

Cellular and Regional Localization of Pentobarbital-2-¹⁴C by Radioautog- raphy of Selected Areas of Mouse Brain at Loss and Return of Withdrawal Response

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Regional and cellular distribution of pentobarbital-2-¹⁴C in mouse brain was determined by frozen-section radioautographic methods. The mice were studied at the times of loss (WRL) and return (WRR) of withdrawal response following a single intravenous dose of either 40 or 50 mg/kg body weight. At WRL, grey matter areas had higher concentrations of pentobarbital-2-¹⁴C than white matter. At WRR grey matter concentrations were not altered, but white matter areas were now similar to the grey. At WRL pentobarbital concentration was 55 per cent higher in large pyramidal cells in the parietal cortex than in surrounding neuropil. At WRL hippocampal pyramidal cell bodies (stratum pyramidale) and glial cells in corpus callosum had pentobarbital levels similar to that of surrounding neuropil. Levels in the neuropil of these three areas were higher at WRR than at WRL. Lipid-rich compartments had higher pentobarbital concentrations at WRR than at WRL. The results suggest that return of consciousness after pentobarbital anesthesia is associated with intracerebral redistribution of pentobarbital even while there is continuing uptake into brain. (Key words: Brain, pentobarbital uptake; hypnotics, barbiturates, pentobarbital; pharmacokinetics, pentobarbital uptake.)

ANESTHETIC LOCALIZATION in brain *in vivo*, with few exceptions,¹⁻⁷ has been inferred rather than measured. There are many known interactions of anesthetics with macromolecules⁸⁻¹¹ which, in addition to the complicated variables of cerebral blood flow, make consideration of the distribution of anesthetics in brain extremely complex. We have presented data² that showed variation in regional pentobarbital concentration in mouse brain after different doses. Regional and total brain pentobarbital concentrations were found to be higher at the time mice recovered from anesthesia than they were at onset of anesthesia.³ Brain-region concentrations were measured on grossly dissected tissues; with this level of resolution, no relationship between concentration and neurologic effects was observed. This strongly suggested that there was an undetected pentobarbital localization that might be rendered visible with better resolution, or that some of the pentobarbital in the brain was not involved in the anesthetic process, or both.

In our previous study, metabolites of pentobarbital-2-¹⁴C were present in the liver, kidney, and blood, but metabolites were not detected by chromatography in brain or brown fat at any time with any dose. Consequently, any radioactivity in brain has been taken in the present study to represent pentobarbital-2-¹⁴C. We have examined its distribution by two separate radioautographic techniques designed to locate soluble compounds within tissues. First, x-ray film radioautography was used for surveying the

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regional distribution of pentobarbital to achieve better anatomic resolution than previously obtained.² Second, high-resolution¹² 3- μ -thick frozen-section radioautography of selected structures was used to determine whether there was a specific cellular distribution of pentobarbital-2-¹⁴C that could account for the changing regional distribution. Two times in the course of pentobarbital anesthesia were chosen, the time when loss of the withdrawal response of the foot to pinprick (withdrawal response loss, WRL) first occurred two out of three times, and the time when the same level of response first returned (withdrawal response return, WRR).

Methods

X-RAY FILM RADIOAUTOGRAPHY

Twelve 42-day-old male Swiss albino mice (CD-1 strain) were each injected via a tail vein with pentobarbital-2-¹⁴C (specific activity 2.75 mCi/mmol, New England Nuclear Corp., Boston, Mass.), final concentration 10 mg/ml saline solution, prepared as previously described.² Six mice received 40 mg/kg body weight and six, 50 mg/kg. Mice were sacrificed by decapitation. Three mice from each group were sacrificed 1 minute after injection; three mice given 40 mg/kg were sacrificed at 6.7 minutes; the three remaining mice given 50 mg/kg were sacrificed at 12.6 minutes. These times were chosen to coincide with WRL and WRR as previously determined by us.²

Following decapitation, the brain from each mouse was removed and cut coronally into two parts at the level of the optic chiasm to facilitate sectioning. Each block was mounted on a cryostat chuck with Tissue Tek and frozen over dry ice. Approximately 4 minutes elapsed from decapitation to freezing. The frozen brains were stored at -30 C in an IEC Harris cryostat until sectioned. Fifteen 16- μ -thick sections, including both hemispheres from each block, were picked up on frozen Scotch Magic Tape, numbered with radioactive ink, and left to dry for two days in the cryostat. The dried sections were then placed on sheets of Kodak No-Screen Industrial x-ray film and exposed for three

days at 4 C over Drierite. Non-radioactive brain sections were used to check for positive and negative chemography and for pressure artifacts. The sections, still adherent to the tape, were separated from the film, fixed with Wohlman's solution for 30 seconds, rinsed, and stained with methylene blue. The radiographs were developed according to manufacturer's instructions.

