

Inhibitory Effects of Different Barbiturates on Lipid Peroxidation in Brain Tissue *in Vitro*:

Comparison with the Effects of Promethazine and Chlorpromazine

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The protective effect of barbiturates in cerebral ischemia has been proposed to be related to inhibition of lipid peroxidation. The present *in-vitro* study was undertaken to test the efficiencies of different barbiturates to inhibit peroxidative reactions in brain tissue, and to compare their effects with established free radical scavengers (chlorpromazine and promethazine). Cortical homogenates, prepared from decapitated rats, were incubated at 37 C with 5 per cent O₂ (in N₂) in the presence of ferrous sulfate (0.01 mM) and ascorbic acid (0.25 mM). During incubation there was extensive lipid peroxidation in the tissue, as evidenced by appreciable production of thiobarbituric acid-reactive material (TBAR), 1.2 μmol malondialdehyde $\cdot\text{g}^{-1}$ cortex in one hour. Thiopental (1.0 mM) caused a 96 per cent inhibition of TBAR production, while other barbiturates (in the same concentrations) had only small (methohexital) or no (pentobarbital and phenobarbital) inhibitory effect. The inhibition of TBAR production by 1.0 mM thiopental was similar to that found with 1.0 mM chlorpromazine or 0.1 mM promethazine. The inhibitory effect of thiopental on lipid peroxidation was confirmed by analysis of fatty acids and phospholipids. Thiopental prevented the peroxidative degradation of polyenoic (20:4, 22:6) fatty acids and of ethanolamine phosphoglyceride that otherwise occurred during incubation.

The marked differences between the tested barbiturates with respect to their abilities to inhibit lipid peroxidation *in vitro* are at variance with the fact that all of these barbiturates have been reported to protect the ischemic brain in various situations *in vivo*. The results imply that the protective effect of barbiturates under such conditions is unrelated to inhibition of lipid peroxidation, and suggest that instead they may act by other mechanisms. (Key words: Ataractics, phenothiazines: chlorpromazine; promethazine. Brain: hypoxia, ischemia, protection. Hypnotics, barbiturates: methohexital; pentobarbital; phenobarbital; thiopental. Hypnotics, benzodiazepines: diazepam. Membrane: lipid peroxidation. Toxicity: free radicals.)

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Received from the Laboratory of Experimental Brain Research, E-Blocket, and the Department of Neurosurgery, University of Lund, Lund, Sweden. Accepted for publication March 7, 1980. Supported by grants from the Swedish Medical Research Council (No. 14X-263 and O3P-4931), from USPHS Grant No. 5 RO1 NS07838, and from the Knut and Alice Wallenberg Foundation. Dr. Smith was supported in part by USPHS Contract No. GM 07612-01 to the Department of Anesthesia, Hospital of the University of Pennsylvania. Presented in part at the Zentralluropäischer Anaesthesiekongress, Innsbruck, September 5-8, 1979.

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BARBITURATES AMELIORATE neuronal damage caused by cerebral hypoxia and ischemia, but the mechanisms by which they act are unknown.¹⁻⁵ Barbiturates do not increase the tissue concentrations of high-energy phosphates, nor do they greatly prolong the availability of these compounds during complete or severe incomplete ischemia. Barbiturates decrease cerebral metabolic rate, yet other agents that do this are not protective. Barbiturates reduce cerebral blood flow and depress intracranial pressure in conditions of intracranial hypertension, but there is no evidence that this effect is important in other forms of cellular hypoxia.

Recently, Demopoulos and co-workers⁶ proposed that damage during cerebral hypoxia and ischemia is related to lipid peroxidation. They hypothesized that the onset of ischemia or hypoxia releases from the electron transport chain normally tightly controlled free radicals, which then react with surrounding phospholipids. These phospholipids are the major membrane lipids in cell organelles, and are highly susceptible to lipid peroxidation because of their high concentration of polyenoic fatty acids. Lipid peroxidation of phospholipids *in vitro* produces conformational changes and degradation that can directly alter or prevent the function of closely associated enzymes.⁷ As hypothesized by Demopoulos *et al.*,⁸ the same events may occur *in vivo*, thus explaining the irreversible damage secondary to a hypoxic or ischemic insult.

