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Uptake, Distribution, and Anesthetic Effect of Pentobarbital-2-^{14}C after Intravenous Injection into Mice

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The uptake and distribution of a single dose of intravenously administered sodium pentobarbital-2-^{14}C (5 ethyl-5-[methylbutyl] barbiturate-2-^{14}C) and its effects on function of reflex response to stimuli were studied in mice. Groups of mice given 30, 40, or 50 mg/kg pentobarbital-2-^{14}C intravenously were sacrificed at intervals from 10 seconds to 1 hour after injection; the level of anesthesia was assessed by testing loss and return of righting reflex and loss and return of withdrawal response to pinprick. There was no obvious relationship between whole brain, or specific brain region, concentration of unchanged pentobarbital and the states of these two reflex responses. No metabolite was detected by chromatography in brain or brown fat with any dose at any time. After each dose three metabolites could be detected by chromatography in the liver, kidney, and blood, as early as 1 minute after injection. (Key words: Pharmacokinetics: pentobarbital; Hypnotics, barbiturate: pentobarbital pharmacokinetics; Brain: pentobarbital distribution; Biotransformation: pentobarbital.)

THERE ARE MANY INCONSISTENCIES between brain barbiturate concentrations and predicted and observed levels of anesthesia.[1-3] Recent studies of the concentrations of barbiturates and volatile anesthetics within the brain have demonstrated that early distribution of anesthetics in the grey matter is determined primarily by circulatory factors,[4] while later, after intravenous injection,[5] distribution in the white matter more closely correlates with lipid content.[4-8] However, several exceptions to these principles were found in different brain areas.[4]

Neither Scherrer-Etienne and Posternak's study of cats,[6] nor Cohen and co-workers' study of monkeys[4] correlated the neurologic state of the animals with drug levels found in different brain areas, nor were the effects of different doses studied. Accordingly, the aim of the present study was to measure pentobarbital levels in different brain regions of mice at different times after different intravenous doses, and to time the loss and return of both righting reflex and withdrawal response to pinprick. The distribution of pentobarbital and its metabolites within the body were also studied.

Methods

One hundred and seventy-two 42-day-old male Swiss albino mice, CD-1 strain, were injected intravenously with pentobarbital-2-^{14}C (5 ethyl-5-[methylbutyl] barbiturate-2-^{14}C) sodium (New England Nuclear Corp., Boston, Mass.) in physiologic saline solution at a final specific activity of 0.35 mCi/mmol and a concentration of 10 mg/ml. The pentobarbital-2-^{14}C was supplied in the acid form and converted to its sodium salt with 1 N NaOH to a *p*H of 9–10. Pentobarbital used had one peak when checked by thin-layer chromatography. Twenty-four mice received a dosage of 30 mg/kg of the drug, 120 mice received 40 mg/kg, and 28 mice received 50 mg/kg. All injections were given as rapidly as possible (less than 2 seconds) intravenously through a tail vein, using a number 26 0.5-inch disposable needle (Becton Dickinson) on a Hamilton 250-μl gastight syringe (#1725). The total volume injected did not exceed 0.18 ml. In three mice, sham injection of this volume of physiologic saline solution at *p*H 10 had no obvious effect. Injection was

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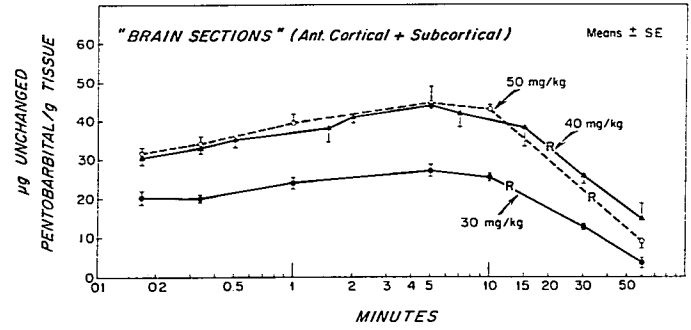


FIG. 1. "Brain section" levels of unchanged pentobarbital in mice given pentobarbital-2-^{14}C intravenously. "Brain section" refers to an anterior cortical and subcortical region which we removed from all mice (see text). Earliest sacrifice time was 10 seconds post-intravenous injection. All values are shown as means ± SE. Comparable points between the 40- and 50-mg/kg curves are not statistically different ($P > 0.9$) despite the observation that the effects of the 40- and 50-mg/kg doses were different. R marks the mean times of righting reflex return.