FROZEN THIN-SECTION RADIOAUTOGRAPHY (APPLETON METHOD¹³)

Each of six male Swiss albino mice (CD-1 strain) was injected via a tail vein with pentobarbital-2-¹⁴C, 50 mg/kg (5.3 mCi/mmol, New England Nuclear Corp.), final concentration 10 mg/ml saline solution. Three mice were decapitated at 1 min (average time of WRL²) and three at 12.6 min (average time of WRR). The brains were removed, cut coronally at the level of the optic chiasm, and the posterior hemispheres separated. One hemisphere was mounted on a cryostat chuck and frozen over dry ice. Four minutes elapsed between sacrifice and tissue freezing. Sections were cut at a setting of 3 microns at -20 C using an I.E.C. Harris Lo-Temp cryostat equipped with a Jung microtome. Sections were picked up without thawing onto frozen NTB-2 emulsion (Kodak) coated slides. Radioautographs were exposed over Drierite for 14 to 17 days at -30 C. Other emulsion-coated slides were developed and the number of developed grains per unit area was determined (background). Fewer than 5 grains/1,000 μ^2 was considered acceptable for use. Silver grains in non-tissue-covered areas were again measured after exposure to determine actual background grain densities for subtraction from tissue grain densities (see below). Tests for latent image fading and positive chemography¹³ were made with brain tissue from a non-radioactive mouse injected with Diabutal (Diamond Laboratories, Inc.), sacrificed by decapitation, prepared, sectioned, and exposed at -30 C for 13, 18, 21, and 27 days like the radioactive sections.

After exposure, sections were brought to room temperature, fixed with Wohlman's fixative, rinsed three times in distilled water,

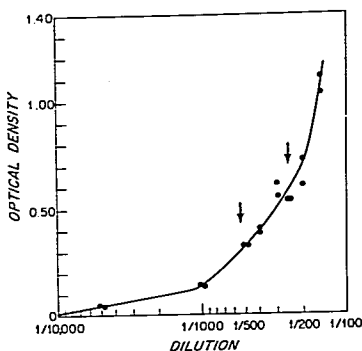


FIG. 1. X-ray radioautographs were made by exposing film to $1 \mu\text{l}$ of various dilutions of pentobarbital-2- ^{14}C for 3 days. The initial concentration of pentobarbital-2- ^{14}C was 10 mg/ml. The x-ray radioautographs were scanned with a densitometer and results plotted as shown. Optical densities from the x-ray radioautographs of brains of mice given pentobarbital-2- ^{14}C could, from this graph, be equated with pentobarbital levels in different brain regions. All optical densities from the x-ray radioautographs of brains were between 0.400 and 0.700 (arrows).

and developed in Dektol (Kodak) 1:1 water at 15 C for 2 minutes. The sections were fixed, washed, and stained with hematoxylin and eosin. The photographs, taken within 24 hours of staining, were enlarged 1,200 and 2,400 \times .

ANALYSIS OF RADIOAUTOGRAPHS

Two analytic methods for radioautography were used. For regional distribution, optical densitometry of x-ray film radioautographs of frozen 16- μ slices of mouse brain was used. For cellular distribution, grain density analysis of the radioautographs of thin sections (3 μ) was performed.

