the drug. It was then recognized that continuous administration of Veronal leads to an addiction similar to that produced by paraldehyde and chloral hydrate. Therefore, work began that was to study the development of barbiturate tolerance and physical dependence. The early literature (1920–1946) pertaining to these phenomena is inconsistent. Many investigators reported little or no development of barbiturate tolerance or dependence (96–99), whereas others reported tolerance or dependence (100–104).

Masuda and his associates (102) noted this discrepancy and related it to the differing experimental procedures used in various laboratories. Because of the discrepancy, Gruber & Keyser (105) used experimental animals in a series of studies on the development of tolerance and cross tolerance to barbiturates. Another substantiation of tolerance development was their finding that induction of tolerance was related to the time interval between barbiturate administrations. They also showed that time intervals between doses could be lengthened by using a long-acting barbiturate to produce tolerance. Based on their results and the available literature, they concluded that the lack of agreement could be attributed to the following factors: (*a*) the imprecise use of the terms *drug tolerance* and *drug addiction;* (*b*) differences in the time intervals between repeated injections of the drug; (*c*) differences in the quantity of drug injected per dose; (*d*) differences in criteria used to determine acquired tolerance; (*e*) different animal species used by the various investigators.

That development of tolerance following repeated barbiturate administration is generally accompanied by development of physical dependence is now recognized. Over the past five decades, numerous methods have been utilized to induce tolerance to and dependence on barbiturates (Table 1). Although most all animal species commonly used in laboratories have been employed as models, rats are used most frequently (30%). Pentobarbital and barbital are the most frequently used barbiturates (32%). The oral route of administration is the most frequently used method (28%).

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Table 1 Methods utilized for inducing tolerance to and dependence on barbiturates in laboratory animals

Route of administration	Type of barbiturate	Species	Dose, mg/kg body weight	Duration of treatment	References
p.o.	Barbital	Cat	217	1–80 days	100
p.o.	Phenobarbital	Cat	21–65	10 weeks	100
p.o.	Barbital	Monkey	143	7–52 days	100
i.g.	Amobarbital	Rabbit	190–650		
i.g.	Barbital	Dog	61.6–100	4.5–38 months	103
i.p.	Pentobarbital	Guinea pig	7.5–20	4–6 weeks	193
s.c.	Phenobarbital	Rat	8–23	7 weeks	104
s.c.	Pentobarbital	Rat	6, 18, 36	7 weeks	104
l.p.	Pentobarbital	Rat	35–40	14–32 days	194
p.o.	Amobarbital	Dog	40	2 months	99
i.v.	Amobarbital	Rabbit	35–40	1–10 days	102
i.v.	Pentobarbital	Dog	28–42	27–83 days	195
i.v.	Pentothal	Dog	20	10 days	101
i.v.	Amobarbital	Rabbit	37.5–40	10 days	105
i.v.	Pentobarbital	Rabbit	50–107	5 days	105
i.v.	Secobarbital	Rabbit	18–22.5	5 days	105
i.p.	Pentobarbital	Rat	29	10 days	105
i.p.	Secobarbital	Rat	69	10 days	105
i.p.	Thiopental	Mouse	50	13 days	196
i.p.	Phenobarbital	Dog	60–100	25 days	197
p.o.	Amobarbital	Dog	55	180–195 days	125
p.o.	Barbital	Dog	106–168	216–339 days	125
p.o.	Pentobarbital	Dog	60–104	180 days	125
p.o.	Secobarbital	Dog	35–42	180–195 days	125
p.o.	Barbital	Cat	61–279	23–217 days	128
p.o.	Barbital	Cat	190–335	106–267 days	129
p.o.	Barbital	Dog	40–150	107 days	126
i.v.	Hexobarbital	Mouse	70	1–4 days	198
i.v.	Pentobarbital	Cat	88–114	1–21 days	130
i.p.	Pentobarbital	Rat	20–30.5	3–96 days	111
p.o.	Barbital	Rat	313–396	111–159 days	135
i.p.	Barbital	Rat	200	25 days	199
i.p.	Pentobarbital	Rat	30–40	4 days	199
p.o.	Barbital	Rat	100–400	32 days	115
i.p.	Thiopental	Rat	25–50	0–72 hours	200
i.v.	Pentobarbital	Monkey	50–400	2 weeks	106
i.m.	Phenobarbital	Monkey	50–100	6 weeks	106
i.m.	Pentobarbital	Monkey	30–45	6 weeks	106
p.o.	Phenobarbital	Mouse	150–350	7 days	144
i.g.	Barbital	Monkey	75	3 months	127
s.c.	Phenobarbital	Mouse	(75 mg pellet/ mouse)	3 days	46
i.g.	Pentobarbital	Cat	30–50	35 days	107
i.c.v.	Barbital	Rat	2.4 mg/rat	12 hours	121
i.c.v.	Phenobarbital	Rat	800 μg/mouse	4–5 days	201
i.p.	Pentobarbital	Rat	85	10 hours	202
i.g.	Barbital	Cat	400	5 weeks	168
i.p.	Barbital	Rat	150	5–15 days	148
s.c.	Barbital	Mouse	(osmotic mini-pump ~50 mg/ mouse)	95–101 hours	120
s.c.	Barbital	Mouse	(16 mg pellet/ mouse)	3 days	203