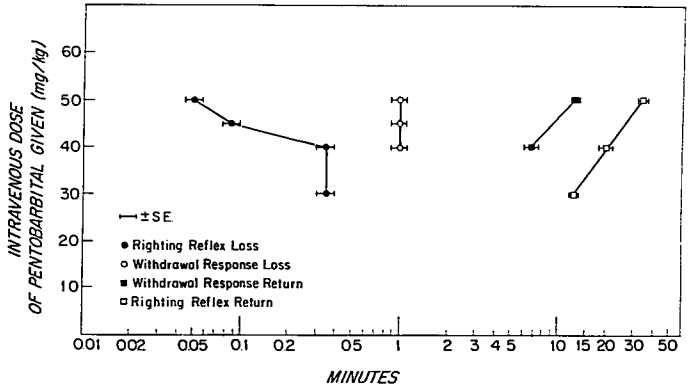


FIG. 2. Effects of intravenous dose of pentobarbital on righting reflex loss and return and on withdrawal response loss and return. Points in time after intravenous injection are shown as means ± SE. There was no significant difference between times of righting reflex loss after doses of 30 mg/kg and 40 mg/kg, nor was the difference in times between the intravenous injection and loss of withdrawal response at any given dose significantly different ($P > 0.9$). After doses of 25 mg/kg neither the righting reflex nor the withdrawal response was lost. At 55 mg/kg two of three mice died. The mice given intravenous doses of 45 mg/kg were sacrificed before awakening.

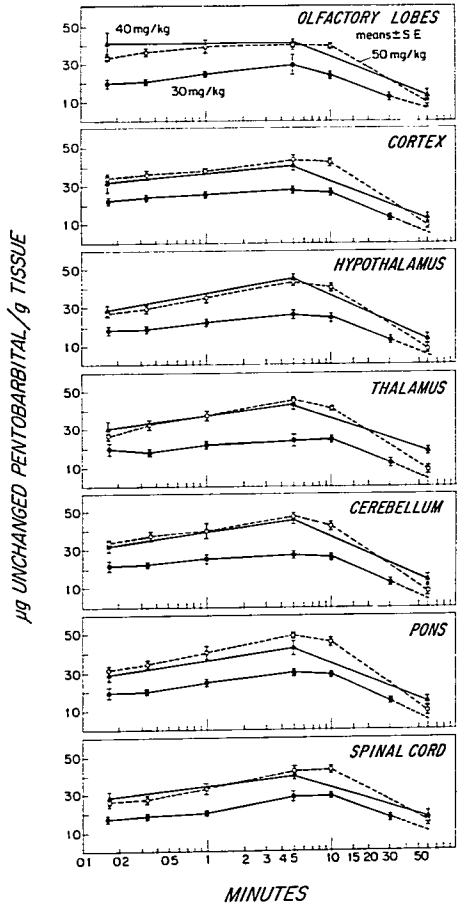


FIG. 3. Levels of unchanged pentobarbital in dissected brain regions from mice given pentobarbital-2-^{14}C in single intravenous doses of 30, 40, or 50 mg/kg. Forty- and 50-mg/kg curves are not statistically different ($P > 0.9$) (all values are means ± SE). Times on the abscissa are post-intravenous injection.

considered intravenous when the tail vein could be observed to flush and immediately refill with blood and no extravasation was noted. All injected animals which failed to meet these criteria were rejected from the study. Injections were made in groups of 12 to 20 animals during the morning at a room temperature of 22 C. The clinical level of general anesthesia was judged using two criteria: loss and return of the righting re-

