INTRACELLULAR DISTRIBUTION OF
PENTOBARBITAL SODIUM IN RAT BRAIN

by

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The undersigned, appointed by the Dean of the Graduate Faculty, have
examined a thesis entitled

**INTRACELLULAR DISTRIBUTION OF
PENTOBARBITAL SODIUM IN RAT BRAIN**

presented by **Carol Jean Oen**

a candidate for the degree of **Master of Arts**

and hereby certify that in their opinion it is worthy of acceptance.

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I. INTRODUCTION

The major effect of the barbiturates is depression of the central nervous system, but the way in which these drugs exert their effect is not yet well understood.

According to Goodman and Gilman (1955), there is evidence to indicate that the barbiturates as a class act at all segmental levels and at all levels of functional organization of the central nervous system. Furthermore, the cerebral cortex and reticular activating system are most sensitive to barbiturate effects, the cerebellar, vestibular, and spinal systems less so, and the medullary system least sensitive.

A sublimation method of barbiturate analysis has been used to give evidence of a differential distribution of barbiturates in the brain (E. Keeser and I. Keeser, 1927, 1935; E. Keeser and J. Keeser, 1927, 1929). Work by Koppanyi, Dille, and Krop (1934) and by Koppanyi and Dille (1935) refutes the Keesers' work, as these workers found no difference in barbiturate concentration in the various divisions of the brain using either the small doses (50-60 milligrams/kilogram) employed by the Keesers or large anesthetizing doses (225-600 milligrams/kilogram). More recent work by Maynert and van Dyke (1950), using N¹⁵-labelled barbital also showed no localization of the drug in any particular division of the central nervous system.

Schneider and Hogeboom (1956) state that the fact that cells are seen to be exceedingly complex structures and not simply homogeneous masses of protoplasm implies that this arrangement must have some functional significance. If all compounds and enzymes were uniformly distributed throughout the cell, there should be no reason for the existing discrete subcellular organization of the cell.

As the biochemical functions of the cell and its various parts become better understood, it is of interest to relate the actions of a drug to some particular function (Reiner and Gellhorn, 1956). If it were found that a barbiturate were localized by a particular cellular component, this might mean that its effect was rendered through some function of that particular component. Consequently, this study was undertaken to determine the intracellular distribution of a particular barbiturate, pentobarbital sodium, within rat brain cells.

II. LITERATURE REVIEW

A. Barbiturates

The barbiturates may be classified in three major ways; 1) structure, 2) length of action, and 3) site of detoxification (Goodman and Gilman, 1955; Sollmann, 1957). Pentobarbital's structure is that of the 5-ethyl-5-(1-methylbutyl) derivative of barbituric acid; it is a short-acting barbiturate, and it is detoxified mainly in the liver (Goodman and Gilman, 1955).

Nutritional conditions such as starvation, anemia, or vitamin C deficiency may alter the sleeping time under barbiturates (Richards and Taylor, 1956). Kinsey (1940) showed that tolerance to pentobarbital develops rapidly in white rats, resulting in a marked reduction of sleeping time.

The concentration of barbiturate in the central nervous system parallels the depth of anesthesia, and the rate of penetration into the brain parallels the time of onset of effects (Butler, 1950). An equilibrium between the brain and the plasma levels of barbiturate is established quickly and remains relatively constant (Richards and Taylor, 1956). The concentration of barbiturates, other than thiopental, in the cerebrospinal fluid is usually very low (Goodman and Gilman, 1955).

In clinical cases of barbiturate poisoning, the concentration of barbiturate in the brain is not very high (Sollmann, 1957). There is no close parallelism between clinical effect and the concentration of barbiturate in the blood (Helldorff, 1955).

In vitro studies using rabbit tissue homogenates have shown that barbiturates are bound in the same order as in vivo, decreasing in amount bound from liver to kidney, to plasma, to brain, to red blood cells, to muscle (Goldbaum and Smith, 1954). In the same study these investigators also established the fact that the pentobarbital penetrates the brain rapidly and attains a higher concentration there than in the plasma.

