A CORRELATIVE STUDY OF PENTOBARBITAL DISTRIBUTION IN DOGS UTILIZING SELECTED TISSUES AND BODY FLUIDS.

by

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A THESIS

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Dedicated to Marthel

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INTRODUCTION

Barbiturate poisoning has become a problem of such magnitude that it is second only to carbon monoxide poisoning[1] as a toxicological cause of death in the United States. As Goldstein[2] has aptly pointed out "while the trend in the United States is lower for suicides and fatal poisoning by all solid and liquid poisons, suicides and fatal poisoning by barbiturates are increasing."

Prior to the early 1930's reports regarding barbiturate overdosage were relatively infrequent[3]. However, with the increasing clinical use of these drugs[2] and the continual introduction of more potent barbiturates[4], innumerable publications regarding their toxicity have appeared[6-26].

The increasing incidence of barbiturate overdosage as a medical problem has necessitated closer attention to diagnostic and therapeutic procedures. The development of more specific techniques for toxicological analysis[27] has been an important factor in stimulating further research in this field. Of particular importance has been the attention paid to gaining more precise information as to the fate and distribution of the barbiturates within the body.

The importance of knowledge regarding the fate and distribution of the barbiturates within the body was clearly demonstrated to this writer during the time he assisted in performing toxicological analyses for the Multnomah County Coroner's Office. A survey of the toxicological

analyses done on this service during the past six-year period showed that approximately one-half of all cases analyzed disclosed the presence of barbiturates of one form or another. In many instances more accurate information could have been provided had reliable data and knowledge of the distribution and time-rate of removal of the barbiturates been available. It was because of these factors and the awareness of the inadequacy of information regarding this particular subject that this study on comparative barbiturate distribution was undertaken.

A. HISTORY OF THE BARBITURATES.

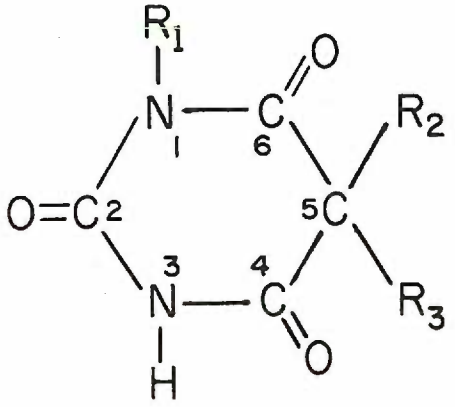
The parent compound of the barbiturate group, di-ethyl barbituric acid (Barbital U.S.P.; Veronal(R)) (figure 1), was introduced into medical usage in 1903 by von Mering and Fischer(28). This was followed, in 1911, by Hoerlein who developed phenyl-ethyl barbituric acid (phenobarbital)(29). By further modification of the side chain at the carbon five position ethyl-1-methyl-butyl barbituric acid (pentobarbital) was synthesized in 1916(30). In 1921 Shonle and Moment(31), after testing a large group of barbituric acid derivatives concluded that ethyl-isoamyl barbituric acid (amobarbital) had clinical merit. Since then hundreds of barbituric acid derivatives(29) have been studied, many introduced for clinical trial, but only a score or so now retained for clinical use.

Initially the barbiturates were only administered in oral form. In 1927 R. Bumm introduced the first intravenous anesthetic named Pernoston(R) (29). This stimulated research into the further possibilities of the barbiturates as intravenous anesthetic agents.