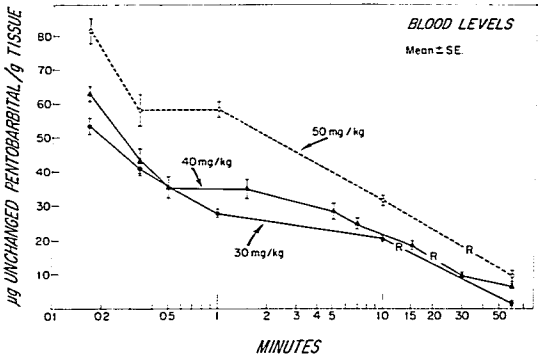


FIG. 4. Whole-blood levels of unchanged pentobarbital taken from mice given single intravenous doses of 30, 40, or 50 mg/kg pentobarbital-2-^{14}C. All values are means ± SE. Times of righting reflex return (R) occurred at approximately the same blood levels. Times on the abscissa are post-intravenous injection.

flex and loss and return of the withdrawal response to pinprick. The righting reflex was considered lost when the mouse failed to right itself after 15 seconds; it was considered returned when the mouse spontaneously righted. The withdrawal response was considered lost when pinprick to the hind foot pad failed to evoke foot withdrawal two out of three times; the withdrawal response was considered to have returned when pinprick to the hind foot pad evoked withdrawal two out of three times. All mice were sacrificed by decapitation at times ranging from 10 seconds to 1 hour after injection. The brains were removed by 30 seconds after decapitation. An anterior cortical and subcortical area which we termed "brain section" was removed from all animals and weighed for ^{14}C assay. In addition, brains from 12 of the mice given 40 mg/kg and from all the mice given 30 mg/kg and 50 mg/kg were dissected over ice into cortex, olfactory lobes, hypothalamic area, thalamic area, cerebellum, pons, and thoracic spinal cord; these portions were always taken in the same order and were weighed. For determination of metabolites, the remainder of the brain, as well as blood and pieces of liver, kidney, and brown fat (in that order) were frozen over dry ice and stored at −20 C. The total time from sacrifice until all the organs were frozen never exceeded 10 minutes. The samples of brain tissue and 0.1-ml amounts of blood for ^{14}C assay were digested in Soluene 100 (Packard) and counted in a toluene-based scintillation fluor (Liquifluor-NEN) in a Packard Tri-carb liquid scintillation counter. Blood was bleached[9] prior to counting. Quenching was corrected by external standardization, and results were expressed as μg pentobarbital/g tissue, wet weight.

Tissue stored at −20 C and the whole brains from four additional mice sacrificed immediately prior to the extraction procedure were used for determination of metabolites. Two of these four additional animals received a 40 mg/kg dose of pentobarbital-2-^{14}C and were sacrificed 1 minute and 6.7 minutes after injection; two received a 50 mg/kg dose and were sacrificed at 1.1 and 12.0 minutes. All tissue was homogenized in saline solution at 4 C. The pentobarbital and metabolites were precipitated by acidification and extracted from the homogenate.[10] Recovery of 99 per cent of radioactivity was achieved by this procedure. Samples of the pentobarbital and metabolite extracts were applied to thin-layer chromatography plates (Gelman ITLC Type SG) and a standard of pentobarbital-2-^{14}C (New England Nuclear Corp.) was applied adjacent to these samples. Plates were developed with benzene:dioxane:NH_4OH (75:20:5, Rf of pentobarbital 0.91) or with benzene:acetic acid (199:1, Rf 0.33), and air-dried. Distribution of radioactivity was determined with a Packard Radiochrom-

