EFFECT OF DIETHYL ETHER ON TISSUE DISTRIBUTION AND METABOLISM OF PENTOBARBITAL IN RATS

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CLINICIANS have long noted an apparent potentiation of effect during the simultaneous administration of a nonvolatile anesthetic and an inhalation anesthetic. Proof that the interreaction represents potentiation rather than a simple additive effect is, however, difficult to obtain. Eckenhoff and Helrich (1) have produced experimental evidence strongly suggesting that in humans nitrous oxide potentiates a combination of a narcotic and thiopental and that the effect of the nitrous oxide is not merely additive. Greene and Whittaker (2) have also found that the administration of 3.6 volumes per cent of diethyl ether to rats given 20 mg./kg. of pentobarbital resulted in an effect which resembled potentiation rather than summation. The present study is an extension of the work of Greene and Whittaker in an attempt to define why ether potentiates pentobarbital anesthesia. The results to be presented are of practical clinical value in view of the frequent use of barbiturates prior to inhalation anesthesia. They are also of value in that they provide information concerning the effect of ether on certain enzyme systems as well as casting light on the cellular site of action of ether.

METHODS

Pentobarbital used in the analytical studies was prepared as follows: U.S.P. pentobarbital sodium was added to water at 60 C. almost to the saturation point, following which the solution was acidified with HCl. The resultant white precipitate was vacuum filtered, washed first with distilled water, then with ether and 95 per cent ethanol, and finally with water again before being dried in a vacuum desiccator. The white crystalline product was insoluble in water but freely soluble in alkali. It had absorption peaks at 240 μ in *p*H 11 phosphate buffer and at 255 μ in 1*N* NaOH as reported by Brodie *et al.* (3). A standard solution of pentobarbital containing 10 μg./ml. was prepared by dissolving 10 mg. of pentobarbital acid in 3 ml. 0.1*N* NaOH and diluting to 100 ml. with distilled water. The standard solutions were stored at 4 C. and were made up fresh each month. The optical density of standard solutions in *p*H 11 phosphate buffer with 10 μg./ml. did not change from 0.40 with time.

The analytical procedure used for estimation of pentobarbital was

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a modification of that described by Brodie *et al.* (3). Pentobarbital was extracted from tissues as the free acid by shaking tissue homogenates or supernatant reaction mixtures with a mixture of 98.5 per cent petroleum ether-1.5 per cent isoamyl alcohol, NaCl, and *p*H 5.5 phosphate buffer. The resulting mixture was centrifuged, aliquots were withdrawn from the nonaqueous phase, and the aliquots then shaken with *p*H 11 phosphate buffer to re-extract the pentobarbital as its sodium salt. Following this the *p*H 11 buffer was drawn off and the sample read at 240 μ in a Beckman ultraviolet spectrophotometer.

Tissue homogenates and plasma aliquots, after being prepared as described under *in vivo experiments,* were mechanically shaken in 120 ml. glass-stoppered shaking bottles. Liver, kidney, fat, and brain aliquots were shaken for 10 minutes with 100 ml. 98.5 per cent petroleum ether-1.5 per cent isoamyl alcohol, to which 3 Gm. NaCl and 3 ml. of 0.2 M *p*H 5.5 phosphate buffer had been added. Plasma aliquots were shaken for only 5 minutes with 50 ml. of solvent to avoid gel formation. Liver, kidney, fat, and brain mixtures were centrifuged for 10 minutes at 4 C. at 2,000 r.p.m. and plasma mixtures for 10 minutes at 2,000 r.p.m. at room temperature. Aliquots of the resulting supernatants (20 ml. for plasma, 75 ml. for other tissues) were withdrawn and shaken for 3 minutes with 5 ml. of *p*H 11 phosphate buffer, following which the contents of the shaking bottles were transferred to 60 ml. separatory funnels, allowed to stand for 10 minutes and the *p*H 11 buffer drawn off into quartz cuvettes and read against *p*H 11 buffer in the ultraviolet spectrophotometer at 240 μ.