Neurophysiological and electroencephalographic studies have established that the barbiturates elevate the threshold of neurons by stabilizing the cell membrane, and that they prolong the time for recovery from stimulation (Goodman and Gilman, 1955). Since there is an increase in the absolute and relative recovery times at cortical levels with increasing depth of pentobarbital anesthesia, Wikler (1952) suggested that part of the effect of barbiturates is due to depressant effects in the thalamus. It is possible that a specific effect is exerted on the central mechanisms which regulate the sleep process (Goodman and Gilman, 1955). Even though variations of concentration in different parts

of the brain have not been shown, a difference in sensitivity to a given level of drug may occur (Richards and Taylor, 1956).

The actions of pentobarbital on spinal cord and peripheral nerve are essentially similar to its effects on the brain centers, except that higher doses are required to depress the spinal cord, and even greater doses are required to depress peripheral nerve (Goodman and Gilman, 1955).

B. Barbiturate Analysis

Earlier quantitative analyses of barbiturates were based upon gravimetric methods in which the barbiturate was extracted into an organic solvent, the solvent evaporated, and the residue purified (Dybing, 1955). This sort of method has the disadvantages of requiring a great deal of time and a large amount of sample.

Colorimetric methods have been developed using colors produced by the reactions of barbiturates and various compounds of cobalt and copper, but application of these methods to biological material is not easy because of the low specificity and sensitivity (Raventós, 1954).

Spectrophotometry in ultraviolet light is now the preferred method. This type of analysis for the barbi-

turates in general appeared in 1948 (Goldbaum, 1948; Walker, Fisher, and McHugh, 1948). Earlier the same principle had been used for particular barbiturates (Jailer and Goldbaum, 1946). Subsequently, there have been published several modifications and improvements of the spectrophotometric method (Dybing, 1955; Brodie, Burns, Mark, Lief, Bernstein, and Papper, 1953; Goldbaum, 1952).

According to Walker, Fisher, and McHugh (1948), the limiting factor in the sensitivity of the spectrophotometric method is the ratio of barbiturates present to other interfering substances concomitantly extracted.

It is well known that barbiturates split in strongly alkaline solutions, but measurements cannot be made in acidic solutions because the absorption of ultraviolet light is too small (Dybing, 1955). Dybing (1955) studied the rate of splitting to determine if it were high enough to influence spectrophotometric determinations. At pH 10, the barbiturates were sufficiently stable to prevent splitting from influencing the determination, while a wide range of stability was found among the various barbiturates in 0.5 N. sodium hydroxide solution. Pentobarbital was not tested by this investigator.

It is claimed now that Goldbaum's method of barbiturate analysis has an accuracy of about 95 per cent for concentrations of barbiturates as low as 1 milligram/100

grams (or 10 micrograms/gram) of blood or tissue (Goldbaum, 1956).

C. Cellular Fractionation

Centrifugation has been used for isolation of particular cellular components for some time, but techniques for complete fractionation of cells by centrifugation have been used extensively only since the later 1940's (De Duve and Berthet, 1954).

Fractionation by means of centrifugation is based on the fact that particles of different size or density sediment at different rates in a centrifugal force field. Conditions for centrifugation are chosen for each fraction so as to cause complete sedimentation of the heavier particles with as little contamination as possible by the lighter ones (De Duve and Berthet, 1954). An approximate formula expressing the rate of sedimentation for a spherical particle in a centrifugal force field is

$$\frac{dx}{dt} = \frac{2r^2(d_p - d_m)}{9\eta} \omega^2 x$$

where x = distance traveled along the direction of the field in centimeters

t = time in seconds

r = radius of particle in centimeters

d_p = density of particle in grams/cubic centimeter

d_m = density of suspension medium in grams/cubic centimeter

η = viscosity of medium in poises
 ω = angular velocity in radians/
second

It is seen in the above formula that the governing factor in a given medium is represented by the term $r^2(d_p - d_m)$. Therefore, it is theoretically possible for smaller particles with higher density to have the same sedimentation rate as larger particles with a lower density. Not included in the above formula is the influencing factor of the particle's shape in sedimentation rates, but this is not appreciable unless there is pronounced asymmetry (De Duve and Berthet, 1954).