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Development of Barbiturate Tolerance

Barbiturate tolerance is defined as a decreasing response to repeated administration of the same dosage of a barbiturate or as a necessary increase of the dosage to obtain the initial response. The indices used to assess the development of barbiturate tolerance have varied, depending on species. Graded ratings for CNS depression induced by barbiturates have been used in rhesus monkeys (106) and in cats (107), and include slowed motion, ataxia, impairment in corneal reflex, nictitating membrane tone, pain withdrawal, respiration, self-righting, standing, and walking. One of the oldest and most widely used indexes is loss of righting reflex (sleeping time or narcosis). The duration of sleeping time is considered to be the time between loss and recovery of righting reflex. The sleeping time index has been used in dog, rabbit, monkey, guinea pig, cat, rat, and mouse. Other barbiturate-induced pharmacological responses such as hypothermia (108) and lethality (108, 109) also have been used.

Kalant and co-workers (110) summarized the three decades of literature relating to the onset and degree of barbiturate tolerance development. Barbiturate tolerance can be detected in laboratory animals within a matter of days, and the degree of development is generally characterized by a 25 to 60% decrease in barbiturate effect. Because the margin of safety is narrow, attempts to study the relationship of change in dose response and the development of tolerance to barbiturates have been limited. The only studies are those of Aston (111) and Ho (108). According to their studies, as animals become tolerant to pentobarbital there is an apparent shift to the right of the log dose response curve.

There are two types of barbiturate tolerance—one dispositional, the other functional (110). Biotransformation of barbiturates (112–115) and the phenomenon of induction of drug metabolizing enzymes by barbiturates and other drugs have been reviewed in detail (116–118). It has been shown in a wide variety of animal species that chronic administration of barbiturates is followed by a reduction in the duration of action (i.e. the period of righting reflex loss), which correlates with an increased rate of barbiturate metabolism by the liver (115, 119). Because of the nature of this induction, it is possible that part of the development of tolerance can be explained by changes in metabolism. It is a fact, however, that drug metabolism alone is not the basis of all barbiturate tolerance; it cannot explain tolerance phenomena related to the recovery of function at plasma and brain drug concentrations associated with marked depression in the nontolerant subject (110). In addition, enzyme induction does not contribute significantly to development of tolerance to barbital, which in most species is metabolized to a very limited extent. This is made clear by the studies of Siew &

Goldstein (120), who found that functional tolerance is displayed by a significant decrease in sleeping time following a challenge barbital dose administered 24 hr after withdrawal. Increased metabolism does not explain tolerance development to barbiturates administered into the cerebral ventricles (19, 121).