Tissue Blanks.—Inasmuch as the tissues studied contained a compound or compounds which were partially or completely extracted by the analytical procedure used for pentobarbital and because these compounds had an appreciable absorption at *p*H 11 and 240 μ, tissue blanks were determined in seven rats which were sacrificed after being deeply anesthetized with ether. Because the methods used to obtain tissues for tissue blank determination were the same as those used in the *in vivo* experiments, they will be described here. An incision was made so as to open widely the peritoneal and pleural cavities. As much venous blood as possible (2 to 10 ml.) was withdrawn from the beating heart and shaken with enough sodium oxalate to prevent clotting. Samples of liver, kidney, brain, and peritoneal fat were then obtained (always in the same order), weighed, and homogenized at room temperature with three times the weight of the samples of unchilled distilled water. The fat samples were emulsified with 0.5 *N* NaOH and the *p*H adjusted to 5.5 with 5 *N* HCl. A 3–4 ml. aliquot of each tissue homogenate was then inactivated by pipetting into a shaking bottle containing a mixture of 98.5 per cent petroleum ether–1.5 per cent isoamyl alcohol, NaCl, and *p*H 5.5 phosphate buffer. The oxalated blood was centrifuged after preparation of the liver homogenate and a 1 ml. aliquot was then inactivated in the same manner as were aliquots of tissue homogenates.

In Vivo Experiments.—In all *in vivo* experiments male Sprague-Dawley rats weighing 275 to 480 grams were used. The concentrations of pentobarbital in liver, kidney, fat, brain, and plasma were determined in 7 rats 40 minutes after the intraperitoneal administration of 25 mg./kg. of pentobarbital. During the 40 minute interval the rats were in a transparent 90-liter airtight container filled with room air. Rats given this amount of pentobarbital lost their righting reflexes in about 5 minutes but usually were moving, although ataxically, after 30–35 minutes.

Seven rats were studied to determine the effect of ether on the tissue distribution of pentobarbital. Each rat was given 25 mg./kg. of pentobarbital intraperitoneally and immediately placed in the 90-liter airtight container to which was added enough ether to provide a concentration of 3.6 volumes per cent (14.4 ml. at room temperature at sea level). All rats became anesthetized within 5 minutes. After 40 minutes in the container the rats were sacrificed and tissues obtained for analysis.

In Vitro Experiments.—The enzymatic oxidation of pentobarbital by rat liver homogenates was studied using the technique described by Cooper and Brodie (4). Three non-fasting male Sprague-Dawley rats were used in each experiment. Smaller rats (140–170 Gm.) were used in the *in vitro* studies than in the *in vivo* studies because it was found that the livers of the younger rats oxidized pentobarbital more completely than did those of more mature rats. The three rats used in each experiment were stunned and exsanguinated; their livers were then removed, placed in a chilled tared beaker, and weighed. The combined weight of the 3 livers ranged from 11 to 14 grams. The weighed livers were homogenized in a chilled homogenizer with three times their weight of chilled 0.1 *M* *p*H 7.4 phosphate buffer. The homogenate was centrifuged at 2,000 r.p.m. (about 9,000 g.) for 30 minutes at 4 C., following which four 2-ml. aliquots of the resultant supernatant were pipetted into 50 ml. beakers preheated to 37 C. The remaining supernatant was kept chilled and saved. The 50-ml. beakers were shaken for 3 minutes in a 37 C. bath to bring the aliquots to 37 C. and then removed to a room of the same temperature where the rest of the procedure was carried out. To two of the four aliquots, 0.25 μM of triphosphopyridine nucleotide (TPN), 50 μM of nicotinamide, 25 μM of $MgCl_2$, and 0.1 *M* *p*H 7.4 phosphate buffer were added. To the other two aliquots the same constituents were added along with 1 μM of pentobarbital. The total volume of each mixture was 4.0 ml. after addition of these substances. The nicotinamide was added to protect the TPN against enzymatic destruction, the Mg^{++} to increase enzymatic activity. The four aliquots were then placed in an airtight container of 9.18 l. capacity and mechanically shaken for 40 minutes following which 2-ml. aliquots were removed and inactivated as described under *in vivo* methods.

Aliquots (2 ml.) of the saved chilled supernatant were preincubated at 37 C., transferred to the 37 C. room and treated exactly as described

above except that 1.51 ml. of ether was added to the 9.18 l. airtight container in order to provide a concentration of 3.6 volumes per cent ether during the 40 minutes of shaking. The possibility that time inactivation of the chilled supernatants would affect the results was avoided by altering the starting order of the runs. A given run was started approximately 4 minutes after preincubation, the second component starting approximately 12 minutes later.