With high-speed centrifuges the total force applied is expressed as an integral, the g-minute. Where the instrument is equipped with a "revolutions-per-minute" indicator, a chart giving the value of the integral for different speeds can be devised easily.

III. METHODS

A. Preliminary

Adult, albino, male rats were used throughout this study since it has been shown that the susceptibility of rats to barbiturates varies with age and sex (Moir, 1937).

It is recommended (De Duve and Berthet, 1954; Schneider and Potter, 1949) that animals whose tissue is to be used for cell fractionation be fasted previously for about 12 hours to reduce the glycogen content. Since the glycogen content of brain is very low (Dawson and Richter, 1950) and because brain glycogen differs from liver glycogen in that it is not altered appreciably by starvation (West and Todd, 1955), the fasting period was eliminated.

Pentobarbital sodium was chosen as the barbiturate for use in this investigation, since it is not stored in significant amounts by the body fat (Brodie, 1952). This eliminates error resulting from different percentages of body fat in the various animals.

The pentobarbital sodium was prepared as a standard anesthetizing solution of 10 milligrams of pentobarbital sodium per milliliter of normal saline solution. The dose used for anesthesia was 40 milligrams per kilogram of body weight (or 40 micrograms/gram), given intraperitoneally.

The time at which the animal could no longer support its weight on its feet was taken to be the beginning of anesthesia. Exactly 20 minutes later the rat was decapitated, its brain removed as quickly as possible and chilled for a few minutes in finely crushed ice (Brody and Bain, 1952). From this point until completion of the cellular fractionation procedure, all solutions, containers, and the tissue itself were kept within a temperature range of 0-5 degrees Centigrade.

The chilled brain was blotted, then quickly and accurately weighed on a Roller-Smith precision balance.

B. Cellular Fractionation

The method employed for the separation of the cellular components was essentially that outlined by De Duve and Berthet (1954). The principle employed is differential centrifugation. A classification for that term was proposed by Anderson (1955), i.e., fractions are collected consecutively at the bottom of the tube. The separation depends on differences in sedimentation rate.

Centrifugations were carried out in an International Portable Refrigerated Centrifuge, Model PR-2, fitted with a high-speed, angle-head attachment. The temperature control was set at 0 to -2 degrees Centigrade in all instances.

Ice-cold isotonic sucrose solution (.25 M.) was used as the homogenizing medium. No additional buffering agents were added since, in the words of De Duve and Berthet (1954),

"it . . . appears that conditions suitable for the successful isolation of one type of cellular component may influence unfavorably the separation of another, and vice versa. At the present time, salt-free solutions would appear to be the best medium for a complete fractionation."

The processes of homogenization and isolation of the nuclei were combined as suggested by De Duve and Berthet (1954). This was said to give a more complete disruption of the cells with minimum damage to the components. The procedure was to add isotonic sucrose to the whole brain--the number of milliliters = 2.5 times the brain weight. This mixture was then placed in a 40-milliliter, glass, Ten-Broeck homogenizer surrounded by a container of ice water, and homogenized for a short time. This type of homogenizer is similar to that described by Potter and Elvehjem (1936), the one recommended by De Duve and Berthet (1954) for use in this procedure.

The mixture from this first homogenization was poured into a 25-milliliter, Lucite centrifuge tube and centrifuged at 10,000 gravity-minutes. The supernatant was pipetted off, using a rubber-bulbed pipette with a 90-

degree angle near the tip.

The isolation of the nuclei was completed in two additional steps, each involving further homogenization and centrifugation. The total number of milliliters of sucrose added in the three steps was equal to 9 times the brain weight. The milliliters of sucrose solution to be added in each of these two additional steps was calculated as follows: $\frac{1}{2} (9 \times \text{brain weight}) - (2.5 \times \text{brain weight}) =$ volume of sucrose solution in milliliters. After the addition of this amount of isotonic sucrose solution to the sediment from the first centrifugation, the mixture was transferred to the Ten-Broeck homogenizer and homogenized again for a short time, then centrifuged at 6,000 gravity-minutes. The supernatant was again pipetted off and added to the supernatant from the first centrifugation. The final portion of isotonic sucrose solution was added to the sediment and the homogenization and centrifugation repeated as described above.