The two aspects of barbiturate tolerance are further differentiated by the "maximally tolerable dose" technique developed by Okamoto et al (107) in cats. Their results distinguished between dispositional and functional tolerance. The maximal dispositional tolerance to pentobarbital developed within one week and was maintained at that level throughout the remainder of the treatment period. Functional tolerance, however, developed more gradually and progressed with continued treatment. The functional aspect of tolerance to pentobarbital is further supported by the results of Ho (108), who used the pellet implantation procedure in the mouse. With intracerebral administration of sodium pentobarbital to the mouse, it was found that when the animals were implanted with pentobarbital pellet for three days the intracerebral LD_{50} of sodium pentobarbital in the pentobarbital pellet-implanted group was significantly increased compared with the control group. Furthermore, in mice implanted with pentobarbital pellets for three days, the hypothermia induced by intracerebral administration of sodium pentobarbital was less and body temperature recovery was faster than in mice implanted with placebo pellets. These results indicate that induction of increased hepatic metabolism does not entirely account for development of tolerance to pentobarbital when animals have become highly tolerant to pentobarbital by pellet implantation. In addition to drug dispositional tolerance, CNS adaptive tolerance occurred.

It is interesting to note that barbiturate tolerance development has been demonstrated in hamster glial cells in culture by Roth-Schechter & Mandel (122). They exposed hamster astroblast glial cells to pentobarbital concentrations ranging from 0.01 to 3.0 mM. Marked morphological changes induced in cultured glial cells by pentobarbital are accompanied by an increase in oxygen consumption. The development of pentobarbital tolerance is evidenced by the fact that glial cells treated for four weeks with barbiturate are less sensitive to the depression of oxygen consumption caused by a challenge dose of pentobarbital. This tolerance may be due to an enhancement of mitochondrial activities (123, 124).

Development of Barbiturate Dependence

Physical dependence on barbiturates has been produced in several species of laboratory animals. Barbiturate withdrawal reactions have been observed in dogs (103, 125, 126), monkeys (106, 127), cats (128–133), rats (134–142), mice (46, 143–147), and hamster glial cells in culture (122). Although

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barbital is the agent most frequently used to produce physical dependence, phenobarbital and pentobarbital also are often used.

The degree of physical dependence has been estimated by grading the withdrawal reaction. Yanagita & Takahashi (106) defined three classes of withdrawal signs in rhesus monkeys, based on studies with pentobarbital, phenobarbital, and barbital. Major abstinence signs observed in their studies are characterized as (*a*) mild: apprehension, hyperirritability, mild tremors, anorexia, and piloerection; (*b*) intermediate: severe tremors, muscle rigidity, impaired motor activity, retching and vomiting, and weight loss of over 10%; and (*c*) severe: grand mal convulsions, delirium, and hyperthermia. Boisse & Okamoto (133) assessed the severity of pentobarbital and barbital withdrawal in cats by counting the number of grand mal convulsions and subjectively rating 20 additional motor, autonomic, and behavioral signs, including tremors, twitches, myoclonic jerks, postural disturbances, and motor incoordination. The incidence of severe physical dependence manifested by withdrawal convulsions has not been shown with short- or intermediate-acting barbiturates (e.g. pentobarbital) in rats and mice. Crossland & Leonard (134) were the first to show that a barbital withdrawal convulsion can be induced in rats. This phenomenon has been substantiated by other investigators (135–142) and phenobarbital-induced withdrawal convulsions have been reported (143, 144, 147).

Other methods have been used to detect pentobarbital physical dependence in rodents. One of the primary characteristics of the barbiturate withdrawal syndrome is increased susceptibility to seizures. Jaffe & Sharpless (130) used the threshold for pentylenetetrazol-induced seizures as the index for pentobarbital physical dependence. This method was successfully used in mice that were continuously administered pentobarbital by pellet implantation (46). Other stimulants (e.g. bemegride and picrotoxin) also have been used (136). Audiogenic seizure susceptibility was also used to assess barbiturate withdrawal in rodents (136, 146, 148).