Alternatively, Nordström *et al.*⁸ speculated that free radical damage might occur not during, but after, an episode of hypoxia or ischemia, *i.e.*, when oxygen returns to the tissue. If so, potentially reversible damage may be converted into irreversible damage after the restoration of blood flow and tissue oxygenation.⁹

With respect to barbiturates, Flamm *et al.*¹⁰ proposed that they protect during cerebral ischemia by inhibiting lipid peroxidation. They showed that methohexital decreases the loss of ascorbic acid (a naturally occurring free radical scavenger) after ligation of the middle cerebral artery in the cat.^{10,11} Further, they have shown that methohexital obliterates coenzyme Q free radicals in certain model systems.¹²

However, investigations of carbon tetrachloride peroxidative damage in the liver suggest that not all barbiturates are efficient free radical scavengers.^{13,14}

The following experiments were designed to examine the hypothesis that barbiturates act as free radical scavengers in the brain. Tissue homogenates and subcellular fractions incubated in the presence of oxygen undergo lipid peroxidation, the rate of which is increased by adding iron and ascorbic acid.^{15,16} In an earlier communication we described such an *in-vitro* model for studying lipid peroxidation in brain cortical homogenates.¹⁷ In that study we showed that changes in three different indices of lipid peroxidation (measurement of thiobarbituric acid-reactive material, diene conjugation, and the accumulation of lipid-soluble fluorescent products) parallel each other during the first 60 min of peroxidation. Furthermore, we showed that changes in these indices of lipid peroxidation occur concomitantly with characteristic changes in brain fatty acids and phospholipids¹⁷ (*cf.* May and McCay¹⁸). In the present study, we used this model for evaluating the antioxidant effect of several barbiturates, of diazepam, and of two classic free radical scavengers, promethazine and chlorpromazine. Lipid peroxidation was assessed by measuring the formation of thiobarbituric acid-reactive material, and in one series of experiments, by measuring changes in fatty acids and phospholipid composition. A preliminary account of some of the findings has been published.¹⁹

Materials and Methods

Male Wistar rats (350–400 g) had free access to food and water prior to each experiment. They were decapitated and their heads immediately frozen in liquid nitrogen. The brains were chiselled out under intermittent liquid nitrogen irrigation and stored at -80°C until use (12–36 hours after decapitation). The technique for preparation of tissue homogenates and for induction of lipid peroxidation has been described elsewhere.¹⁷ In brief, just prior to each experiment the brain was brought to -22°C , and a 400-mg sample of cortical tissue was removed. The cortical tissue was homogenized in a deoxygenated, 0.05 M, phosphate buffer at 0°C using a glass pestle homogenizer. The buffer stock solution contained 15 mM Na^{+} and 145 mM K^{+} and was adjusted to pH 7.0 with 1 M HCl.

The resulting brain homogenate was split into equal volumes and placed into paired tonometers equilibrated with 5 per cent oxygen in 95 per cent nitrogen. The drug tested for its antioxidant capacity was

added to one tonometer, and an equal volume of its solvent to the other. Following this the free radical initiators ferrous sulfate (0.01 mM) and ascorbic acid (0.25 mM) were added. The final tissue concentration was $80\text{ mg}\cdot\text{ml}^{-1}$.

Unless otherwise stated, the homogenates were incubated for one hour at 37°C and samples were taken for analysis after 0, 30, 45 and 60 min of incubation. In a separate series of experiments, rats received injections of phenobarbital, $150\text{ mg}\cdot\text{kg}^{-1}$, ip, one hour prior to decapitation. No additional lipid peroxidation inhibitors were added at the time of incubation, but the homogenates were incubated with and without added free radical initiators.

Lipid peroxidation was estimated using the thiobarbituric acid test as modified from Slater and Sawyer.^{17,20} This test measures certain lipid peroxidation products which react with thiobarbituric acid (TBA). These products (thiobarbituric acid-reactive; TBAR) are mainly, but not entirely, malondialdehyde (MDA).²¹ In one series of experiments, effects of lipid peroxidation were also estimated by analyzing brain homogenates for total fatty acids, fatty acid composition, total free fatty acids, free fatty acid composition, total phosphorus, and phospholipid composition according to methods described previously.¹⁷ For these analyses brain homogenates from decapitated rats were incubated for 0 or 45 min prior to extraction in chloroform–methanol (aliquots for TBA testing were also taken). At the onset of incubation, thiopental (1 mM) or its solvent was added just prior to the addition of the free radical initiators, ferrous sulfate and ascorbic acid.

The following drugs were tested for their ability to inhibit lipid peroxidation: thiopental (Abbott, USP for injection), methohexital (Lilly, USP for injection), pentobarbital, phenobarbital, promethazine, chlorpromazine, and diazepam. All drugs except diazepam were dissolved in double-distilled water. Diazepam was dissolved in propylene glycol. Except for chlorpromazine, all drug solutions (including ferrous sulfate and ascorbic acid) were freshly made daily, and stored on ice in subdued light. The chlorpromazine solution, because of its allergenic potential, was prepared at the hospital pharmacy, stored in a dark container, and used within two weeks. It showed no sign of discoloration at the time of use. All concentrations referred to are the final concentrations in the tonometer.