The combined supernatants were then ready for further fractionation. Mitochondria were the main constituent of the sediment which was centrifuged down from the supernatants at 160,000 gravity-minutes. The supernatant from this was pipetted off as before. The sediment was washed by resuspending it in a volume of isotonic sucrose equal to the brain weight, followed by centrifugation

at 130,000 gravity-minutes. Resuspension was accomplished by using a "micro-homogenizer", the rounded end of a Folin-Wu tube, which fits directly into a centrifuge tube, thus eliminating a transfer of the solution for mixing. A second wash of the mitochondrial sediment was performed in the same manner. The supernatants from each wash were combined with that from the first centrifugation of the mitochondria-containing solution.

Three basic fractions were thus obtained, "nuclei", mitochondria, and the final supernatant. The "nuclear" fraction consisted of two fairly distinct layers; a dense, fatty bottom layer fitting Brody and Bain's (1952) description of a gel-like residue, and hereafter referred to as "cellular debris", and a less dense, grayish, upper layer fitting De Duve and Berthet's (1954) description of the nuclear layer. These could be separated quite easily by carefully pipetting off the upper layer. These four final fractions were then weighed accurately on the Roller-Smith precision balance. Weighing was done directly in the tared centrifuge tubes by using a wire basket especially made to hold these tubes.

The weights of the various fractions were found to total considerably more than the original whole brain weight. Each retains some of the sucrose solution. Also some material is lost in the fractionation process, since

the total weights do not equal the weight of the brain plus the weight of the sucrose added.

Some criterion was needed to compare the relative efficiencies of the various fractionations. It was decided that the weight of each final fraction expressed as a percentage of the original whole brain weight would give a simple measurement for this comparison.

The fractions were transferred to glass-stoppered bottles, using 2 milliliters of isotonic sucrose solution to aid in the transfer. These were then stored in a refrigerator at 1 degree Centigrade until time for analysis.

Microscopic examination of the various fractions obtained was made in 4 trial runs. As the particles were so dense, 1 drop of sedimented fraction was diluted with 2 drops of sucrose solution for easier examination.

The cellular debris consisted of some apparently unbroken cells, many red blood cells, some particulate matter, and a great deal of jelly-like material forming a matrix.

The nuclear fraction was found to be fairly uniform in larger particles, but some smaller particles were also evident.

The mitochondrial fraction contained a fairly wide range of particle sizes. Attempts were made to differen-

tiate the true mitochondria from other material present in this fraction. A mixture of acetic acid and alcohol is known to dissolve mitochondria. One drop of a mixture of acetic acid and absolute alcohol in a 1:1 ratio was added to 1 drop of mitochondrial sediment on a slide. Only larger particles could be seen. When 1 drop of sucrose was added to 1 drop of sediment, the large particles were visible, but there was a high concentration of the smaller ones also.

The procedure for staining mitochondria with Janus Green B given by Guyer (1950) was tried. Janus Green B was made up in mammalian Ringer's solution in a dilution of 1:20,000. One drop of mitochondrial sediment was added to one drop of this solution on a slide, a cover glass was applied, and the slide was examined. No staining was detected.

The Janus Green B was then made up in 1:5,000, 1:10,000, 1:15,000 and 1:20,000 in mammalian Ringer's solution. These various dilutions were tried on hemolyzed human blood, but again no staining was found to occur. The same dilutions of dye in normal saline instead of Ringer's solution gave an even medium-blue stain to spherical particles in the blood preparation. The stain in saline was then tried on the mitochondrial fractions. Some staining could be obtained only with the 1:5,000 dilution, and then only when 1 drop of sediment was added to 3 drops

of dye solution. According to Cowdry (1918) Janus Green B is specific for mitochondria only in very dilute solutions.

The supernatant fraction was found to be almost devoid of particulate matter, and even though not diluted for microscopic examination, it must be remembered that this fraction is greatly diluted with the homogenizing medium. In this work the microsomes would also be expected to be present in the supernatant, though they are not easily differentiated microscopically.

A group of trials was made also on unanesthetized animals to serve as controls for the experimental determinations. The same procedures were followed throughout, with the exception of anesthetizing the animals.