It is interesting that in man a long-acting barbiturate (149–151) manifested less severe withdrawal convulsions than short- or intermediate-acting barbiturates (152–154). Barbital, however, has been the drug of choice for the induction of barbiturate physical dependence in laboratory animals (125, 126, 128, 129, 134–136, 155–167). Only a few studies have dealt with pentobarbital (106, 125, 131). The rationale for barbital preference is unclear. Boisse & Okamoto (133, 168) suggest that the slow elimination rate of barbital favors drug accumulation, and hence, prolonged time-action. This reduces the frequency of barbiturate administration necessary to produce physical dependence. Based on comparative studies of pentobarbital and barbital in cats, Boisse & Okamoto (133) concluded that compared with pentobarbital, barbital withdrawal signs are less severe, surface later, develop more slowly, and last longer.

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Biochemical Aspects of Tolerance to and Physical Dependence on Barbiturates

The development of tolerance to and physical dependence on barbiturates has been established as being a consequence of repeated or continuous administration of this group of sedative-hypnotic drugs. The biochemical mechanisms involved in tolerance and physical dependence processes have been difficult to pinpoint. During the past decade, an increase in the number of studies pertaining to barbiturates has resulted in some understanding of how neurotransmitters, macromolecules, and ions might be involved in barbiturate tolerance and dependence. Although monkeys and cats are ideal models for quantitative pharmacologic characterization of barbiturate dependence, they are impractical for studying the biochemical mechanisms involved in barbiturate tolerance and dependence; therefore, of the biochemical studies reported, all used rodent models. The following section highlights attempts to relate various neurotransmitters, nucleic acids, proteins, and ions to barbiturate tolerance and physical dependence.

NEUROTRANSMITTER MECHANISMS IN TOLERANCE AND DEPENDENCE Table 2 summarizes the available evidence, which is discussed below.

ACETYLCHOLINE (ACh) Several studies indicate that cholinergic mechanisms may be involved in the tolerance and physical dependence that develops after long-term barbiturate administration. Single doses of sodium pentobarbital administered to mice have been reported to increase endogenous ACh in whole brain (169), and it has been observed that the endogenous ACh content is not significantly altered when mice become tolerant to barbital (141, 163). In contrast, in rats receiving chronic oral administration of barbital, the endogenous ACh content in the striatum is most markedly decreased on the third and twelfth day of abstinence (141). It has been postulated that tolerance and physical dependence induced by chronic treatment with barbital may be due to a change in sensitivity to central cholinergic stimulation. Atropine, a cholinergic antagonist, reduced the development of barbital tolerance and suppressed barbital withdrawal convulsions (140). Along with tolerance, an increased sensitivity to the temperature-reducing effects of pilocarpine has been found in the rat (170). A decrease in muscarinic receptor binding has been observed in the cerebella of animals repeatedly treated with phenobarbital and withdrawn for 24–48 hr (171). In contrast, Nordberg et al (172) found an increase in brain muscarinic receptor binding sites in rats chronically treated with barbital and withdrawn for three days.

The regional biosynthesis of ACh in brain following chronic barbital

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Table 2 Functional states of neurotransmitters in barbiturate tolerance and dependence[a, b]

	Acetylcholine		Norepinephrine		Dopamine		γ-Aminobutyric acid	
	Tolerance	Withdrawal	Tolerance	Withdrawal	Tolerance	Withdrawal	Tolerance	Withdrawal
Content	0 (163) (141)	↓ (141)	Intact NE system seems to be necessary for tolerance (147, 176)	↓ (139) 0 (138)		↓ (139)	0 (192, 136) ↓ (179)	0 ↓
Receptor Sensitivity	↑ (170) (140)	↑ (170) (140)				0 (171)		
Binding		↓ (171) ↑ (172)	↑ (175)			0 (171)	↓ (171) (181)	
Turnover Biosynthesis	↑ (142)	↑ (142)		↑ (138)				
Breakdown						↑ (139)	↓ (178)	
Uptake			↓ (81)	0 (81)	↓ (81)	0 (81)	↓ (181)	0 (181)
Release								
Related enzymes	Choline acetyltransferase 0 0 (163, 142) Acetylcholinesterase 0 0 (163, 142)						Glutamate decarboxylase 0 (178) ↓ (180) ↓ (179) 0 (180) GABA-transaminase 0 (178) 0 (180)	

[a] Numbers in parenthesis refer to items in the Literature Cited section.
[b] ↑ = increase; 0 = no change; ↓ = decrease.