Concentrations are given as $\mu\text{mol}\cdot\text{g}^{-1}$ of cortical wet weight. Values are means \pm SEM of results from four to six brains. Differences between groups were statistically evaluated by using the Student *t* test.

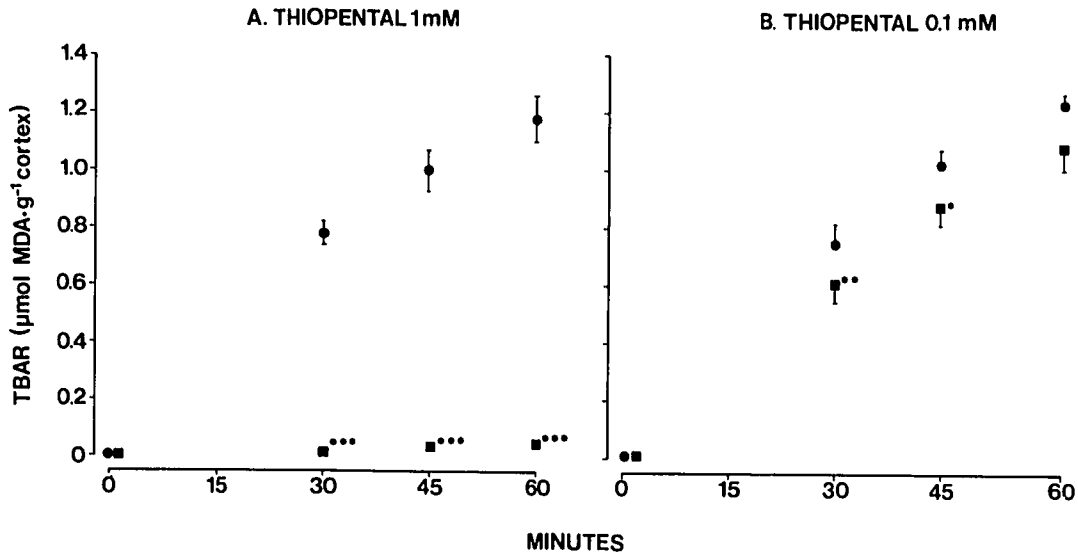


FIG. 1. The inhibitory effect of thiopental on lipid peroxidation in brain tissue, *in vitro*. Squares represent thiopental-treated and circles represent nontreated aliquots from the same homogenate. Lipid peroxidation was measured by the accumulation of thiobarbituric acid-reactive material (TBAR) during incubation at 37 C with ferrous sulfate (0.01 mM) and ascorbic acid (0.25 mM) in the presence of 5 per cent O₂ (in N₂). Each symbol represents the mean \pm SEM of four to six paired experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (paired-sample *t* test).

Results

INHIBITION OF THE PRODUCTION OF THIOBARBITURIC ACID-REACTIVE MATERIAL (TBAR)

In some models of ischemia a protective effect of barbiturates was observed only when very large doses were used.¹ The first drug tested in the present study was phenobarbital. In anesthesia induced with phenobarbital, brain tissue concentrations approach 1 mM.²² We chose to use this dose in the *in-vitro* system. In order to facilitate comparisons, other drugs were added in equal concentration. However, in the event TBAR

formation was inhibited, a tenfold lower concentration was also employed.

Since we have shown that the production of TBAR in the present model correlates well with other techniques for measuring lipid peroxidation,¹⁷ the TBA test was used primarily to assess the inhibitory effects of the drugs tested.

There was no TBAR present at the onset of incubation, suggesting that lipid peroxidation did not occur before or during preparation of the homogenates (fig. 1A). During incubation with ferrous sulfate and ascorbic acid in the presence of 5 per cent oxygen