C. Barbiturate Analysis

The spectrophotometric method of Goldbaum (1952) was used to determine the concentration of pentobarbital in the various samples. In this procedure the concentration is a function of the difference in optical density between barbiturate in sodium hydroxide and barbiturate in alkali buffered to pH 10.5. Pentobarbital in 0.9 N. sodium hydroxide exhibits an absorption peak at 255 millimicrons. In pH 10.5 buffered solution, its peak is at 240 millimicrons (Goldbaum, 1952).

In preliminary work unextracted standard solutions were compared with extracted ones. The same absorption peaks were found, but the numerical values were considerably different. It was decided that extracted standards would give a better comparison with extracted tissue values, so all were carried through the extraction procedure in experimental work.

The absorption spectrum of a solution of commercial pentobarbital sodium was compared with a solution of pentobarbital sodium made from the free acid in the manner described by Brodie, Burns, Mark, Lief, Bernstein, and Papper (1953). For purposes of comparison, the amount of sodium pentobarbital used was 1.097 times the amount of pentobarbital acid called for in the above paper. The curves obtained were, for all practical purposes, identical. Therefore, the commercial product was used for all standards as well as the anesthetizing solution.

Ethylene dichloride (1,2-dichloroethane) was used to extract the barbiturate from the fractions, from a known standard in sucrose solution, and from sucrose solution alone. The ethylene dichloride needed for one set of extractions (about 400 milliliters) was washed in a separatory funnel with approximately 1.0 N. sodium hydroxide solution using 1 milliliter sodium hydroxide to 10 milliliters ethylene dichloride. This initial wash was

followed by two washings with redistilled water, using the same volume ratio as for the sodium hydroxide.

A known standard of pentobarbital sodium in sucrose solution was made, using 100 micrograms of barbiturate per milliliter of isotonic sucrose. As each fractionation was run, 1 milliliter of this standard was diluted with 1 milliliter of isotonic sucrose for use as a working standard to be analyzed along with the various fractions of that run. This working standard was stored in the same type glass-stoppered bottle as the fractions and for the same length of time.

Two milliliters of isotonic sucrose solution were used as a "blank" solution to be extracted and analyzed with each set of standard barbiturate and cellular fractions.

Thirty milliliters of ethylene dichloride were used to extract each blank and standard, 50 milliliters for each of the three sedimented fractions, and 100 milliliters for the supernatant fraction. These mixtures in 125-milliliter separatory funnels were shaken 70 times per minute for three minutes on an Eberbach mechanical shaker. Then the ethylene dichloride (lower) layer in each separatory funnel was passed through Whatman Number 41 filter paper into a second 125-milliliter separatory funnel.

Next, 4 milliliters of 0.9 N. sodium hydroxide were

added to each sample. The normality of the sodium hydroxide had been previously adjusted such that, when diluted with a borate buffer (1 M. each--boric acid and potassium chloride) in equal volumes, the pH would be 10.5 as measured on a Beckman Model G pH meter. The borate buffer was made according to the directions given by Goldbaum (1952). The concentration of the buffer was found to change with time, so the adjustment of the sodium hydroxide normality was made each time an analysis was to be performed. Also, a fresh buffer solution was made at least every two weeks.

The sodium hydroxide-ethylene dichloride mixture was shaken for three minutes as before. Askevold and Løken (1956) found that shaking beyond 3 minutes during reextraction did not increase the recovery of barbiturate. The ethylene dichloride (lower) layer was discarded and the sodium hydroxide layer was run into 15-milliliter centrifuge tubes. These were centrifuged at the maximum velocity (3030 revolutions per minute) of a Clay-Adams Safety-head centrifuge for 5 minutes.

The upper layers from the solutions in the centrifuge tubes were pipetted off, and 3 milliliters of each were transferred to calibrated, 1-centimeter, silica cuvettes. The optical density readings were then taken on a Beckman Model DU Spectrophotometer with ultraviolet

accessories. The wave length setting for all readings was 260 millimicrons. The samples were then diluted 1:1 with borate buffer and the optical density readings were taken again. Dilution was accomplished by pipetting off 1.5 milliliters from each cuvette and replacing it with 1.5 milliliters of borate buffer solution. The contents of each cuvette were stirred with individual, small, clean, stirring rods to ensure thorough mixing. It was also found advantageous to allow the diluted contents to stand a few minutes before taking the final readings. These final optical density readings were multiplied by 2 to correct for dilution of the barbiturate.