All solutions added to the supernatants were prepared freshly each week, refrigerated until used, and preheated to 37 C. immediately prior to being added to the supernatants. In order to prevent decomposition of the TPN, it was stored frozen in an acid solution, thawed just before use, and discarded after use.

Due to evaporation at 37 C., volume controls were run in each experiment. These showed that 0.3 ml. of the starting volume of 4 ml. evaporated after 40 minutes of shaking. The loss due to evaporation was used in calculating results.

Methods of Calculation.—Extraction of pentobarbital from tissues by the added solvents was found to be neither complete nor constant. This was contrary to the experience of Brodie *et al.* (3) who found complete extraction. Because of this, it was necessary to run control studies in which 50 μg. of pentobarbital were added to tissues analyzed in the *in vivo* experiments, and to run these tissues with the added pentobarbital through the entire analytical procedure along with the tissue unknowns. The pentobarbital levels in the tissue unknowns were then calculated using the average of the optical densities of the controls.

The petroleum ether used in the first portion of the *in vivo* experiments could be easily purified so that there were no optically absorbing impurities at 240 μ. However, the petroleum ether used in the last two sets of *in vivo* experiments contained optically absorbing impurities which were at least partially extracted by the extraction procedures used for pentobarbital. These impurities could not be entirely removed despite vigorous treatment of the petroleum ether. Accordingly, in the last 2 experiments reagent blanks were run in duplicate along with the tissue samples.

In the *in vitro* experiments, four 10-minute controls and four 10-minute reagent blanks were run in quadruplicate along with the 40-minute reaction mixture aliquots, and their average values were used in calculating the amount of pentobarbital remaining in the reaction mixture after 40 minutes.

The methods of calculation were as follows:

In vivo:

$$\mu\text{g. PB/Gm. tissue} = \frac{OD - RB_{10}}{C_{10} - RB_{10}} \times \frac{V_1}{V_2} \times \frac{1}{W} \times 50$$

$$\mu\text{g. PB/ml. plasma} = \frac{OD - RB_5}{C_5 - RB_5} \times \frac{1}{V_2} \times 50$$

In vitro:

$$\mu M \text{ PB remaining} = \frac{OD - RB_{10}}{C_{10} - RB_{10}} \times \frac{V_f}{V_a} \times 50,$$

where PB = pentobarbital acid,
OD = observed optical density of unknown,
C_{10} = observed optical density of 10 minute control,
C_5 = observed optical density of 5 minute control,
RB_{10} = optical density of 10 minute reagent blank,
RB_5 = optical density of 5 minute reagent blank,
V_1 = volume of homogenate,
V_2 = volume of homogenate taken for analysis,
V_3 = volume of plasma taken for analysis,
W = weight of tissue sample,
V_f = residual volume,
V_a = volume of reaction mixture taken for analysis.

Statistical Methods.—The statistical significance of the difference between pentobarbital tissue levels found in animals given pentobarbital alone and those levels found in animals given pentobarbital and ether was determined by use of the pooled t test where

$$t = \frac{\bar{X}_{(PB+E)} - \bar{X}_{(PB)}}{\sqrt{S^2_{\bar{X}(PB)} + S^2_{\bar{X}(PB+E)}}},$$

PB = pentobarbital,
$\bar{X}_{(PB)}$ = mean tissue level of animals treated with pentobarbital alone,
$\bar{X}_{(PB+E)}$ = mean tissue level of animals treated with pentobarbital and ether,
$S^2_{\bar{X}(PB)}$ = dispersion of tissue level of animals treated with pentobarbital alone,
$S^2_{\bar{X}(PB+E)}$ = dispersion of tissue level of animals treated with pentobarbital and ether,
$S_{\bar{X}} = \sqrt{S^2_{\bar{X}}}$ = standard deviation from the mean $\bar{X}$.

The difference in tissue levels was considered to be significant if t corresponded to a value of p equal to or less than 0.05.

Ninety-five per cent confidence limits, L, were also computed for the mean of each tissue level, where $L = \bar{X} \pm t_{0.05}S_{\bar{X}}$, and t is the value of t corresponding to a value of $p = 0.05$ and to the number of degrees freedom, $N = n\text{-}1$ (n = number of observations) found in standard tables of probabilities.