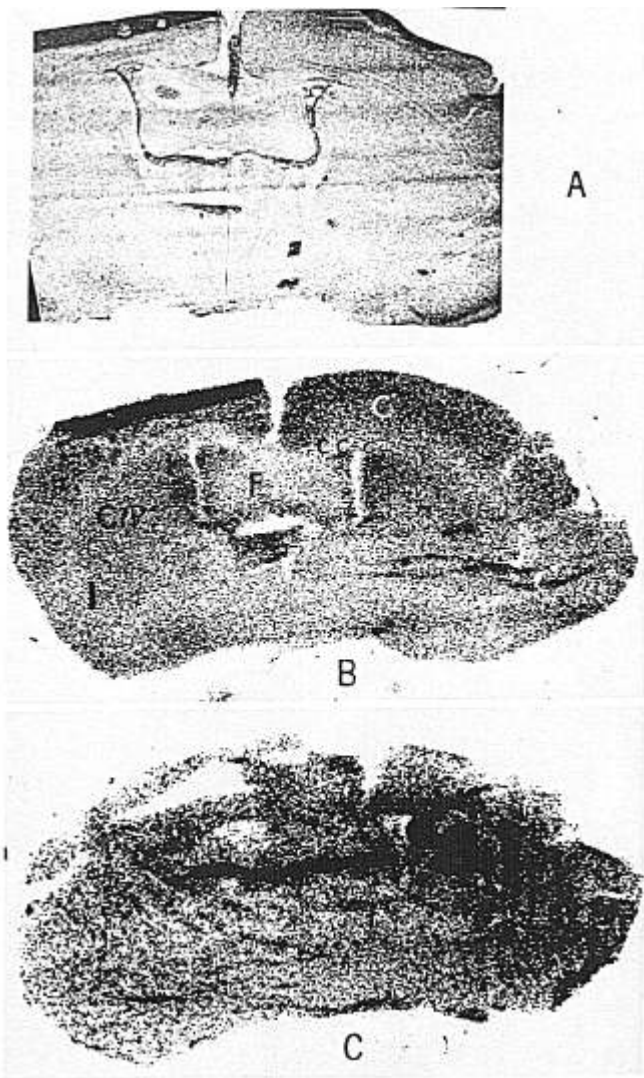
X-RAY FILM RADIOAUTOGRAPHY

Optical density of x-ray film radioautographs was measured with a densitometer (Photovolt Corp.) attached to a Leitz microscope. Densitometric field size, illumination source intensity, and magnification were kept constant for all determinations. Five optical density measurements were made in a random manner by using predetermined coordinates within each section in which structures could be positively identified (see below). The optical density measurements were corrected for background (determined in areas adjacent to tissue slices), averaged, and standard errors calculated. Obvious artifacts such as cracks in the tissue were excluded from measurement.

Optical density was correlated with tissue drug level by spotting $1 \mu\text{l}$ each of several known pentobarbital-2- ^{14}C concentrations onto filter paper. Each concentration was spotted in duplicate. Radioautographs were made by exposing x-ray film to the filter paper for 3 days at 4 C, as done for the thick-sectioned (16 μ) tissue. Radioactive spots thus produced are known to be relatively uniform in size and concentration.¹⁴ The area of each spot was measured and five O.D. readings were taken across each radioautograph. A graph plotting optical density against pentobarbital dilution (fig. 1) was constructed. Optical density readings from the x-ray film radioautographs occurred on a portion of the curve that was relatively linear, indicating that the x-ray film emulsion is sufficiently sensitive for quantitative optical densitometry in the range used in these studies.⁵

⁵ The shape of this curve also shows indirectly film emulsion sensitivity to β particles in the 160 Kev energy range under exposure conditions reported above. (For a full discussion of the theoretical basis of this curve and method see reference 14).

FIG. 2. Sixteen- μm coronal sections of mouse brain at the level of the fornix (F) (ref. 15, pp 33-34) (12 \times magnification). A, hematoxylin and eosin stained section adjacent to that of B, showing structures delineated on B. B, x-ray film radioautograph at time of withdrawal response loss. Note that all grey structures contain more pentobarbital-2- ^{14}C than do white structures. In particular, note labeling of cortex (C) and caudate/putamen (C/P) and lack of label in corpus callosum (cc) and internal capsule (I). C, x-ray film radioautograph at time of return of withdrawal response: Note almost uniform labeling of all structures. The lateral ventricles and median fissure are clearly visible.



Tissue sections were matched with corresponding radioautographs by comparing identification numbers made with radioactive ink. Anatomic areas to be densitometered were independently identified by four investigators using a mouse-brain atlas.¹⁵ All identifications were made from duplicate photomicrographs of tissue sections by outlining areas identified with a marking pencil (figs. 2A and 3A). These outlines were transferred to clear plastic overlays, and agreement between the areas identified by all four investigators was judged by superimposition of the overlays. Points within identified areas of the overlays were assigned coordinates. These coordinates were then applied to the x-ray film radioautographs (figs. 2B and 3B) of that particular section.

Thin frozen-section radioautographs were quantified by a standard grain density analysis method.^{16,17**}

Standard deviations of grain densities were calculated by the formula:

$$SD = \frac{G}{P} \sqrt{\left(\frac{1}{G}\right)^2 + \left(\frac{1}{P}\right)^2}$$

where G = number of grains and P = number of points.††

** For grain density analysis a series of points random with respect to morphology are superimposed over the photomicrograph of the developed and stained radioautograph. In this case the photomicrographs were perforated using a grid made from tacks placed in a board at 1-cm intervals. Thus, the number of points counted over a given compartment or structure represents the relative area of that compartment. Next, developed silver grains are counted over the same compartment. The ratio of grains to points is the grain density and is related to the concentration of radioactivity in a selected compartment after correction for background. Discussion of difficulties in relating grain density to absolute concentration are beyond the scope of this paper, but for more detailed explanation and discussion of grain density analysis applied to radioautography see references 13 and 17.

†† This form of analysis, based on Chi-square analysis, is valid for compartments that are greater than 1 per cent of total area analyzed.¹⁷ In our analysis the smallest compartment, glial cells in the corpus callosum (WRR), comprised 9.0 per cent of the area analyzed.