After completion of the analytical procedure, the silica cuvettes were placed in an approximately 3 N. hydrochloric acid bath for about 1 hour, then rinsed several times in distilled water as suggested by Brodie, Burns, Mark, Lief, Bernstein, and Papper (1953) to minimize the reagent blank.

IV. RESULTS

The formula given by Goldbaum (1952) was used to determine the concentration of barbiturate in the various fractions.

$$\text{Micrograms barbiturate/gram of sample} = \frac{a \times b \times c \times d}{e \times f \times g}$$

where

a = difference of unknown extract in alkali and in pH 10.5 at 260 millimicrons

b = volume of ethylene dichloride used to extract sample

c = volume of alkali used to extract ethylene dichloride aliquot

d = micrograms of barbiturate per milliliter of known alkaline extract

e = difference of known extract in alkali and in pH 10.5 at 260 millimicrons

f = ethylene dichloride aliquot

g = amount of sample

The results of the analysis of the control group of animals are shown in Table I. All fractions exhibit absorption at 260 millimicrons. In all cases, however, the absorption in the buffered solution was greater than that in the 0.9 N. sodium hydroxide, thus giving negative values rather than the "ideal" condition of being zero in the absence of barbiturate.

The standard deviations found in the control group for the cellular debris, nuclei, mitochondria, and super-

Table I

PENTOBARBITAL EQUIVALENT (MICROGRAMS/GRAM) CORRECTIONS
FOR ISOLATED CELLULAR FRACTIONS FROM CONTROL ANIMALS

Trial Number	Cellular Debris	Nuclei	Mitochondria	Supernatant
A	-1.303	-1.460	-5.035	-.230
B	-1.114	-1.453	-4.003	-.311
C	-1.552	-1.200	-3.202	-.462
D	-2.016	-0.977	-4.404	-.282
E	-2.534	-1.609	-4.351	-.306
F	-2.637	-1.476	-5.002	-.246
G	-1.863	-1.211	-3.535	-.340
H	-1.962	-1.015	-3.994	-.291
I	-1.343	-1.343	-4.901	-.198
$\bar{x}$	-1.814	-1.305	-4.270	-.296
σ	.537	.218	.649	.076
S.E.M.	.190	.077	.229	.027

$\bar{x}$ = mean
 σ = standard deviation
 S.E.M. = standard error of the mean

natant were .537, .218, .649, and .076 micrograms of pentobarbital per gram of fraction, respectively (Table I). The standard errors of the mean for these same fractions were found to be, respectively, .190, .077, .229, and .027 micrograms of pentobarbital per gram of fraction (Table I).

In a few experimental determinations the optical density difference was also found to be negative. A correction factor was applied to each experimental result. This factor was the average of the control group for that fraction as determined in Table I. This resulted in a net positive reading in all experimental determinations which had previously been negative. These corrected values for the experimental group are given in Table II. The means and the standard errors of means for the experimental group have been plotted in Figure 1. The standard deviations for the experimental groups of cellular debris, nuclei, mitochondria, and supernatant were 6.037, 9.022, 4.36, and 3.80 micrograms of pentobarbital per gram of fraction, respectively (Table II). The standard errors of the mean for these groups were found to be 1.67, 2.50, 1.26, and 1.05 micrograms of pentobarbital per gram of fraction (Table II).

It will be noted that the value for mitochondria was omitted in trial number 5 (Table II). This was eliminated on the basis of Chauvenet's criterion for the rejection of suspected observations (Jarrett, 1949).