Figure 1

Basic structural formula for the barbituric acid nucleus. Note that in the basic nucleus substitution occurs primarily at the Carbon number 5 location. Also, substitution occasionally occurs at Nitrogen number 1. The following are the substitutions made for the barbiturates mentioned in this report:

U.S.P. Name	R_1	R_2	R_3
1. Barbital	H-	C_2H_5-	C_2H_5-
2. Phenobarbital	H-	C_2H_5-	
3. Pentobarbital	H-	C_2H_5-	$CH_3-CH_2-CH_2-CH(CH_3)-$
4. Amobarbital	H-	C_2H_5-	$CH_3-CH(CH_3)-CH_2-CH_2-$
5. Hexobarbital	CH_3-	CH_3-	


R1
N1
C6
O
R2
O=C2
5C
3
N
H
4
C
O
R3

Many of the early results were discouraging, for the barbiturates then used were all of the long or intermediate duration of action type and gave rise to many unwanted after-effects. In 1930-31, both Fitch et al and Lundy reintroduced pentobarbital sodium as an intravenous hypnotic (29, 30). In the following year came the discovery of Beta-N-methyl-5, 5'-methyl-phenyl barbituric acid (hexobarbital)(29), and with it the first satisfactory intravenous agent of the barbiturate class. This discovery also ushered in a new group of barbiturates, namely the so-called ultra-short acting class.

While there is widespread clinical application of the ultra-short barbituric acid derivatives in the field of anesthesiology, these particular agents do not represent a problem to the toxicologist since their route of administration confines them to hospital usage. Thus, the very infrequent death attributable to them is usually because of hospital accident.

B. METHODS OF BARBITURATE IDENTIFICATION.

1. Early Methods of Isolation and Identification.

During this same period of time (1903-1930), only one report(32) regarding barbiturate distribution appeared in the literature. The paucity of information, according to Koppanyi and co-workers(33), was due to inadequate methods of quantitatively measuring the drug once it was isolated.

Several methods have been devised for isolation and identification of barbituric acid derivatives in biological material. One of the first was the modified Stas-Otto procedure(34) which used organic solvents for separation and a very crude colorimetric means of

detection. This was soon replaced with a method introduced by Koppanyi, Murphy and Krop[35] which utilized the formation of a cobalt complex with the barbituric or thio-barbituric acid as the means of identification. This represented a modification of Zwikker's[36] method of 1931, the final result being determined colorimetrically. However, such methods, though still being used[39, 38], have the disadvantage of low specificity and sensitivity[39, 40]. It has also been demonstrated by Iverson[41] and by Riley et al[42] that "false-positive" reactions may occur due to impurities present in the biological extracts used as the initial sample specimen. Even with attempts to remove such impurities, as by the application of aluminum or charcoal[43], many interfering substances still persist and continue to give positive color reactions.

Of the many methods used at the present time, such as: 1. - chemical[37-38]; 2. - titration[44, 45]; 3. - x-ray[46-48]; 4. - infra-red[49-50]; 5. - paper chromatography[51-56]; and, 6. - ultra-violet spectrophotometry[57-65], the last two have gained the greatest popularity because of their sensitivity and specificity.

2. Use of the Ultraviolet Absorption Method.

The ultraviolet absorption characteristics of the 5,5 substituted barbiturates (figure 1) have been well established. This characteristic was first demonstrated in 1926 by MacBeth et al[66], and later in 1928 by Castille and Ruppol[67], and again in the early 1940's by Stuckey[68-70]. However, it was not until the introduction by Hellman[65], in 1943, of a method for the identification and quantitative estimation of the ultra-short acting thiopental that any

practical use had been made of such a physical property as the ultraviolet absorption band characteristic for the barbiturates. In 1946 Jailer and Goldbaum[64] introduced a modification of Hellman's procedure and in 1948 Goldbaum[60] and Walker, Fisher, and McHugh[58], introduced methods to be used for barbiturates other than the ultra-short acting type.

Since then many modifications of the ultraviolet absorption method have been introduced all of which utilize the basic characteristic of the barbiturates. This is the occurrence of an absorption band in the ultraviolet spectrum with a maximum density at 240 millimicrons or 255 millimicrons in alkaline media, depending upon whether the pH is 9.5 to 10.0[59] or 11.0[60], and a maximum density at 220 millimicrons in acid media[60]. Lous[59] has shown that the intensity of absorption can then be measured quantitatively and used to determine the concentration of barbiturate present in the sample under investigation by utilizing the Bouguer-Beer equation (figure 2).