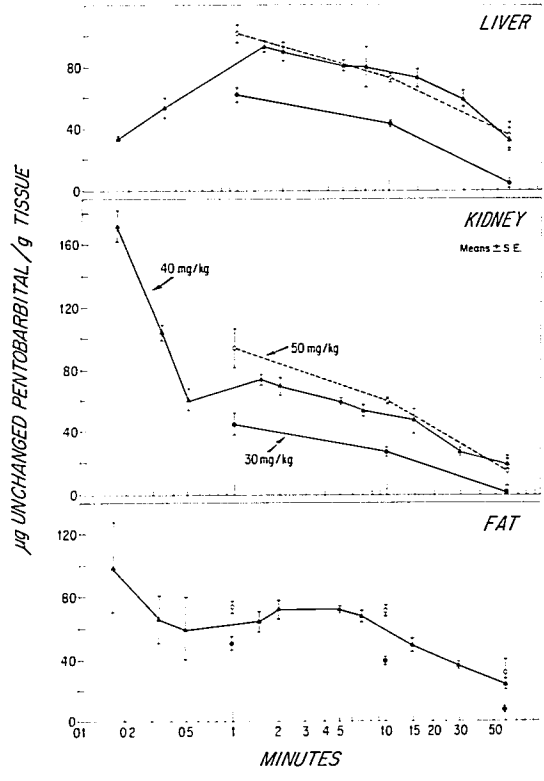


FIG. 5. Levels of unchanged pentobarbital-2-^{14}C in liver, kidney, and brown fat after intravenous injection of a single dose of 30, 40, or 50 mg/kg pentobarbital. All values are means ± SE. Times on the abscissa are post-intravenous injection.

scanner (model 7201). The peak corresponding to the standard was considered unchanged pentobarbital; all other peaks were considered metabolite. Areas under the curves above background obtained by scanning were measured by planimetry (K & E 62-0015 compensating polar planimeter). To calculate the amount of metabolites in μg, the molecular weights of the metabolites were estimated to be the same as that of unchanged pentobarbital (226.3).§

The effects of intravenous doses of pentobarbital Na on righting reflexes and withdrawal responses at doses of 25, 45, and 55 mg/kg were also investigated in 24 additional mice. Rectal temperatures of representative mice were monitored with a thermistor probe. Measurements of tail-blood *p*H were made on representative mice after warming the distal half of the tail. Arterialized blood was then drawn into glass capillary tubes.

§ The major metabolic pathway of pentobarbital is side-chain oxidation. Formation of an alcohol or ketone would add only 16-17 molecular weight units to pentobarbital's molecular weight, or less than a 10 per cent increase. Our calculations of concentration of metabolites in figure 7 are probably within 10 per cent of true values.

Results

Brain

"Brain section" concentrations of unchanged pentobarbital at different times are shown in figure 1. The 30-mg/kg dose "brain section" concentration curve was separate and distinct from the 40- and 50-mg/kg curves. Peak brain concentrations were achieved for all three doses at 5 minutes (27.5 ± 1.2 SE $\mu g/g$ wet weight for 30 mg/kg; 44.3 ± 0.6 $\mu g/g$ for 40 mg/kg; 44.7 ± 4.5 $\mu g/g$ for 50 mg/kg). The rates of decrease in brain concentrations after 5 minutes were the same for the 40- and 50-mg/kg curves in spite of differing blood levels for the two doses.

The times for loss and return of righting reflex and withdrawal response in relation to dose given are shown in figure 2. Although the concentrations in brain with 40 and 50 mg/kg were essentially the same at all measured points, the times of righting reflex loss and return were clearly different (fig. 1). For mice given 30 mg/kg pentobarbital, the extrapolated range of "brain section" concentrations at which the righting reflex is likely to return is 26–23 $\mu g/g$; for 40 mg/kg, 37–30 $\mu g/g$; for 50 mg/kg, 23–18 $\mu g/g$ (fig. 1).

Brain Regions

The concentrations of unchanged pentobarbital in the cortex, olfactory lobe, hypothalamus, thalamus, cerebellum, pons, and spinal cord at different times are shown in figure 3. Comparisons of the 40- and 50-mg curves showed no significant difference ($P > 0.9$) at any point. Peak concentrations were reached at 5 minutes in all brain regions except the spinal cord, which showed a peak concentration at 10 minutes. The differences between regions at any time to 1 hour after the same dose never exceeded 25 per cent. These brain-region pentobarbital concentrations after each dose of pentobarbital did not allow prediction of when the righting reflex would be lost or would return. Moreover, similar concentrations were measured in a given brain region when the mice were clearly in different neurologic states.