For frozen section radioautographs the following tissue compartments were defined:

- 1) All elements in pyramidal cell layer of parietal cortex (layer V).
- 3) All elements of the corpus callosum.
- 3) Pyramidal cells in layer V of the parietal cortex.
- 4) Cortical neuropil (included tissue between pyramidal neurons excluding all other cell bodies and nuclei).
- 5) Glial cells (included all cells in the corpus callosum. These cells are mostly oligodendrocytes, with a minority being astrocytes).
- 6) Fiber tracts of the corpus callosum (the remainder of corpus callosum tissue excluding the cell bodies).
- 7) Hippocampus (including all parts).
 - a) Superficial hippocampus included all elements in the stratum oriens and alveus.
 - b) Stratum pyramidale hippocampi.
 - c) Deep hippocampus included all elements in strata radiatum and lacunosum.

Total areas analyzed by grain density analysis were: 1) pyramidal cell and surrounding neuropil, 8,600 μ^2 at WRL, 6,000 μ^2 at WRR (final magnification $\times 2,400$); 2) glial cells and surrounding neuropil, 8,000 μ^2 at WRL and 8,600 μ^2 at WRR ($\times 2,400$); 3) corpus callosum, 23,000 μ^2 at WRL and the same area at WRR ($\times 1,200$); 4) hippocampus, 57,300 μ^2 at WRL and 44,480 μ^2 at WRR ($\times 1,200$).

Rectal temperatures of representative mice were monitored with a thermistor probe. Measurements of tail-blood pH's of representative mice were made after warming the distal half of the tail. Arterialized blood was then drawn into glass capillary tubes.

Results

X-RAY FILM RADIOAUTOGRAPHS

Distributions of pentobarbital-2-¹⁴C at WRL and WRR were different (table 1, figs. 2

Fig. 3. Sixteen- μ m coronal sections of mouse brain at the level of the thalamus and hippocampus (see ref. 15, pp 43-44 (A), pp 41-42 (B), and 47-48 (C) (12 \times magnification). A, hematoxylin and eosin stained section for comparison with B. B, x-ray film radioautograph at time of loss of withdrawal response. Note labeling of cortex, (C) hippocampus, (h) and thalamus, (T) and lack of label in corpus callosum (cc) and internal capsule (I). C, x-ray film radioautograph at time of return of withdrawal response. Differences in amounts of pentobarbital-2-¹⁴C in different structures are difficult to detect.

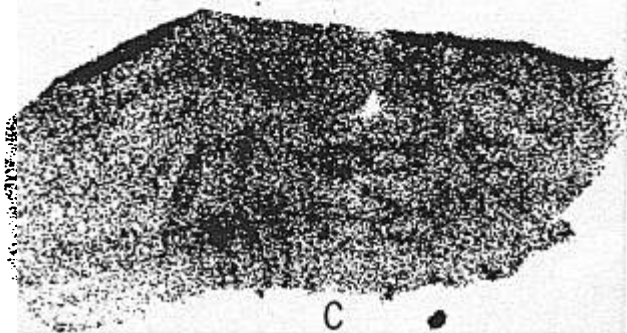
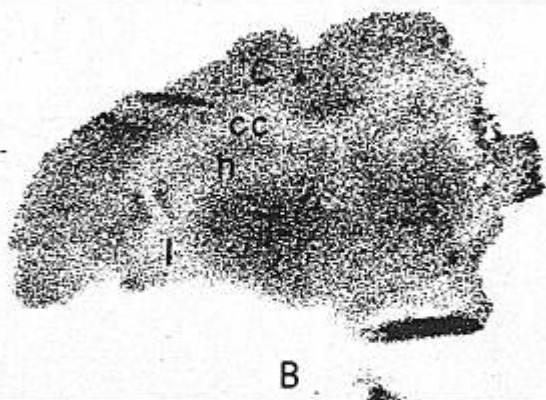
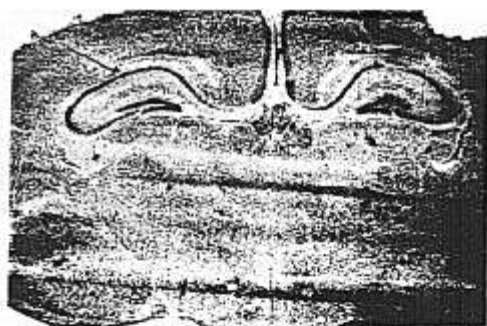


TABLE I. Concentrations of Pentobarbital in Mouse Brain by X-ray Film Radiointerography (Means \pm SE)