However, as Raventos[43] has pointed out the ultraviolet spectrum of all barbituric and thiobarbituric acids, and some of their metabolites, is identical as long as their ring structure remains intact. Thus, although the sensitivity of the method is the best now available[43], its specificity still leaves much to be desired.

3. Paper Chromatography Methods.

In an attempt to overcome this lack of specificity, paper chromatographic separation was introduced by Raventos[71] in 1946. By this procedure he was able to remove many of the impurities

THE BOUGUER—BEER EQUATION

(For determination of the concentration of barbiturate present in a sample using the intensity of absorption as measured by ultraviolet spectrophotometry.)

Bouger—Beer equation:

$$c = \frac{\log 10 \ 1/Ti}{kb}$$

c = concentration

Ti = transmittance (I/Io)

k = extinction coefficient

b = the thickness of the layer of the absorbing solution

Bouguer's Law - Each layer of equal thickness absorbs an equal fraction of the radiant energy which traverses it.

Beer's Law - The absorptive capacity is directly proportional to the concentration of the solute.

(Excerpt from Mellon, M. G. Analytical Absorption Spectroascopy. John Weley & Son, New York, 1950.)

FIGURE 2

present in biological extracts used for analysis, and in addition was able to separate barbituric and thio-barbituric acids. An extension of this principle was utilised by Bush, Butler, and Dickison[72] in 1953 with the application of a silica column to separate metabolites of hexobarbital. But with the attempt to gain specificity, it was soon discovered that sensitivity had to be compromised; unfortunately, most procedures which employ chromatographic separation require a concentration of at least 50 micrograms before a positive result can be obtained[43]. Thus, it was not until the introduction of a combined paper chromatographic-ultraviolet spectrophotometric method by McBay and Algeri[51], in 1954, that sensitivity and specificity were combined into a one-stage procedure.

C. PHARMACOLOGICAL ASPECTS OF THE BARBITURATES.

1. Absorption of the Barbiturates.

The major gastrointestinal absorption, according to Weese[73], occurs in the stomach. Most investigators feel that the rectal administration is far more efficient than oral, apparently since the drug does not pass immediately to the liver[74]. This experimental observation has been substantiated by Werner, Pratt and Tatum[75] who showed that the oral versus intravenous LD_{50} ratio in rabbits given sodium pentobarbital (Nembutal) was in the ratio of 6 to 1, while the rectal versus intravenous LD_{50} was only 1.4 to 1.

Mulinos[76] demonstrated that it requires between one and two hours following the subcutaneous injection of amobarbital before anesthesia was obtained in animals. However, when amobarbital was

given by the intraperitoneal route anesthesia occurred within twenty to thirty minutes. The latter time interval represented an average since at any given time the individual animal's response to a given dosage of amobarbital varied. Page and Corylloís[77], after investigating the intravenous administration of amobarbital in animals, concluded that intravenous administration was the best route. However, these workers pointed out that very little difference could be demonstrated between the depth of anesthesia with either intravenous or intraperitoneal injection except for the more rapid onset of anesthesia with intravenous administration.