Blood

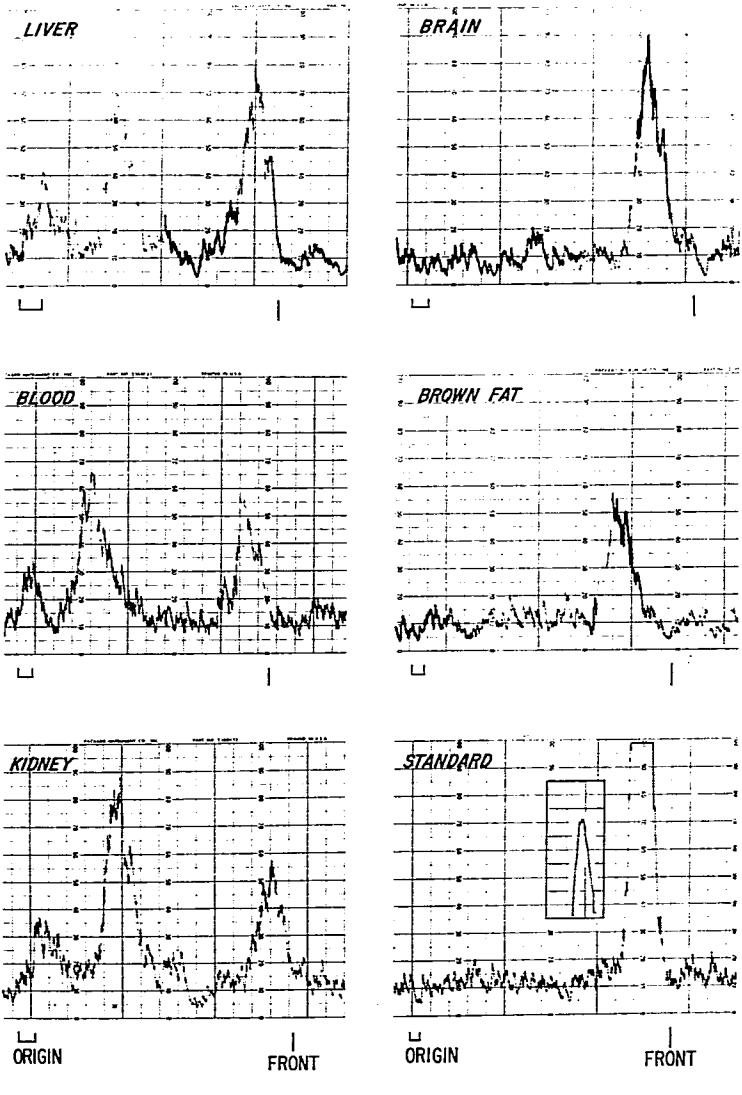
The blood contained both unchanged pentobarbital and metabolites of pentobarbital. Figure 4 shows blood levels of unchanged pentobarbital at different times after 30-, 40- and 50-mg/kg doses. Peak values were obtained 10 seconds after iv injection, and the concentration of unchanged pentobarbital was dose-dependent: for 30 mg/kg, 53.2 ± 2.4 (SE) $\mu g/g$; for 40 mg/kg, 62.8 ± 2.3 $\mu g/g$; for 50 mg/kg, 81.4 ± 3.5 $\mu g/g$. These groups of values were all significantly different ($P < 0.001$) from each other. After these initial peaks, each curve appeared to follow its own course. The 50-mg/kg curve remained significantly higher at 1 minute than either the 40- or the 30-mg/kg curve. All three curves show continuous declines to minimum concentrations at 1 hour of 1.7 ± 0.7 $\mu g/g$ (30 mg/kg), 6.7 ± 0.4 $\mu g/g$ (40 mg/kg), and 9.6 ± 1.8 $\mu g/g$ (50 mg/kg).