	40 mg/kg Dose						50 mg/kg Dose						
	Loss of Withdrawal Response (1 Min.)		Return of Withdrawal Response (6.7 Min.)		P	µg/g*	Loss of Withdrawal Response (1 Min.)		Return of Withdrawal Response (12.0 Min.)		P	µg/g*	
	Optical Density	µg/g*	Optical Density	µg/g*			Optical Density	µg/g*	Optical Density	µg/g*			
Grey matter													
Cortex	.519 \pm .02	31 \pm 1.43	.419 \pm .03	26 \pm 1.61	N.S.†	26 \pm 1.61	.639 \pm .02	39 \pm 1.34	.409 \pm .04	31 \pm 2.30	<.05	31 \pm 2.30	<.05
Cortex cinguli	.531 \pm .02	32 \pm 0.06	.406 \pm .03	25 \pm 1.60	<.05	25 \pm 1.60	.676 \pm .03	40 \pm 1.54	.488 \pm .05	30 \pm 2.95	<.05	30 \pm 2.95	<.05
Hippocampus	.439 \pm .04	27 \pm 2.46	.404 \pm .06	24 \pm 3.44	N.S.	24 \pm 3.44	.406 \pm .02	31 \pm 1.25	.540 \pm .04	32 \pm 2.25	N.S.†	32 \pm 2.25	N.S.†
Caudate/putamen	.521 \pm .05	31 \pm 2.91	.407 \pm .05	31 \pm 3.12	N.S.	31 \pm 3.12	.629 \pm .03	38 \pm 1.57	.498 \pm .11	31 \pm 7.16	N.S.	31 \pm 7.16	N.S.
Thalamus	.539 \pm .03	32 \pm 1.30	.475 \pm .06	29 \pm 3.97	N.S.	29 \pm 3.97	.675 \pm .04	40 \pm 2.19	.585 \pm .05	35 \pm 3.17	N.S.	35 \pm 3.17	N.S.
Mean	.510		.440				.623		.525				
White matter													
Corpus callosum	.328 \pm .01	20 \pm 0.85	.444 \pm .02	27 \pm 1.15	<.05	27 \pm 1.15	.409 \pm .07	31 \pm 4.47	.547 \pm .04	33 \pm 2.59	N.S.	33 \pm 2.59	N.S.
Internal capsule	.325 \pm .01	20 \pm 0.92	.425 \pm .04	26 \pm 2.69	<.05	26 \pm 2.69	.393 \pm .01	25 \pm 0.57	.598†	36†		36†	
Fornix	.423 \pm .12	26 \pm 7.50	.477 \pm .03	28 \pm 2.13	N.S.	28 \pm 2.13	.638 \pm .08	30 \pm 4.64	.469 \pm .06	20 \pm 3.65	N.S.	20 \pm 3.65	N.S.
Anterior commissure			.336 \pm .05	21 \pm 2.94		21 \pm 2.94	.463 \pm .11	29 \pm 6.64	.454†	28†		28†	
Mean	.359		.413				.498		.517				

* Pentobarbital concentration corresponding to mean optical density. Standard errors are calculated from the variance of the O.D. measurements.

† One value.

N.S. = not significant.

TABLE 2. Pentobarbital Concentrations in Cortex and Thalamus*

	Pentobarbital, 40 mg/kg		Pentobarbital, 50 mg/kg	
	Scintillation Counted	Calculated from Optical Density	Scintillation Counted	Calculated from Optical Density
Cortex	34 ± 2 µg/g	31 ± 1 µg/g	37 ± 2 µg/g	39 ± 1 µg/g
Thalamus	37 ± 2 µg/g	32 ± 2 µg/g	37 ± 2 µg/g	40 ± 2 µg/g

* All values ± SE are expressed per gram wet weight. Concentrations measured with scintillation counting from grossly dissected areas⁵ show good agreement with concentrations calculated from O.D. measurements on radioautographs. All values at WRL (1 minute after injection.)

and 3). The average grey matter concentration was higher than that of white matter at WRL after both 40 mg/kg and 50 mg/kg. At WRR the concentrations of pentobarbital-2-¹⁴C in grey and white matter were similar (table 1, figs. 2C and 3C). The absolute levels of pentobarbital-2-¹⁴C were always higher after 50 mg/kg than after 40 mg/kg. The change in distribution seems to be accounted for by a decrease in grey matter concentration from WRL to WRR and an increase in white matter concentration, at least at a dose of 40 mg/kg. At 50 mg/kg the overall white matter concentration was marginally but not significantly higher at WRR compared with WRL, but it was previously found that the highest overall brain levels of pentobarbital occurred at 5 minutes; by 10 minutes, the overall brain concentration had begun to decrease.⁵ Therefore, the small increase in white matter concentration from WRL to WRR following 50 mg/kg could be accounted for by the long interval, 12.6 minutes, between injection and WRR. Note the agreement of drug levels in areas that are easily defined and contain only grey matter (table 1, figs. 2 and 3).

Pentobarbital-2-¹⁴C levels in the hippocampus were slightly lower than the mean grey-matter levels ($P < 0.2$) at WRL after both 40 and 50 mg/kg. The hippocampus contains both grey and white matter. The fornix and anterior commissure are small, and could be influenced by adjacent grey-matter structures. To help further explain these results, cellular localization of pentobarbital-2-¹⁴C was undertaken.