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administration to rats was studied recently (142). Compared with controls, rats abstinent for three days or receiving barbital until death had a significantly higher content of newly synthesized ACh in the cerebellum, medula oblongata, and midbrain. The newly synthesized ACh content was significantly increased in the hippocampus and cortex of rats abstinent for three days. These results suggest an increased turnover of ACh (142). The effect of long-term barbital treatment on the enzyme activity of brain choline acetyltransferase and acetylcholine esterase was studied, but no significant effect was found (142, 163). The high affinity sodium-dependent uptake of choline by nerve terminals, which has been shown to be one of the rate-limiting steps in ACh synthesis (173), is increased 12 days after withdrawal of barbital, but not at 3 days after withdrawal (174). These studies substantiate the idea that there is an enhanced activity of cholinergic function as a result of chronic barbiturate treatment, and that this enhanced activity persists during the abstinence phase and may contribute to withdrawal symptoms. Both pre- and postsynaptic mechanisms appear to be involved in the enhanced cholinergic sensitivity.

NOREPINEPHRINE (NE) Statistically significant decreases in NE concentration were observed in the rat cerebral cortex and thalamus following one-day withdrawal, whereas NE concentration in the hypothalamus was significantly reduced during the second day of withdrawal (139). No changes in NE concentration were observed in the telencephalon and brain stem of barbital-dependent rats (139). On the other hand, when compared to the same parameters in control rats, the depletion of NE produced in the telencephalon of rats by pretreatment with α-methyl tyrosine or FLA-63 was significantly greater following one day of barbital withdrawal. Compared with nondependent animals, NE depletion after FLA-63 pretreatment was significantly greater in the brain stem of rats one and two days following barbital withdrawal. These results suggest that NE turnover in the telencephalon increases during the first day following withdrawal of barbital from dependent rats (138). In terms of pharmacologic manipulation of the NE functional state, 6-hydroxydopamine pretreatment enhances the degree and onset of spontaneous convulsive seizures in barbital-dependent rats (137). Recently it has been demonstrated that when mice are treated both acutely and chronically with pentobarbital, there is a significant attenuation of NE uptake into synaptosomal preparations (81). The decrease in NE uptake during the course of pentobarbital tolerance development was demonstrated to be time dependent. The NE uptake process was restored by abrupt withdrawal of pentobarbital. Chronic phenobarbital ingestion has been shown to increase β-adrenergic receptors in mouse brain (175), which may reflect a decreased release of NE during chronic treatment. Recent

evidence presented by Tabakoff et al (147, 176) demonstrates that mice treated with 6-hydroxydopamine prior to chronic phenobarbital feeding do not develop functional barbiturate tolerance, as measured by duration of hypothermia and the loss of righting reflex. Injection of 6-hydroxydopamine causes significant depletion of brain NE, whereas brain dopamine levels are not significantly depleted. These workers concluded that intact brain NE systems are necessary for development of tolerance to the hypnotic and hypothermic effects of phenobarbital. This was the first study demonstrating that the development of tolerance to certain effects of barbiturates can be prevented without changing the development of physical dependence. These results suggest that the effects of barbiturates on NE may be more important for the development of tolerance than for dependence.

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DOPAMINE (DA) Morgan et al (139) have shown that the concentration of DA in the telencephalon of barbital-dependent rats is significantly decreased on the second day of barbital withdrawal when compared to the levels in control animals. On the second day following barbital withdrawal, a significant decrease in DA content in the telencephalon of rats is observed. This is consistent with an increase in DA utilization. A significant difference in DA uptake by synaptosomes in mice continuously exposed to pentobarbital for different periods of time has recently been demonstrated (81). Compared with the group treated chronically with pentobarbital, the control group had a higher DA uptake with increasing concentrations of DA. The effect of continuous pentobarbital treatment on DA uptake was shown to be time dependent. The uptake of DA returned toward the control value one day after the abrupt withdrawal of pentobarbital. Mohler et al (171), however, report that DA receptor binding is unchanged in the corpus striatum of rats that receive 30 mg/kg of intraperitoneal phenobarbital for 30 days.