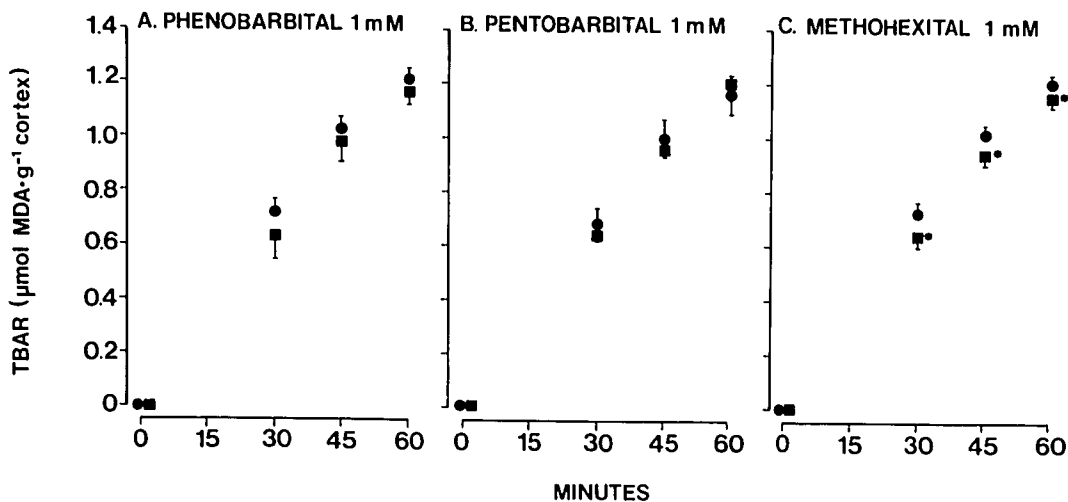


FIG. 2. The effects of phenobarbital, pentobarbital, and methohexital on lipid peroxidation in brain tissue *in vitro*. Incubation conditions and symbol representation are the same as in figure 1.

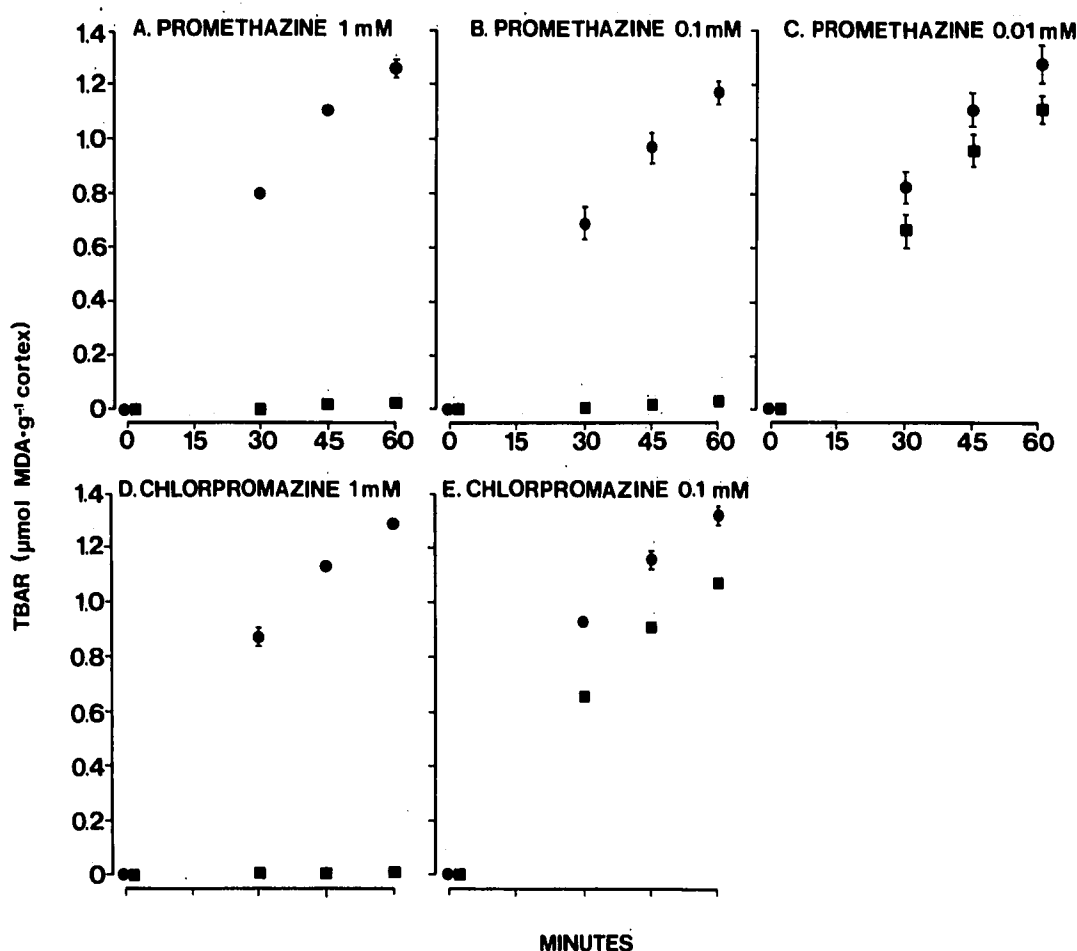


FIG. 3. The effects of promethazine and chlorpromazine on lipid peroxidation in brain tissue *in vitro*. Incubation conditions and symbol representation are the same as in figure 1. For panels A, B, D, E: $P < 0.001$ for 30–60 min of incubation compared with controls. For panel C: $P < 0.05$ for 30–60 min of incubation compared with controls.

there was rapid formation of TBAR. The addition of thiopental (1 mM) at the beginning of incubation produced a 96 per cent inhibition of TBAR formation (fig. 1A). Thiopental (0.1 mM, fig. 1B) produced a 14 per cent inhibition, but this decrease in TBAR was statistically significant for the first 45 min of incubation only.