Correlation between pentobarbital concentration calculated from optical densitometry and previously measured⁵ tissue pentobarbital concentration is shown in table

2. Pentobarbital concentration calculated from measured O.D. in the cortex and thalamus at WRL (1 minute) agreed well with unchanged pentobarbital concentrations measured by scintillation counting.⁵

FROZEN-SECTION RADIOAUTOGRAPHY

No evidence of latent image fading or positive chemography was found in radioautographs exposed for as long as 27 days at -30 C. The interval between staining and photography had been previously found to be crucial: exposure of the developed silver grains to hematoxylin and eosin stained tissues for several months resulted in loss of developed grains.

Grain densities over, and thus the pentobarbital levels in, pyramidal cell bodies (perikarya) were 55 per cent higher than those over the surrounding neuropil at WRL ($P < 0.001$), but levels in the cell bodies did not change between WRL and WRR (figs. 4 and 5). Between WRL and WRR, in contrast to the decrease of pentobarbital in the cortical band taken as a whole and measured from the x-ray film radioautographs, the overall grain density of layer V increased. Glial grain densities were similar to those of the adjacent fibers at WRL, but at WRR the adjacent fiber tracts were twice as heavily labeled ($P < 0.001$) (fig. 4). The increase in grain density over the general corpus callosum coincides with the observations from the x-ray film radioautographs. The glia were less labeled than pyramidal cell bodies at both WRR and WRL. The neuropil of the pyramidal layer of parietal cortex contained a 21 per cent higher concentration of pentobarbital than the corpus callosum at WRL, but 33 per cent less than the corpus callosum at WRR

(fig. 5). Layer V pyramidal cell bodies and corpus callosum white matter had similar grain densities at WRR.

At WRL the three subcompartments of the hippocampus had equivalent pentobarbital-2-¹⁴C concentrations (figs. 6 and 7). The overall hippocampal grain density increased between WRL and WRR ($P < 0.001$). The grain densities of the deep and superficial layers had also increased, that of the superficial layer by 46 per cent ($P < 0.001$). At the time of WRR the superficial layer had a higher concentration of pentobarbital-2-¹⁴C than stratum pyramidale or the deep layer ($P < 0.001$). Stratum pyramidale (essentially all neuronal cell bodies) labeling did not change significantly. Thus, the shifts in pentobarbital concentration within many areas of the brain are relatively large and in certain areas of the brain, such as the superficial layer of the hippocampus, they are nearly 50 per cent higher at awakening (WRR) than at loss of withdrawal response.

Rectal temperature just before WRR, 12 minutes after pentobarbital, 50 mg/kg, iv, was less than 0.5 C below normal awake temperature. Arterialized tail-blood pH values ranged from 7.37 to 7.46 just before WRR; no significant difference between pH values for the mice given 40 and those given 50 mg/kg pentobarbital iv was found.

Discussion

The distribution of pentobarbital-2-¹⁴C in mouse brain following a single intravenous injection has several interesting features. Pyramidal neuronal cell bodies in cortical layer V and in the hippocampus are labeled at WRL, but the level is unchanged at WRR. Glial cells are not differently labeled from their surroundings at WRL but are distinctly less labeled than their surroundings at WRR; the absolute level of label is unchanged. The level of label over the neuropil increases markedly from WRL to WRR. Regional distribution, as computed from x-ray film radioautographs, reflects these changes. The structures that have large numbers of neurons were labeled at WRL, and either the levels of label decreased or they did not change by WRR. The structures that have no neurons and large numbers of axons and myelin sheaths were less labeled than grey matter at WRL but similarly labeled at WRR, the result being that at WRR the average level of labeling of all brain structures is the same as at WRL.

The absolute levels of pentobarbital-2-¹⁴C, calculated from the optical densitometry readings (table 1, fig. 1), corresponded fairly closely to the data derived from scintillation spectrometry of the same brain regions.⁵ In

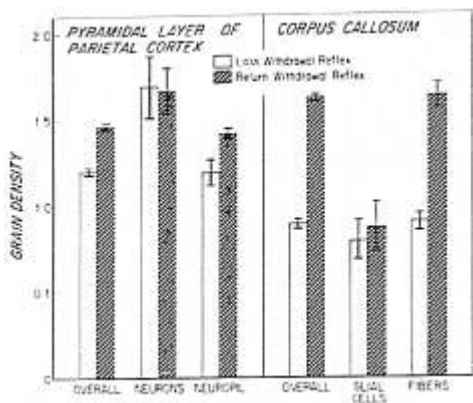


FIG. 4. Histogram of grain densities \pm SD over pyramidal cell layers of the parietal cortex and over the corpus callosum in 3- μ m frozen sections of mouse brain exposed to NTB-2 for 14 days at -30 C. The analyses were performed on photomicrographs enlarged 2,400 \times . Note changes in grain densities between time of loss and return of withdrawal reflex. Note also that the SD's in these analyses and those in figure 6 are affected more by the size of the tissue compartment than by the number of grains. The minimum number of points counted in one compartment was 71 and the average, 420.