Pentobarbital levels in blood at which the righting reflex would be likely to return were extrapolated from figure 4 and were found to be 20–17 $\mu g/g$ (30 mg/kg), 18–13 $\mu g/g$ (40 mg/kg), and 19–13 $\mu g/g$ (50 mg/kg). These levels are nearly identical, and thus it appears that the blood level can be used to predict the point of righting reflex return.

Fat

Figure 5 shows the concentrations of unchanged pentobarbital in brown fat at different times after 30-, 40-, and 50-mg/kg doses.

FIG. 6. Radioscan of thin-layer chromatograms (benzene:dioxane:NH_4OH solvent system) of tissue extracts from liver, kidney, brain, brown fat, and blood of the same mouse one hour after injection of 40 mg/kg pentobarbital-2-^{14}C. Despite the noise level, three metabolite peaks can be seen in the liver and kidney. Similar patterns were observed in radioscans of tissue extracts from mice given 30 and 50 mg/kg. Recognizable metabolite peaks were absent from brain and brown fat at all times with all doses. Peak of pentobarbital standard is shown in inset. Other solvent systems (see text) were used to confirm the validity of these chromatograms.



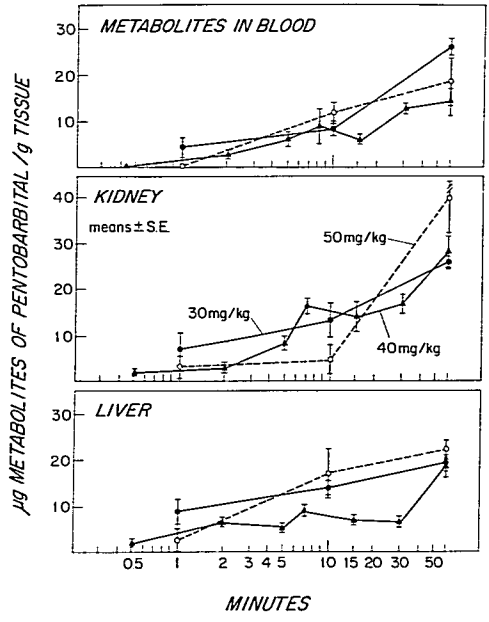


FIG. 7. Levels of metabolites of pentobarbital-2-^{14}C in liver, kidney and blood for mice given 30, 40, or 50 mg/kg pentobarbital-2-^{14}C iv. Metabolite levels were calculated on the assumption that their molecular weight was the same as that of unchanged pentobarbital—an assumption probably accurate to within 10 per cent (see footnote to text). The pentobarbital-2-^{14}C was pure and the label stable as determined by repeated chromatography. Times after intravenous injection are shown on the abscissa.

KIDNEY

Kidney contained both metabolite and unchanged pentobarbital. The levels of unchanged pentobarbital at different times after the three doses are shown in figure 5. The concentration in kidney was highest at 10 seconds (171.7 ± 9.2 μg/g), decreased rapidly to 61.4 ± 7.7 μg/g by 30 seconds, was 73.8 ± 3.1 μg/g at 1.5 minutes, and thereafter decreased continually to 19.9 ± 2.4 μg/g at 1 hour. The kidney pentobarbital levels 1 hour after either 40 or 50 mg/kg were not significantly different ($P > 0.9$).

LIVER

The levels of unchanged pentobarbital in liver at different times after 30-, 40-, and 50-mg/kg doses are shown in figure 5. Both unchanged pentobarbital and its metabolites were found in liver (figs. 5 and 6). The 40- and 50-mg/kg levels appeared identical 1 hour after injection, while the 30-mg/kg curve was clearly different ($P < 0.01$).

METABOLITES

Radioscans of thin-layer chromatograms of liver, kidney, and blood (fig. 6) which were taken an hour after injection of 40 mg/kg demonstrate three major metabolite peaks. Similar patterns were seen after 30- and 50-mg/kg doses. No metabolite was detected in brain or brown fat at any time after any dose level. Actual metabolite levels in μg/g at different times are shown in figure 7. We cannot calculate the rate of metabolism *in vivo* from our data since the metabolite levels measured indicate concentrations only, with no indication of turnover rates.