Results

Rats given 25 mg./kg. of pentobarbital and exposed to 3.6 volumes per cent ether vapor for 40 minutes were found to have higher concentrations of pentobarbital in liver, fat, brain, and plasma than did rats given 25 mg./kg. pentobarbital and sacrificed after 40 minutes. No

TABLE 1

EFFECT OF ETHER ON TISSUE DISTRIBUTION OF PENTOBARBITAL

Tissue	Rats Treated with 25 mg./kg. Pentobarbital			Rats Treated with 25 mg./kg. Pentobarbital and Exposed to 3.6 Volumes Per Cent Ether		
	L	Mean Pentobarbital Level	L	L	Mean Pentobarbital Level	L
Liver	16.0	17.3	18.6	35.6	36.8	38.5
Kidney	22.7	27.2	31.7	26.3	28.5	30.7
Fat	14.0	22.8	31.6	33.0	46.1	59.2
Brain	10.4	15.6	20.8	20.8	22.9	25.0
Plasma	0.4	9.5	18.6	12.4	14.0	15.6

Tissue levels expressed as μg./gm., plasma levels as μg./ml. L = 95 per cent confidence limits.

difference was found in kidney pentobarbital levels. These data are summarized in table 1 which also shows computation of 95 per cent confidence limits. The increase in liver and plasma pentobarbital levels is statistically significant. The changes in fat and brain pentobarbital levels are not statistically significant.

In the *in vitro* studies, the presence of 3.6 volumes per cent ether vapor was associated with a 69, 45, and 96 per cent inhibition of pentobarbital metabolism (table 2). No statistical analysis was made of the *in vitro* results because of the small number of experiments. However, it is believed that the inhibition associated with ether is real because of the very small variations found in duplicate samples. Both the reaction mixture blanks and the samples with pentobarbital added were run in duplicate and showed deviations as small as 0.003 per cent and no greater than 1 per cent. The average deviation of a given duplicate was less than 0.1 per cent.

DISCUSSION

The report of Greene and Whittaker (2) shows that ether potentiates the effect of pentobarbital in rats. This potentiation could theoretically be due to 1) changed tissue distribution of pentobarbital; 2) inhibition of the metabolic degradation of pentobarbital; 3) decrease in the brain level of pentobarbital necessary to maintain a state of anesthesia; or 4) altered permeability of the blood-brain barrier to pentobarbital.

TABLE 2

EFFECT OF ETHER ON THE *IN VITRO* METABOLISM OF PENTOBARBITAL FOLLOWING THE ADDITION OF 1μ*M* PENTOBARBITAL TO 2 ML. LIVER SUPERNATANT

μ*M* Pentobarbital Metabolized in 40 Minutes		Per Cent Inhibition of Metabolism
Without Ether	With Ether	
0.32	0.10	69
0.49	0.27	45
0.28	0.01	96

The present *in vivo* tissue distribution studies show that the levels of pentobarbital are significantly higher in rats given 25 mg./kg. of pentobarbital and exposed to 3.6 volumes per cent ether than they are in rats given 25 mg./kg. of pentobarbital alone. The increase in liver pentobarbital levels might be explained on the basis of respiratory acidosis secondary to ether anesthesia, on the basis of decreased hepatic blood flow caused by ether and pentobarbital, on the basis of a direct inhibitory effect of ether on the metabolic breakdown of pentobarbital by the liver (hypothesis 2), or, finally, by altered tissue distribution (hypothesis 1).

The effects of high concentrations of carbon dioxide on the depth of anesthesia have been demonstrated by Beecher and Moyer (5). They found that elevations of carbon dioxide increased the depth of pentobarbital anesthesia in dogs. Similarly, Barbour and Seevers (6) found that the sleeping time of rats anesthetized with pentobarbital was prolonged in atmospheres containing 10 or 20 per cent carbon dioxide. However, respiratory acidosis cannot be the only explanation for the increased liver pentobarbital levels observed in the present *in vivo* experiments because in the *in vitro* studies inhibition of pentobarbital metabolism occurred in rat liver homogenates in the absence of increased carbon dioxide tensions. Similarly, the decrease in hepatic blood flow which has been shown to occur with cyclopropane (7, 8) and which might be hypothesized as occurring also with ether could not be the sole explanation for the high liver pentobarbital levels found with ether because of the results of the present *in vitro* experiments.