2. Distribution of the Barbiturates.

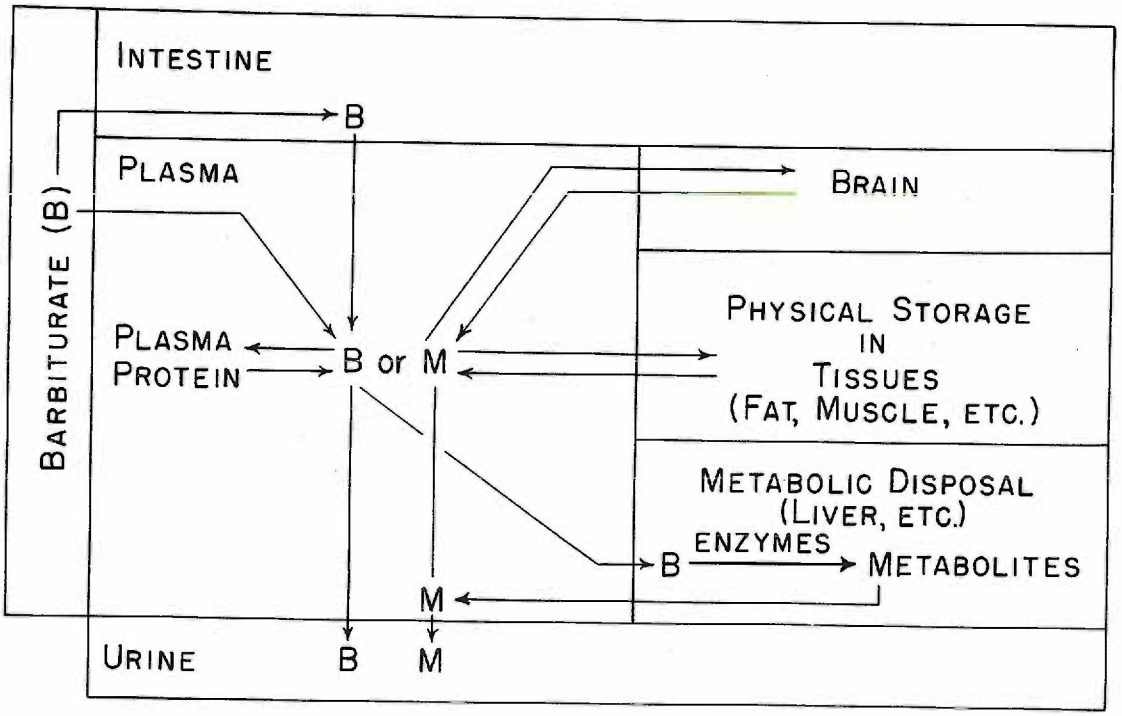
With the development of more specific methods for identifying and quantitating the amount of barbiturate present in biological tissue and body fluids, reports regarding distribution within the body soon followed. For example, Maynert[78] found that the biological distribution of a drug (referring to the barbiturates) was determined by two factors. One was the capacity of the drug to permeate the capillary and cellular membrane. The other was rather loosely referred to as a "binding" factor and included protein binding, localization in fat and binding to other cellular elements such as nucleic acids. Toman and Taylor[79] have graphically portrayed the general scheme of barbituric distribution within the body (figure 3).

a. Distribution of the barbiturates in body fluids.

In man the maximum drug concentration in the blood occurs immediately after injection[43, 80, 81, 82]. Following a single intravenous injection of a short-acting barbiturate, the rapid

Figure 3

A generalized, schematic diagram of the major pathways of the distribution and excretion of a barbiturate and its metabolite: B = barbiturate; M = metabolite. Arrows indicate direction of movement (adapted from Toman and Taylor[79]).


INTESTINE
B
PLASMA
BRAIN
BARBITURATE (B)
PHYSICAL STORAGE
IN
TISSUES
(FAT, MUSCLE, ETC.)
PLASMA
PROTEIN
B or M
METABOLIC DISPOSAL
(LIVER, ETC.)
ENZYMES
B
METABOLITES
M
URINE
B
M

decline in blood barbiturate concentration over the first twenty to thirty minutes is assumed to represent tissue distribution. The second and more gradual fall represents the rate of metabolism (43, 80, 83). This same pattern has been observed in animals given pentobarbital, by Brodie et al(84, 85), Kahn(86), Axelrod(87), and Taylor et al(88). Further evidence to the effect that the decline noted in the first fifteen to thirty minute period following a single parenteral injection was due simply to distribution and did not represent metabolism was offered by Kozelka et al(81). These workers were able to recover almost all of the administered pentobarbital unchanged, fifteen minutes after intravenous administration to rats. However, in cases where the pentobarbital was given by continuous infusion, it was found that a much slower rate of drug-blood level decline was noted. The slower rate so observed was probably more closely representative of the metabolic rate(84).