To exclude the possibility that thiopental inhibited the TBA reaction without actually altering lipid peroxidation,²³ thiopental (1 mM) was added at the end of 45-min incubations in five experiments. In samples taken before addition of thiopental, TBAR was $0.995 \pm 0.046 \mu\text{mol}\cdot\text{g}^{-1}\text{ cortex}$; in samples taken after addition of thiopental, $1.010 \pm 0.070 \mu\text{mol}\cdot\text{g}^{-1}\text{ cortex}$. Thus, thiopental did not affect the TBA reaction.

Unlike thiopental, neither phenobarbital (1 mM) nor pentobarbital (1 mM) altered TBAR formation (fig. 2, A and B). Methohexital (1 mM) produced a slight but significant decrease in TBAR formation (fig. 2C). This decrease, however, was markedly less than that seen with thiopental. In fact, the non-barbiturate

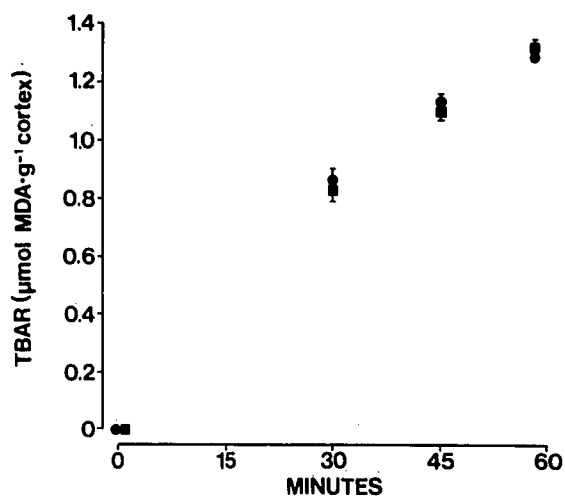


FIG. 4. The effect of phenobarbital ($150 \text{ mg}\cdot\text{kg}^{-1}$ given ip an hour prior to decapitation) on lipid peroxidation in brain tissue, *in vitro*. Incubation conditions are the same as for figure 1. Squares represent homogenates from phenobarbital-treated and circles represent homogenates from non-treated rats. Each symbol is the mean \pm SEM for four rats. There was no statistically significant difference between the two groups (Student *t* test).

TABLE 1. Effects of Thiopental on Peroxidative Changes in Brain Cortical Fatty Acids*

	Thiopental	Control
Fatty acids		
Total	106.5 ± 6.2	96.0 ± 3.8‡
Individual		
16:0†	25.8 ± 1.2	24.9 ± 0.6
18:0	22.6 ± 1.3	20.5 ± 0.7‡
20:0	1.5 ± 0.04	1.5 ± 0.1
16:1	1.6 ± 0.04	1.5 ± 0.04
18:1	20.9 ± 1.5	20.0 ± 1.0
20:1	1.5 ± 0.2	1.5 ± 0.1
18:2	1.0 ± 0.2	0.8 ± 0.2
20:3	0.6 ± 0.1	0.7 ± 0.1
20:4	11.0 ± 0.6	9.6 ± 0.5‡
22:4	2.4 ± 0.3	1.9 ± 0.1
22:6	17.6 ± 1.1	13.1 ± 0.9§

* To paired samples of cortical homogenates, thiopental (1 mM) or an equivalent volume of water was added just prior to the addition of ferrous sulfate (0.01 mM) and ascorbic acid (0.25 mM). The values ($\mu\text{mol}\cdot\text{g}^{-1}$ wet weight cortex) are means \pm SEM from four experiments.

† Number of carbon atoms:number of double bonds.

‡ $P < 0.05$, § $P < 0.01$ (paired-sample t test).

TABLE 2. Effects of Thiopental on Peroxidative Changes in Brain Cortical Phospholipids *in Vitro**

	Thiopental	Control
Phospholipids		
Total	60.3 ± 1.4	55.3 ± 2.1†
Individual		
Ethanolamine phosphoglyceride	23.9 ± 0.7	19.4 ± 1.1†
Choline phosphoglyceride	25.5 ± 0.8	24.9 ± 1.1
Serine + inositol phosphoglyceride	8.5 ± 0.1	7.6 ± 0.4
Sphingomyelin	1.9 ± 0.2	2.1 ± 0.3

* Conditions are the same as for table 1. The values are means \pm SEM of three experiments.