γ-AMINOBUTRYIC ACID (GABA) In 1963, Essig (126) demonstrated that aminooxyacetic acid (AOAA), an inhibitor of GABA metabolism (177), prevents convulsions in barbital-dependent dogs that are abruptly withdrawn from sodium barbital. He suggests that barbital withdrawal seizures might be caused by a deficiency of GABA in the brain, and that the deficiency occurs during the development of physical dependence or during the period immediately following drug withdrawal (126). Biochemically, contradictory data exist concerning the chronic effects of barbiturates on the brain GABA system in rodents. It has been reported that no changes occur in the GABA content (136, 192) or glutamic acid decarboxylase (GAD) and GABA-transaminase (GABA-T) activities (136) in barbiturate tolerant-dependent animals. The turnover of GABA, however, is decreased

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by chronic administration of pentobarbital (178). In the mouse, chronic administration of pentobarbital results in a decrease of both GABA and glutamate levels (179). There was a concomitant 30% decrease in GAD activity, which is confirmed by the finding (179) that the rate of brain GABA accumulation induced by AOAA administration in tolerant mice is lower than that in nontolerant animals. Although brain GABA remains at significantly lower levels after an abrupt withdrawal from pentobarbital, brain glutamate levels show no significant difference when compared to the control group. The involvement of the GABA system in pentylenetetrazol-induced convulsions in pentobarbital-dependent mice (180) has been studied further. GABA levels in these dependent animals are significantly lower than those of the placebo-implanted mice. The further decrease in GABA is also observed in dependent mice that convulse after administration of pentylenetetrazol, as compared with those that do not convulse. In addition, the activity of GAD measured in convulsed dependent mice is significantly lower than that in nonconvulsed dependent mice.

Regarding the GABA receptor, a decrease in striatal GABA binding in phenobarbital-treated rats has been reported (171). Chronic administration of pentobarbital has recently been shown to reduce significantly the maximum amount of GABA bound at synaptosomal sites (181). These results suggest that after chronic treatment the enhancement of GABAergic transmission produced by barbiturates may be offset by a subsensitivity of GABA receptors. In view of the importance of GABA as an inhibitory CNS transmitter, it is tempting to speculate that the initial enhancement of GABAergic transmission produced by barbiturates may be offset by pre- and postsynaptic alterations which return excitability to normal and result in functional tolerance. After barbiturate withdrawal, however, these homeostatic alterations result in a hypofunctional GABA system, leading to the CNS excitability characteristic of barbiturate abstinence. This unitary hypothesis of tolerance and dependence is difficult to reconcile with the observation that 6-hydroxydopamine lesions affect tolerance, but not dependence (147, 176). It is possible that a NE deficit could mask the expression of tolerance but not affect (or potentiate; 137) the expression of dependence. Several experiments, however, suggest that NE depletion affects the development, not the expression, of tolerance (147, 176).

Membrane Lipids in Tolerance and Dependence

Few studies have evaluated the effects of chronic barbiturate treatment on the lipid composition of membranes. The acute effects of pentobarbital on brain phospholipid turnover are greatly attenuated in barbiturate-tolerant rats (182, 183), but whether chronic barbiturate exposure alters the lipid composition of brain membranes is not known. Exposure of *E. coli* and Chinese hamster ovary cells to pentobarbital increases the amount of 18:0

fatty acid while decreasing 16:0 and decreasing the degree of unsaturation of the membrane fatty acids (184). A change in fatty acid composition is a mechanism frequently used by cells to regulate the fluidity of their membrane lipids in response to changes in temperature (homeoviscous adaptation) (185, 186). It has been proposed that ethanol tolerance and dependence are related to homeostatic responses to membrane effects of the drug. These responses may involve changes in brain membrane fatty acids (187). Similar mechanisms could be involved in barbiturate tolerance and dependence, but have yet to be demonstrated.