(1) Blood and Plasma Distribution.

Raventos(43) found that the actual barbiturate concentration within the tissues was a function of the blood concentration, and that following the initial distribution period a direct proportionality existed between the two concentrations. This observation was further substantiated by Kahn(86) in his work with rats given pentobarbital tagged with C-14. However, neither of the above authors reported the cyclic fluctuations in pentobarbital blood content which were observed by Anderson and Essex(89).

In Goldbaum's(60) report on rabbits given various

barbiturates, including pentobarbital, it was concluded that the plasma level was approximately equal to whole blood content. While Fretwurst and Voss[90] concluded that the erythrocytes, in cases of human poisoning, contained virtually no barbiturate.

Relative to some of the factors influencing barbiturate distribution within the intravascular compartment, Klimesch[91] and Starkenstein and Klimesch[92] concluded that in the case of those barbiturates producing immediate narcosis, the lipid-soluble free barbituric acid was relatively more abundant in the plasma. However, Bush[93], after estimating the ratio of free acid to sodium salt, was unable to substantiate this finding.

Bennhold[94] demonstrated that binding of Evipal(R) by plasma albumin and globulin occurred during intravascular transportation, while Brodie[85] showed that 55 per cent of pentobarbital was bound to plasma protein. Of the seventeen barbiturates studied by Goldbaum and Smith[95], all showed some plasma binding, with the percentage for pentobarbital being 50 per cent. The shorter acting barbiturates were more strongly bound than the long acting barbiturates, and such binding was reversible and dependent upon both the concentration of the albumin and of the drug in the ultrafiltrate. Taylor et al[96] further demonstrated that the concentration of unbound drug in the ultrafiltrate represented physiologically active barbiturate since, twenty-four hours after bilateral nephrectomy, rabbits given barbiturates slept longer and awoke at a higher total plasma level than controls without nephrectomy. Interestingly

enough, analysis of both groups showed no significant difference in the plasma ultra-filtrate barbiturate concentration. The importance of this last mentioned finding cannot be stressed too much and will be discussed in light of the present work later.

(2) Cerebrospinal Fluid Distribution.

The concentration of barbiturate in the cerebrospinal fluid has been the subject of many conflicting reports[74]. Kozelka and Tatum[97], using the copper-complex colorimetric method of detection, recovered less than 0.1 milligram of barbiturate per animal eight hours after intravenous injection. The same authors reported a barbiturate content of 0.2 milligrams per cent in cerebrospinal fluid, thirty to forty-five minutes after ingestion of phenobarbital by humans. However, Purves-Stewart and Willcox[98], while reporting barbiturate concentrations as high as 7.7 and 9.0 milligrams per cent in patients sampled sixteen to thirty-five hours after oral administration of the drug, were forced to conclude that they could find no correlation between dosage and amount of barbital present in the cerebrospinal fluid of the patients and animals in their series. These results differ from Fretwurst and Voss[90] who found very little change between plasma concentration and the concentration of barbiturate present in cerebrospinal fluid. On the other hand, Vogt[99] found consistently less barbiturate in cerebrospinal fluid than in any part of the brain.

Recently Brodie et al[84] reported that the concentration of thiopental within the cerebrospinal fluid was 20 per cent lower

than the amount present in the plasma, provided a fifteen minute distribution interval was allowed between injection and sampling. Further, these workers noted that this was a linear relationship which continues throughout the remainder of the animal's anesthesia. Lous[100], using ultra-violet spectrophotometry as a means of detection and quantitation, correlated the barbiturate concentration of the serum with the amount present in the cerebrospinal fluid, both samples being drawn simultaneously. From the seventeen humans so sampled, he reported 52.9 ± 4.2 per cent as much phenobarbital present in cerebrospinal fluid as present in serum. Of greater importance was his observation that the amount of barbiturate present in the plasma ultra-filtrate and that in the cerebrospinal fluid were quantitatively equal.

b. Distribution of the barbiturates in body tissues.