Functional Activity of Nervous System

An expected relationship between the intravenous dose of pentobarbital and sleep time (time period between righting reflex loss and righting reflex return) was observed (fig. 8). Doses of 25 mg/kg pentobarbital, iv, or less did not cause righting reflex loss, nor did doses of 35 mg/kg or less cause withdrawal response loss. Doses of 55 mg/kg or more given rapidly iv resulted in respiratory arrest and cardiac collapse. Peak concentrations in the brain were all reached at the same point in time in spite of widely different blood levels. The slopes of the declines in brain concentrations for the 40- and 50-mg/kg doses were the same in spite of lower blood levels for the 40 mg/kg dose (figs. 1 and 4). Thus, the decline in brain concentration appears to be time-related and not directly related to blood level. The anesthetic effects observed and their termination did not appear to be closely related to tissue concentration and distribution. Rectal temperature 30 minutes after 50 mg/kg iv pentobarbital dropped less than 0.5 C. Arterialized tail-blood *p*H values ranged from 7.37 to 7.46 just before return of the withdrawal response; no significant difference between *p*H values for the mice given 30 and those give 50 mg/kg iv pentobarbital was found.

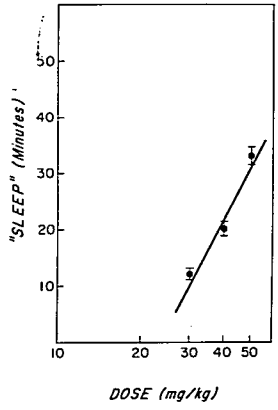


FIG. 8. Effect of intravenous dose of pentobarbital on sleep time (time from loss of righting reflex to return of righting reflex). Dose is shown in log scale and shows an expected curve for the relationship of dose to anesthetic effect. Means (± SE) are shown.

Discussion

We have been unable to demonstrate any obvious correlation between brain area concentration of unmetabolized pentobarbital and anesthetic effect as judged by observations of the righting reflex and withdrawal response. Although care was taken to define carefully the responses we observed, we must stress that the righting reflex is the sum of at least five known reflexes,[11,12] and it may also be altered by non-neurologic factors such as muscular effectiveness.

In spite of large differences between blood levels of unchanged pentobarbital 10 seconds after the 40- and 50-mg/kg doses, no significant difference between brain pentobarbital levels resulted. However, at this time, 10 seconds after completion of intravenous injection the mice that had been given 40 mg/kg pentobarbital had not yet lost their righting reflexes, but the mice given 50 mg/kg had. Detailed information about circulation to different parts of the mouse brain is not available, but recently circulation to ten areas in rat brain has been investigated by indicator fractionation in conscious and pentobarbital-anesthetized rats.[13] The initial distribution of pentobarbital in different areas of the mouse brain was, in general, proportional to the regional blood flows in corresponding areas in the conscious rats.[13] After intraperitoneal injection of a 40-mg/kg dose of pentobarbital, blood flows to all areas of the rat brain with the exception of the olfactory lobes decreased in amounts proportional to the initial perfusion of given areas.[13] In the mice pentobarbital levels in all brain areas except the olfactory lobe continued to increase for approximately 5 minutes. Since areas with the greatest initial perfusion rates contained more drug and also had the greatest decreases in perfusion rates with pentobarbital anesthesia, it is possible that the similarity of brain pentobarbital levels after 40- and 50-mg/kg doses in the mice is related to a greater depression of blood flow caused by the effect of pentobarbital on blood pressure after the higher dose.

Vascular and metabolic stability of the mice during deepest anesthesia is suggested by the fact that core temperature and blood *p*H were normal during that period. Initially, after the intravenous injection, there may have been significant cardiac and vascular depression which we could not measure or control. This may have played a significant role in altering the neurologic state of the mice as well as the distribution and uptake of pentobarbital. Yet the retention of the withdrawal response during the first minute of anesthesia while the righting reflex was absent suggests that some neurologic function remained intact. Furthermore, the very rapid rate of decline of blood levels from flow-dependent distribution of pentobarbital suggests also that the circulation was not severely depressed.