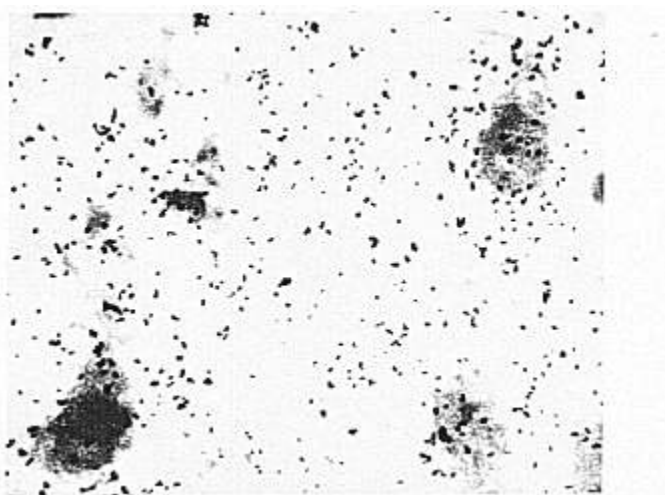


FIG. 5. Radioautograph of large pyramidal cells from the parietal cortex at the time of loss of the withdrawal response following a single intravenous injection of 50 mg/kg pentobarbital-2-¹⁴C. Grain density analysis demonstrates 55 per cent higher grain density over neurons compared with surrounding neuropil ($P < 0.001$). Note also the difficulty in differentiating small neurons from glial cells (hematoxylin and eosin, 1,800 \times).

the previous study no attempt was made to separate grey from white matter.⁵ It should be noted that the area containing the largest relative amount of white matter, the spinal cord, was the region where the peak concentration was reached latest, again confirming our observation that the white matter levels are higher at the time of WRR, *i.e.*, 6.7 and 12.6 minutes after injection of 40 or 50 mg/kg, respectively, than at WRL.

Interpretation of radioautographic results as well as liquid scintillation spectrometry depends on several factors: 1) The knowledge or ascertainment of the compound that carries the radioactive label in the tissue. Metabolites of pentobarbital-2-¹⁴C were not found in brain at any of the times studied.⁵ Therefore, it can be concluded that all the label seen in these experiments reflected radiation from pentobarbital-2-¹⁴C. 2) The material being examined is not extracted or moved around during processing. Because

pentobarbital sodium is water-soluble, the tissue used in these studies was frozen as soon as possible and kept frozen until the radioautographs were completed.^{7,12,13,17}

The mechanisms whereby the neurons are rapidly labeled, but still have the same level of label at WRR when the neuropil has caught up with the neurons, are not known, but several speculations can be introduced. It is realized that we do not know whether the interim levels would have been higher if we had looked. In many species blood flow and number of capillaries are greater in grey matter than in white matter.¹⁸ This fact could account perhaps for the observed differences in regional labeling at 1 minute (*i.e.*, at WRL), but could not account for the pattern of diffuse labeling at later intervals. At 1 minute the pyramidal neurons were decidedly more labeled than their surrounding neuropil, and at 12.6 minutes (WRR) the label in the surrounding neuropil had in-

creased somewhat. In contrast, the concentration of label in glial cells at WRR was not different from that at WRL, whereas their surrounding neuropil at WRR not only was more labeled than the glial cells but was more labeled than the pyramidal neurons.

If pentobarbital were taken up preferentially by neuronal perikarya and subsequently distributed throughout the nerve cell to dendrites and axons, the pattern of labeling observed could be predicted provided that the neuron continued to take up pentobarbital from the blood over the time course of our experiment. It is not known how or where drugs such as pentobarbital cross the blood-brain barrier and gain access to neurons.⁷ All blood vessels in the central nervous system are surrounded by glial foot processes, so in order to travel from blood to neuron the drug must either diffuse or be transported through the extracellular space or through astrocytes. That glial cells were less labeled than neurons could be interpreted to suggest that glial cells are not involved, but higher-resolution methods at earlier sacrifice intervals would be needed to determine this point. Such methods are not currently available but are being developed.^{15,13}

The role of barbiturate solubility in various cell components needs further clarification, since it appears likely that distribution of pentobarbital may be largely dependent upon composition, structure and complex physical-chemical interactions not necessar-

ily related to solubility in lipid. The later higher concentration of pentobarbital in white matter has been equated with its lipid solubility; however, the presence of pentobarbital in myelin sheaths has not been demonstrated. Certain amino acids can freely cross the myelin sheath into the axon and vice versa,¹⁹ and maybe anesthetic agents could behave similarly.