The increased levels of pentobarbital found *in vivo* when ether is administered are best explained on the basis of a direct inhibitory effect of ether on the metabolic degradation of pentobarbital. Since pentobarbital is metabolized only by the liver (4), the present *in vivo* findings suggest that ether potentiates pentobarbital in rats by inhibiting its metabolism in the liver, a conclusion supported by the *in vitro* studies.

As Cooper and Brodie have demonstrated (4) the enzyme involved in the metabolism of pentobarbital is present in liver microsomes. Microsomes are by themselves ineffective, however, in metabolizing pentobarbital, for the presence of the soluble (nonparticulate) fraction of the cell is also required. The soluble fraction is necessary in order to maintain the coenzyme triphosphopyridine nucleotide hydrogenase (TPNH) in a reduced form. This in turn also requires the presence of glucose-6-phosphate dehydrogenase. Ether could, therefore, theoretically inhibit pentobarbital metabolism either by interfering with the activity of the microsomal enzyme or by interfering with the coenzymes responsible for the activity of this microsomal enzyme (TPN, TPNH, or glucose-6-phosphate dehydrogenase). Since the enzyme preparation used in the present *in vitro* studies contained a mixture of microsomes and soluble fraction, it cannot definitely be stated on the basis

of the present work where or how ether interferes with pentobarbital metabolism. However, it has been shown that during ether anesthesia oxidative metabolism via the tricarboxylic acid cycle is normal (9, 10). TPN is essential in the tricarboxylic acid cycle as the coenzyme for isocitric dehydrogenase in the conversion of isocitric acid to *alpha*-ketoglutarate. Since TPN activity is unaffected by ether anesthesia to the extent that oxidation in the tricarboxylic acid cycle is unimpaired, it may be assumed that in the case of pentobarbital metabolism ether exerts its effect not by impairing TPN activity but rather by inhibiting the activity of the as yet unidentified microsomal enzyme itself.

Since ether inhibits the metabolism of pentobarbital in the liver, increased pentobarbital levels would be expected to be reflected first in the blood passing through the liver. The rise in plasma pentobarbital levels during ether is statistically significant. The failure to obtain significant elevations of pentobarbital tissue levels in other organs could be explained on the basis of alterations in tissue distribution secondary to changes in cell membrane permeability or tissue solubility, or they could be a reflection of the time necessary for the blood-borne pentobarbital to equilibrate with these tissues. The natural variation due to the small populations sampled or, in the case of fat, differences in sampling technique, might also explain the failure to observe changes in other tissues which were comparable to those changes observed in liver and plasma. It is unlikely, however, that ether anesthesia significantly altered the permeability of the blood-brain barrier to pentobarbital. The average plasma:brain ratio for pentobarbital was 1.6 when pentobarbital alone was given and remained 1.6 when ether was administered. Hypothesis (4) therefore appears untenable as an explanation for the potentiation of pentobarbital anesthesia by ether.

The results of the present studies neither confirm nor refute hypothesis (3), namely that ether decreases the brain level of pentobarbital necessary to maintain a state of anesthesia.

The anesthetic action of ether is, in all probability, due to the effect of ether on cell membrane permeability. It has not been proven, however, whether ether also penetrates the cell to act on its interior and the metabolic effects of ether could be due either to an extracellular or to an intracellular site of action. In the present *in vivo* experiments, the metabolic effects of ether were studied in the presence of intact cell membranes. The results of these experiments coincided with and could best be explained on the basis of *in vitro* experiments in which cell membranes had been destroyed. This suggests, although does not provide final proof, that ether does in fact cross the cell membranes of rat livers.

Summary

Ether anesthesia potentiates pentobarbital anesthesia in rats by inhibiting the metabolic degradation of pentobarbital. It does so by

impairing the microsomal liver enzyme responsible for the metabolism of pentobarbital. Ether does not alter the permeability of the blood-brain barrier to pentobarbital. Changes in tissue distribution of pentobarbital associated with ether anesthesia are more readily ascribed to factors other than the presence of ether than they are to the ether itself. Evidence is presented suggesting that ether crosses the cell membrane and acts intracellularly.

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