Protein Synthesis in Tolerance and Dependence

Hitzemann & Loh (188) report that functional barbiturate tolerance is blocked by intraventricular injection of cycloheximide, an inhibitor of protein synthesis. The effects of acute and chronic pentobarbital treatments on incorporation of [H^3]-lysine into the protein of various subcellular fractions of the cortex and subcortex were also studied. In the subcortex, chronic pentobarbital treatment significantly stimulated protein synthesis 40–50% in the microsomal, soluble, and mitochondrial fractions. Both acute and chronic pentobarbital treatments significantly increased [H^3-lys]-protein accumulation in a fraction of synaptic plasma membranes derived from a population of GABA-enriched nerve ending particles (189). These results suggest that the synthesis of proteins (or neuropeptides) is somehow required for the process of tolerance development.

Ions in Barbiturate Tolerance and Dependence

Chronic administration of barbiturates has been shown to alter the localization of magnesium and calcium in brain tissue. Belknap et al (145) demonstrated that C57BL/6J mice made physically dependent on phenobarbital exhibited significantly lower brain and serum magnesium concentrations than those of the control mice. The signs of withdrawal from phenobarbital were similar to those seen in magnesium-deficient mice. These workers suggested that brain magnesium deficits produced by chronic phenobarbital withdrawal could contribute to the observed phenobarbital withdrawal syndrome. Regarding calcium, the synaptic membrane content of this ion was decreased by implantation of pentobarbital pellets for three days (48). Whether this change is relevant to barbiturate tolerance and dependence is questionable, since this decrease was also produced by acute injection of pentobarbital (48). As discussed above, the depolarization-stimulated uptake of calcium by synaptosomes is inhibited by in vitro exposure to pentobarbital. Synaptosomes from barbiturate-tolerant animals are resistant to the inhibitory effects of an in vitro pentobarbital challenge on calcium transport (42, 190). This indicates a homeostatic adaptation of synaptic

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calcium transport resulting from chronic barbiturate exposure. Similarly, synaptosomes from alcohol-tolerant mice are also resistant to the in vitro effects of pentobarbital on calcium uptake (191). Thus, synaptosomal calcium transport may serve as a useful system for the study of membrane mechanisms underlying barbiturate and alcohol tolerance.

CONCLUSION

In terms of the biochemical event, most investigations of barbiturate intoxication, tolerance, and dependence have been concerned with the role of neurotransmitters. The evidence at present is strongest for GABA involvement in the acute effects of barbiturates and for NE and GABA involvement in the development of tolerance. Because of the possible regulatory mechanisms of neuroeffector substances of the transmission process, it is important to investigate further the roles of neurotransmitters as they may relate to the biochemical mechanisms of barbiturate intoxication, tolerance, and dependence. Since many neurotransmitter systems are affected by barbiturates, these actions may be mediated by some basic mechanism common to neuronal function in general. Such a mechanism may involve the interaction of barbiturates with membrane lipids. Such an interaction has been demonstrated, but it is not clear if it can provide the specificity required to explain the anesthetic, anticonvulsant, and convulsant effects of structurally related barbiturates.

Functional adaptive responses, such as tolerance, are generally recognized as being protein dependent. The synthesis of enzymes and structural proteins depends in part on the cellular biochemical machinery which is directed by DNA. The genetic information stored in the nucleus of each cell is expressed by the complex mechanisms of DNA to RNA transcription and of RNA translation into protein. Since barbiturates are known to be structurally similar to pyrimidines, the next step toward understanding the biochemical mechanisms of barbiturate tolerance and dependence should be directed toward the involvement of macromolecules such as nucleic acids and proteins, which are related to synaptic membrane function. Furthermore, the important roles of ions related to neurotransmitters and the synaptic event must not be overlooked. Because neurotransmitters, membrane lipids, macromolecules, and ions are intimately related in terms of synaptic transmission, they will be the subject of future biochemical investigations pertaining to the mechanisms of barbiturate tolerance and dependence.

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