It is probable that pentobarbital is distributed to many components of cells whose exact roles in producing anesthesia are unclear.²⁰ Obviously, many other factors could contribute to the distribution we observed. Alterations in local pH or intracellular pH and ionic or protein composition are only a few variables that we could not measure or control.²¹ Despite data indicating reasonable physiologic stability during this level of anesthesia,⁵ the possibility that alterations in intracellular milieu may be significant cannot be excluded.

Although, unfortunately, we did not examine the cerebellum radioautographically, pentobarbital concentrations measured from dissected cerebellum⁵ showed pentobarbital levels that were not much different from those of the cortex, in spite of a cell density of cerebellar cortex which, McEwen *et al.* estimate,²² could account for nearly 50 per cent of total extractable DNA from rat brain. Thus, we would expect by proportion a much higher total pentobarbital level in the cerebellar cortex than we measured for the entire cerebellum if

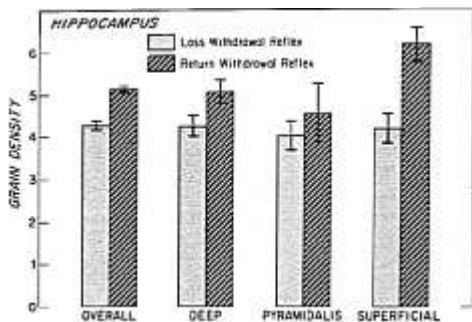
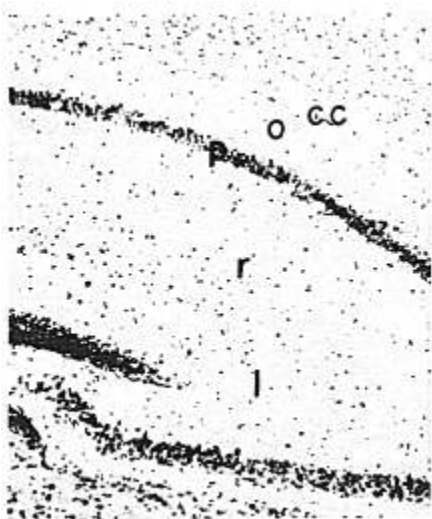


FIG. 6. Histogram of grain densities \pm SD overlying the hippocampus in radioautographs of 3- μ m frozen sections of mouse brain exposed to NTB-2 for 14-17 days at -30°C , magnified 1,200 \times . For subdivisions of the hippocampus see figure 7. Note not only the increase but the change in the relative concentrations of pentobarbital- $2\text{-}^{14}\text{C}$ in the deep and superficial compartments of the hippocampus at the time of return of the withdrawal response. The numbers of points counted per compartment ranged from 378 to 523. Dose 50 mg/kg.

FIG. 7. Radioautograph of the hippocampus at the time of loss of the withdrawal reflex. The areas analyzed were the corpus callosum (cc), superficial hippocampus (stratum oriens [o], and alveus), stratum pyramidale (p), and deep hippocampus (stratum radiatum [r] and stratum lacunosum moleculare [l]). Silver grains are not visible at this magnification. Hematoxylin and eosin, 98x.



pentobarbital localized preferentially to neuronal areas, as our radioautographic data suggest. However, cell nuclei in the granular cell layer of the cerebellum are quite distinct from those in other areas of brain.²² McEwen *et al.* reported an RNA/DNA ratio of only 0.08, in contrast to ratios in the cerebral cortex (0.41) and hippocampus (0.36²²); total protein/DNA ratio was only 2.2 in the cerebellum, compared with 4.8 in the cerebral cortex.²² Cells of the cerebellar granular cell layer have small amounts of Nissl substance, less RNA polymerase, and are less active in protein synthesis than other neurons in brain.²³ Possibly these peculiarities explain why Cohen *et al.* found relatively low levels of inhalational anesthetics in this layer.¹

The mechanism of action of pentobarbital is not known. One possibility is an effect on synaptic transmission. Areas with high synaptic density that were examined were the deep layers of the hippocampus; however, the observed higher level of pentobarbital-2-¹⁴C at WRR than at WRL in these layers might suggest that if there were an effect on

synaptic transmission it would be overcome during continued exposure to the drug. Since metabolites have not been found in the brain,⁵ it seems unlikely that this compensation is the result of drug metabolism, and one must postulate that alternate pathways for function of the brain are rapidly developed or that pentobarbital is not specifically involved in disrupting brain function *in vivo*.

In summary, radioautography of 3- μ -thick frozen sections of brain is a time-consuming but satisfactory method of systematically investigating the distribution of radiolabeled drugs. What role the changing regional and cellular distribution plays in anesthesia is not yet understood. However, speculation about probable cellular and subcellular anesthetic distribution from assumptions based on whole-body distribution dynamics is unwise. Understanding actual anesthetic drug localization may help to relate some of the many effects of anesthetics known to occur *in vitro* to the anesthetic process and toxicities *in vivo*. Further work on the intracellular distribution of anesthetics within the CNS is needed.

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