Table II

**CONCENTRATION OF PENTOBARBITAL SODIUM IN MICROGRAMS/GRAM OF
ISOLATED CELLULAR FRACTION OF RAT BRAIN**

Trial Number	Cellular Debris	Nuclei	Mitochondria	Supernatant
1	11.43	13.32	5.93	16.73
2	9.11	14.64	7.08	19.52
3	13.39	10.67	3.73	14.05
4	7.85	10.19	10.16	12.01
5	17.40	16.35		21.61
6	9.06	36.15	3.58	21.93
7	1.03	.84	5.27	10.95
8	16.23	.98	9.91	11.38
9	1.58	6.68	3.34	12.31
10	1.48	12.26	10.47	13.38
11	15.86	10.66	8.15	14.48
12	11.11	24.49	28.88	20.02
13	1.10	14.10	6.11	14.43
14	15.13	18.25	7.89	14.18
$\bar{x}$	9.41	13.54	8.50	15.50
σ	6.04	9.02	4.36	3.80
S.E.M.	1.67	2.50	1.26	1.05

$\bar{x}$ = mean

σ = standard deviation

S.E.M. = standard error of the mean

Figure 1

The "t" test was applied to the values for each possible pair of experimental fractions (McNemar, 1949). The probability that the difference in value between each pair of fractions is due to chance was found from the "t" value on a table compiled by Treolar (1951). The following Table III summarizes these tests for significance.

Table III

A COMPARISON OF PENTOBARBITAL SODIUM CONTENT
IN THE CELLULAR FRACTIONS OF RAT BRAIN

Cellular Debris	Nuclei	Mitochondria	Supernatant	t	P
X	X			1.093	>.281
X		X		.375	>.68
X			X	3.20	.004
	X	X		1.65	>.102
	X		X	.75	>.431
		X	X	3.42	.002

From Table III there is seen to be a statistically significant difference between the values for the concentration of barbiturate in the cellular debris versus the supernatant ($P = .004$) and the mitochondria versus the supernatant ($P = .002$).

V. DISCUSSION

Studies of subcellular structure and function are still in their infancy (Schneider and Hogeboom, 1956) and methods available are far from ideal.

Anderson (1954, 1955, 1956) has done a series of studies on isolated cell components including the effects of pH, density, viscosity, and composition of suspending medium, as well as methods of preparing homogenates. Anderson (1955) also studied the effects of centrifugation artefacts and has devised a stroboscopic illumination method for obtaining very accurate centrifuge speeds.

In the work reported here, the efficiency of the separation of the cellular components is not great. Due to the very nature of the principle of differential centrifugation, however, a perfect separation would be impossible (De Duve and Berthet, 1954). The separation of fractions may also be influenced by the addition of a drug in large quantities or addition of a substance having a very large molecular weight (Reiner and Gellhorn, 1956), thus increasing the density of the particles to which it might be bound.

Aldridge (1957) compared liver and brain mitochondria from rats. This study showed that brain mitochondria were less stable than liver mitochondria.

Aldridge (1957) states that it is very difficult to obtain a "pure" preparation of mitochondria from brain and attributes discrepancies in published information on brain mitochondria to this fact. According to Brody and Bain (1952), with studies on rats and rabbits, the sizes and shapes of the intracellular particles vary to a much greater degree than they do in liver. From this fact it would be expected that the fractions would not be as uniform or "pure" as those obtained from liver.

All fractions retained some of the sucrose homogenizing medium. It was not known whether or not this retention was proportional to the amount of sample. It might be expected that mitochondria, for example, would retain much more since these particles are capable of expanding to many times their normal size (Siekevitz, 1957). It has been shown by Jackson and Pace (1956), however, that the rate of diffusion of sucrose into mitochondria was considerably slower than diffusion of sucrose into a sphere of water of the same size. It was also shown by Jackson and Pace (1956) that sodium and potassium enter the mitochondria more rapidly than sucrose. Entrance of all three of these substances occurs until an equilibrium concentration is reached.

All tissues show some absorption at all wavelengths, thus making control values necessary for finding

the true relationships between "blank" and barbiturate-containing tissue. Blank curves are especially desirable if the tissue to be examined contains a high percentage of fat (Goldbaum, 1956). Brain has a high lipid content--12 to 15 per cent in fresh adult human brain (West and Todd, 1955). It is interesting to note (Table I) that the blank values vary for the different fractions. Goldbaum (1956) found an optical density value of .12 at 255 millimicrons for "blank" rabbit brain tissue.