† $P < 0.05$ (paired-sample t test).

drug, diazepam, had greater inhibitory effects on TBAR production than did methohexital. Diazepam, at a concentration of 1 mM, decreased TBAR formation by about 50 per cent (not shown). However, diazepam (0.1 mM) had no significant effect on TBAR.

To evaluate the relative antioxidant efficiency of thiopental, its effect was compared with those of the established free radical scavengers promethazine and chlorpromazine (fig. 3). Promethazine produced almost complete inhibition of TBAR formation even at a concentration of 0.1 mM. A concentration of 1 mM chlorpromazine produced complete inhibition of TBAR formation, and a 0.1 mM concentration produced about 25 per cent inhibition. Thus, chlorpromazine had an antioxidant effect similar to that of

thiopental (*cf.* fig. 1), while promethazine was clearly more efficient.

As lipid peroxidation occurs near the hydrophobic regions of cellular and subcellular membranes,²⁴ it is possible that differences in lipid solubility, and consequently the rates of drug diffusion to the active site, may account for the marked differences in inhibitory effects of the barbiturates tested. We therefore incubated brain homogenates with phenobarbital (1 mM) for 15 min prior to adding the ferrous sulfate and ascorbic acid. This preincubation had no inhibitory effect on TBAR formation. Additionally, rats were anesthetized with phenobarbital, 150 $\text{mg}\cdot\text{kg}^{-1}$, ip. This dose of phenobarbital has been previously shown to ameliorate some of the effects of prolonged incomplete ischemia.⁸ As shown in figure 4, brain homogenates from these phenobarbital-treated rats had a TBAR production similar to that of brain homogenates from non-treated controls. This was also true for TBAR production in homogenates to which no free radical initiators had been added (not shown).

EFFECTS OF THIOPIENTAL ON PEROXIDATIVE CHANGES IN FATTY ACIDS AND PHOSPHOLIPIDS

We have reported elsewhere that lipid peroxidation in brain homogenates is accompanied by characteristic changes in cortical fatty acids and phospholipids, notably by losses of the polyenoic fatty acids, arachidonic (20:4) and docosahexaenoic (22:6) acids, and of ethanolamine phosphoglyceride.¹⁷ In order to ascertain that thiopental inhibits not only TBAR formation but also the associated changes in fatty acids and phospholipids, these compounds were measured in homogenates after 45-min incubations with or without added thiopental (1 mM).

Values for TBAR obtained in the samples used for fatty acid analysis were 0.004 ± 0.002 and $0.977 \pm 0.140 \mu\text{mol MDA}\cdot\text{g}^{-1}$ rat cortex (thiopental and solvent control, respectively). As shown in table 1, total fatty acid recovery was 9 per cent greater in samples incubated with thiopental (1 mM) compared with solvent controls. In particular, samples incubated with thiopental had a markedly greater recovery (26 per cent) of the highly unsaturated docosahexaenoic acid (22:6). There were also significantly increased recoveries of arachidonic (20:4) and stearic (18:0) acids.

A major fraction of the total fatty acid loss seen during incubation without thiopental is accounted for by loss of phospholipids. As shown in table 2, significantly less of the phospholipids was recovered when samples were incubated without thiopental. Most of this phospholipid loss (90 per cent) was due to decreased ethanolamine phosphoglyceride, the major polyenoic phospholipid in rat brain.

Free fatty acid content was also analyzed. Total free fatty acid concentrations were increased in both thiopental-incubated and non-thiopental-incubated samples ($1.55 \pm .19$, $1.38 \pm .14 \mu\text{mol FFA} \cdot \text{g}^{-1}$ cortex, respectively) compared with non-incubated controls ($.4 \pm 0.3 \mu\text{mol} \cdot \text{g}^{-1}$). This fourfold increase in total free fatty acids during the incubation period is probably related to phospholipase activity.²⁵ There was, however, no statistically significant difference between thiopental-incubated and non-thiopental-incubated samples with respect to total free fatty acids or free fatty acid compositions. This confirms the fact that the decrease in ethanolamine phosphoglyceride in control homogenates was actually due to a breakdown of this compound and not merely secondary to hydrolytic cleavage. These findings suggest that the effect of thiopental was to inhibit peroxidative loss of fatty acids and phospholipids.