Many other factors can affect cerebral blood flow and its distribution, including alterations of sympathetic activity, changes in regional as well as overall cerebral perfusion pressures, and local vascular effects of changes in P_{aCO_2}. We were unable to control these factors or measure them; thus, we cannot determine the relative roles they played in producing the similar brain levels we observed with the 40- and 50-mg/kg doses. It is therefore possible that these factors may ultimately explain our findings. Regardless of the cause of the similarity of the brain levels, the presence or absence of the reflexes we observed did not appear to correlate with concentration of drug at the morphologic level as sampled in these experiments.

The difficulties in assessing drug effects on the CNS are well known. We chose to observe two types of reflexes. The righting reflex is actually several complicated reflexes acting together. This reflex was chosen since it is a well-recognized reflex commonly used to assess anesthesia in small animals. Sleep times are defined in small animals by measuring the period between loss and return of righting reflex. Furthermore, the neuroanatomy has been well worked out in several species—we have attempted to extrapolate this to the mouse insofar as we have attempted to select areas of the brain that would be likely to be involved in maintenance of this reflex. The withdrawal response was chosen to assess response to a painful stimulus. Both reflexes were easily defined and separated in time of disappearance and of reappearance.

Brain areas sampled by direct dissection undoubtedly overlapped slightly; the standard errors, however, were relatively small, suggesting that these areas were relatively consistent and representative. The lack of correlation between brain area levels or obvious patterns of levels and the presence or absence of reflexes observed suggests that the overall concentration in any sampled area of mouse brain was not the only factor in determining whether a reflex was present.

Scherrer-Etienne and Posternak[6] measured by autoradiographic methods the concentrations of pentobarbital in the optic tract, white matter, pyramid, reticular formation, hypothalamus, hippocampus, amygdala, thalamus, cortex, medial and lateral geniculate bodies, corpora quadrigemina, caudate nucleus, and superior olive at various times after intravenous injection of 20 mg/kg pentobarbital in cats already anesthetized with ether. One minute after the intravenous pentobarbital, the highest concentrations of pentobarbital, approximately 72 μg/g wet brain, were found in the lateral geniculate bodies; after 76 minutes this value had decreased to 26 μg/g. Brain levels in these cats after 20 mg/kg pentobarbital were approximately twice those found in our mice after 30 mg/kg at identical times to 30 minutes after injection; for instance, for cortex at 1 minute, 55 versus 22, at 4.5 minutes, 40 and 24, and at 18.5 minutes, 35 and 18 μg/g. At later times after injection, brain levels were negligible in mice, but in cats they were still above 10 μg/g 6 hours after injection. However, in general, we found a proportional distribution of brain pentobarbital uptake similar to that reported by Scherrer-Etienne and Posternak, but one minute after intravenous injection there were slightly greater differences between the various brain regional pentobarbital levels in their cats as opposed to our mice. Scherrer-Etienne and Posternak studied one dosage.

In our studies, metabolites were present in the liver, kidney, and blood, but no metabolite could be detected by chromatography in brain or fat at any time with any dose. It is generally accepted that the metabolism of pentobarbital occurs principally in the liver

in vivo. *In vitro*, many diverse tissues, including brain, can metabolize pentobarbital.[14] The rapidity of metabolism can be appreciated with the appearance of metabolites in blood as early as 1 minute after injection. The decrease with time in brain pentobarbital concentration may have been due in part to metabolism in brain and immediate excretion of the metabolized pentobarbital, but this seems unlikely in view of our inability to demonstrate the presence of any metabolite in brain at any time with any of the three doses.

The results of Scherrer-Etienne and Posternak[6] and Cohen et al,[4] as well as the results of this study, suggest that while brain blood flow and lipid content explain to some extent the distribution of anesthetic agents, the resulting distribution into different brain areas does not correlate with the reflex state of the animal. Although the brain concentration in some critical area, or areas, may indeed be correlated with neurologic state, available data give few clues as to possible locations of such critical areas.

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