Chloroform is used as a solvent in barbiturate extractions from blood and from most tissues, but ether or ethylene dichloride is preferable for brain because emulsions with interfering substances do not give as much trouble (Goldbaum, 1956).

Sulphonamides and salicylic acid are known to interfere with barbiturate determinations (Dybing, 1955), but neither of these occur normally and would not be expected as a source of error in this study.

An apparent difference was found among all the cellular fractions isolated. The supernatant had the highest concentration of pentobarbital sodium and the mitochondria least, with cellular debris and nuclei second and third, respectively (Table II).

Since the microsomal fraction was not isolated, these particles would be expected to appear in the super-

natant.

Koppanyi, Dille, and Krop (1934) noted that the barbiturates were bound to lecithin in the brain, making analysis by their method difficult. Assuming that lipids do hold pentobarbital sodium, it would be expected that the cellular debris fractions would contain a high concentration of the drug since it seemed to be very fatty. If the barbiturate adheres to the cellular membrane where it seems to have an effect in decreasing permeability of the membrane (Sollman, 1957), then too, a higher concentration would be expected in the cellular debris fraction which would contain the cell membranes as well as some unbroken cells.

The effect of barbiturates is in uncoupling the oxidation-phosphorylation mechanism according to Brody and Bain (1951). Since the enzymes for oxidative phosphorylation are located primarily in the mitochondria (Schneider and Hogeboom, 1956), it might be expected that a high concentration of barbiturate be found in that fraction.

There is a possibility that barbiturates may be bound to lipids. As lipids have a specific gravity less than 1 (Kleiner, 1951) and consequently are less susceptible to sedimentation, they may occur in greatest concentration in the supernatant fractions. This then may be related to the fact that the concentration of pentobarbital

is highest in the supernatant fraction. Also, because the supernatant fraction is probably diluted with the sucrose solution to a considerably greater degree than are any of the other fractions, the concentration of barbiturates in the ground substance of the cell, and in this case, the microsomes also, is actually greater than the values indicate.

Recent investigations on binding and distribution of other ions and substances using similar methods have appeared in the literature. For example, Reiner and Gellhorn (1956) studied the binding of quinaesine by liver cell constituents in tissue homogenates, by isolated nuclei, mitochondria, and cytoplasm. It was found that distribution among cell constituents was similar when the drug was administered in vivo and when added to homogenates. From their results it was concluded that numerous cell constituents are capable of binding quinaesine and that no single chemical entity is responsible for the binding. Griswold and Pace (1956) studied the intracellular distribution of metal ions in liver tissue. Sodium was found in greatest concentration in the supernatant, least in mitochondria, with microsomes and nuclei ranking second and third. In studies of the intracellular distribution of catecholamines in the brain (Weil-Malherbe and Bone, 1957), adrenaline and hydroxytyramine were found in greatest concentration in the

supernatant. Mitochondria were not greatly smaller in value, however, and were the highest in localization of noradrenaline.

Further work would seem desirable to relate the concentration of pentobarbital sodium in the cellular fractions to various depths of anesthesia. It would also be interesting to investigate the intracellular distribution of the drug in the various anatomical divisions of the brain.

Work should also be done with thiopental, the sulfur analog of pentobarbital, to investigate its intracellular distribution at various time intervals.

VI. CONCLUSIONS

1. After 20 minutes of anesthesia at a dose of 40 micrograms of pentobarbital sodium per gram of body weight, this barbiturate was found in varying concentrations in the cellular debris, nuclei, mitochondria, and supernatant fractions of rat brain cells.
2. There is a significant difference ($P = .004$) between the pentobarbital content of the supernatant fraction (mean = 15.50 micrograms/gram) and the content of the cellular debris fraction (mean = 9.41 micrograms/gram).
3. There is a significant difference ($P = .002$) between the pentobarbital content of the supernatant fraction (mean = 15.50 micrograms/gram) and the content of the mitochondrial fraction (mean = 8.50 micrograms/gram).
4. There is no significant difference between the pentobarbital content of the cellular debris and the nuclear fraction ($P > .281$), cellular debris and mitochondrial fraction ($P > .68$), nuclear and mitochondrial fraction ($P > .102$), and between the nuclear and supernatant fraction ($P > .431$).

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