Discussion

Many tissues or subcellular fractions of tissues readily undergo free radical-induced lipid peroxidation *in vitro*.²⁶ Based on *in-vitro* findings, free radical attack or lipid peroxidation has been suggested as the mechanism for carbon tetrachloride-induced hepatic damage, radiation damage, drug-induced hemolysis, vitamin E deficiency disease, oxygen toxicity, ethanol-induced fatty liver, and paraquat toxicity in the lung.²⁶⁻²⁸ But in each case adequate *in vivo* evidence has been difficult to obtain, partly because of the difficulty in measuring lipid peroxidation intermediates or free radicals. Thus, demonstration of lipid peroxidation *in vitro* may be only tentatively extrapolated to explain *in-vivo* pathologic processes. However, *in-vitro* studies of lipid peroxidation can be used to estimate the likelihood of peroxidation *in vivo*.²⁹

LIPID PEROXIDATION IN BRAIN TISSUE IN VITRO

Previously, we showed that brain homogenates incubated together with ferrous sulfate and ascorbic acid in 5 per cent oxygen produce TBAR, and that the increase in TBAR is accompanied by increases in diene conjugation and fluorescent products.¹⁷ These changes are associated with a concomitant decrease in the polyenoic fatty acids arachidonic (20:4) and docosahexaenoic (22:6) acid. There is no increase in TBAR or decrease in polyenoic fatty acids during incubation in 100% nitrogen.¹⁷ Oxygen consumption of these brain homogenates alone is low ($0.06 \mu\text{mol O}_2 \cdot \text{g}^{-1}$ cortex $\cdot \text{min}^{-1}$), but is increased fivefold ($0.31 \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$) with the addition of ferrous sulfate and ascorbic acid (unpublished ob-

servations). All of these findings, though indirect, are consistent with and considered to be evidence of lipid peroxidation.^{18,20,26}

Since our previous results showed an excellent correlation between TBAR formation, diene conjugation, changes in fluorescence and loss of polyenoic fatty acids during the first 60 min of incubation,¹⁷ we chose to evaluate lipid peroxidation by measuring TBAR formation.

In the current experiments, thiopental strongly inhibited TBAR production. Thiopental also prevented the degradation of polyenoic fatty acids and ethanolamine phosphoglyceride that otherwise occurs during incubation. Together these data suggest that thiopental inhibits iron- and ascorbic acid-catalyzed lipid peroxidation in brain homogenates. The results with thiopental are obviously consistent with the hypothesis that barbiturates ameliorate brain damage in some forms of ischemia by acting as free radical scavengers. However, since methohexital had a very small antioxidant effect, and both pentobarbital and phenobarbital none, it seems less likely that barbiturates protect against ischemic brain damage by acting as free radical scavengers (see below).

In the present *in-vitro* study, differences between barbiturates with respect to certain physical properties may have "masked" a possible antioxidant effect. However, differences in lipid solubility do not appear to be contributory, since thiopental and methohexital have similar lipid solubilities as measured by their oil-water partition coefficients.³⁰ Neither is the ratio of ionized to unionized form of the barbiturate a factor, since thiopental and phenobarbital have similar pK_a values.³¹ Finally, neither extended time for the equilibration of phenobarbital with the tissue by preincubating it with the homogenates prior to initiating lipid peroxidation nor administration of the drug *in vivo* prior to decapitation revealed any antioxidant effect. Notice that the dose of phenobarbital administered was previously shown to ameliorate certain aspects of neuronal damage seen during pronounced incomplete ischemia⁸ (see below).

Promethazine and chlorpromazine also inhibited lipid peroxidation in the homogenates. These two compounds are classified as free radical scavengers and are thought to inhibit lipid peroxidation by accepting the unpaired electron from free radicals, forming, in turn, resonance-stabilized and less reactive free radicals.³² This activity depends on both the nitrogen and the sulfur present in the heterocyclic ring of the phenothiazine. Substitution of oxygen for the sulfur, for example, eliminates free radical scavenging activity.³³ Though our experiments do not allow us to conclude that thiopental, chlorpromazine, and

promethazine are acting by the same mechanisms, the presence of a thioketone instead of a ketone on the pyrimidine ring of thiopental may account for its greater effectiveness, compared with the oxybarbiturates, with respect to inhibiting lipid peroxidation *in vitro*.

Others have also found that some barbiturates are relatively ineffective inhibitors of lipid peroxidation *in vitro*. Phenobarbital has minimal inhibitory effects on hepatic microsomal lipid peroxidation induced by carbon tetrachloride, in direct contrast to the marked inhibition seen with chlorpromazine or promethazine.^{13,14} Amobarbital, an oxybarbiturate, inhibits mitochondrial swelling secondary to electron transport,³⁴ but has no effect on swelling secondary to lipid peroxidation.³⁵

IMPLICATION OF PRESENT FINDINGS FOR IN-VIVO EFFECTS OF BARBITURATES

Extrapolation of the present results to *in-vivo* conditions, notably to those prevailing during hypoxia and ischemia, presents certain difficulties. First, the nature of the radicals released *in vivo* (if any) is not known. Second, antioxidants or scavengers may be relatively selective in their efficacy. In other words, if a substance does not quench radicals formed in one system it cannot be concluded that it is without effect on radicals generated in another system. Third, the kinetics of free radical production *in vitro* due to ascorbic acid-ferrous ions may be different from any production *in vivo*. Conceivably, a substance that is without effect in the *in vitro* system could therefore quench radicals *in vivo*.

We chose to induce lipid peroxidation *in vitro* by ascorbic acid and ferrous ions since the system generates hydroxyl radicals (OH[•]), the species which is usually incriminated in radical damage *in vivo* (e.g., Willson³⁶). We assume that any brain damage induced during (or following) hypoxia and ischemia is due to formation of hydroxyl radicals. The assumption appears reasonable in view of the fact that any univalent reduction of oxygen will, via the Haber-Weiss reaction, generate OH[•].^{36,37} The free radical hypothesis of Demopoulos *et al.*⁶ states that the radicals generated emanate from the electron transport chain, possibly involving pathologic cycling of coenzyme Q. This is at present only an assumption. Besides, it seems that such reactions also lead to OH[•] formation.³⁶ Presumably, the kinetics of free radical formation *in vitro* (present data) and *in vivo* are not too dissimilar. This assumption is supported by results showing a 21 per cent reduction in tissue ascorbic acid levels

an hour after ligation of the middle cerebral artery in the cat.¹¹

A potential drawback of the present study is the fact that drug effects were tested in only one radical-generating system. However, preliminary data obtained by mixing the drugs (0.3 mM final concentration) with 1,1 diphenyl-2-picrylhydrazyle (DPPH, 0.07 mM final concentration), a stable free radical that changes color upon reaction with free radical scavengers,³⁸ support the present conclusions. Thus, phenobarbital and pentobarbital produced little decrease in the amount of DPPH free radical, methohexital had some activity, thiopental produced a considerable decrease, and promethazine had the largest effect (Smith, Rehncrona and Siesjö, unpublished observations).

Our results suggest that thiopental could scavenge radicals generated in hypoxia and ischemia. By the same token, the brains of animals given promethazine and chlorpromazine (and diazepam) should be more resistant to the deleterious effects of hypoxia. Conversely, treatment with phenobarbital, pentobarbital, or methohexital should have little effect. Since not only thiopental, but also phenobarbital, pentobarbital, and methohexital, have been shown to protect the brain in ischemia,^{5,8,39} there is a lack of correlation between protection *in vivo* and free radical scavenging properties *in vitro*. Furthermore, we have failed to observe any beneficial effect of promethazine in incomplete ischemia (unpublished results), in spite of the fact that this drug is the most efficient scavenger *in vitro*. Obviously, the present results fail to support the hypothesis that barbiturates in general protect the brain in hypoxia and ischemia by acting as free radical scavengers.

The results obtained by Flamm *et al.*¹⁰ in a study of the *in-vivo* effect of methohexital appear to be at variance with the present *in-vitro* results. The proposition of these investigators that methohexital protects ischemic brain tissue *in vivo* by acting as a free radical scavenger was mainly based on three findings. First, methohexital prevented the loss of cortical ascorbic acid (an endogenous free radical scavenger) that otherwise occurred after five hours of occlusion of the middle cerebral artery in the cat. Second, methohexital prevented the loss of polyenoic fatty acids (20:4 and 22:6) in the same experimental situation. Third, methohexital was found to scavenge radicals generated by irradiation of liposomes with ultraviolet light.¹²

The interpretation of the first finding is somewhat hampered by the fact that the total concentration of a scavenger (oxidized plus reduced form) may

decrease by mechanisms other than oxidation. For example, our results have shown that ischemia may be accompanied by a reduction in the tissue concentration of reduced glutathione (GSH), but since no increase occurred in the oxidized form (GSSG), one cannot conclude that the reduction in GSH concentration was due to oxidation.⁴⁰ The third finding provides rather indirect evidence of a scavenging effect *in vivo* due to the difficulty of extrapolating from *in-vitro* results obtained in artificial membrane systems. Thus, the strongest evidence is provided by the observed, selective loss of polyenoic fatty acids. However, until further data are available, the possibility remains that the suggested peroxidative degradation of tissue constituents is a late phenomenon that is the result of irreversible tissue damage, rather than its cause.

In summary, the present results show that several barbiturates that have been shown to afford protection to the brain in hypoxia and ischemia do not scavenge the free radicals that are produced in iron- and ascorbic acid-induced lipid peroxidation in brain tissue *in vitro*. The results therefore suggest that the protective effects of barbiturates are due to mechanisms other than free radical scavenging. However, due to the difficulty of extrapolating from *in-vitro* to *in-vivo* situations, the provocative hypothesis of Demopoulos *et al.*⁶ deserves further testing.

The authors are grateful to Lena Sjöberg and Eva Westerberg for excellent assistance.

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