That the drug is distributed throughout the body tissues as well as body fluids after introduction is a well-accepted fact (43, 74, 78, 80). Barbital has been recovered from gastric and pancreatic secretions[101] and in fetal tissues[102, 103] indicating transportation through secretory tissue. Phenobarbital has been isolated from human milk[104], as have other barbiturates[105]. In Pucher's[32] report of 1925, tissue analysis of a person dying of barbiturate overdosage disclosed detectible drug in the kidney, brain, liver and spleen. A similar finding was reported by Andrews and Neubeuer[106].

Experimental studies designed to delineate barbiturate distribution in the body were first introduced by Koppanyi and

Dille[107] in 1934. However, it was not until Kozelka and his colleagues[81, 97] applied gravimetric analysis to the problem that reliable quantitative data regarding barbiturate distribution became available[74].

The distribution of pentobarbital has been studied by Goldbaum [60], using rabbits, and by Brodie et al[85] using dogs. Since the results of Brodie and his co-workers are the yardstick by which this writer measured his experimental results, a tabulation of their work was included in detail (table I, figure 4). Also, the results of Kahn[86] on the distribution of radioactive pentobarbital in rats has been included, where appropriate, in the section entitled "Discussion".

Other reports regarding the distribution of pentobarbital in tissues are those of Dille, Linegar, and Koppanyi[82]; Koppanyi and Dille[108]; and Koppanyi, Linegar, and Dille[109]. However, Maynert and Van Dyke[74] consider these results as unreliable due to the inadequacy of the methods employed.

In another series of experiments, Brodie and his co-workers[110], using dogs, compared the tissue distribution of pentobarbital with that of the ultra-short acting barbituric acid derivatives. After evaluating the results, they were unable to demonstrate any preferential distribution for pentobarbital within the body fat, a finding which is so characteristic for the ultra-short acting barbituric acid derivatives[84]. The question of the influence of fat on pentobarbital's action has been further studied by Stavinoha and Davis[111] who demonstrated that the sleeping time in rats fed

TABLE I

Distribution of Pentobarbital in a Dog.

Tissue Analyzed	Conc. of Pentobarbital mg./kg.
1. Plasma	34.4
2. Plasma water	18.7
3. Cerebrospinal fluid	18.2
4. Red cells	36.0
5. Liver	64.4
6. Brain	42.3
7. Muscle	27.5
8. Kidney	45.8
9. Heart	38.4
10. Lung	20.8
11. Spleen	41.4
12. Lumbodorsal fat	37.3

The above studies were made 3 hours after the intravenous administration of 0.43 gram of the drug to a dog weighing 10.7 kilograms. The pentobarbital concentration in the fatty tissue is expressed in terms of total lipid content of the tissue.

(From an article entitled "The Fate of Pentobarbital in Man and Dog." A method for its estimation in biological material by Brodie, B.B., Burno, J.J., Mark, L.D., Lief, P.A., Bernstein, E., and Papper, E.M. The Journal of Pharmacology and Experimental Therapeutics, 109: 26-34, 1953.

FIGURE 4

corn oil prior to receiving pentobarbital was not reduced; whereas when the same animals were given an ultra-short acting barbiturate, thiamylal, after receiving corn oil, the sleeping time was markedly reduced.

3. Correlation between pharmacological action of barbiturates and the blood concentration.

The possibility of a correlation between depth of anesthesia produced by the short acting barbiturates and their concentration in the blood has been subject to much debate. Carrington and Raventos[83] and Peterson et al[112] feel that such a correlation does exist. They are supported by the work of Tatum, Nelson and Kozelka[113] who found in rabbits given amobarbital that while a wide difference in duration of anesthesia was encountered, the righting reflex returned at approximately the same blood concentration (2.9 milligrams per cent) in all animals.

Contrary to the views of Carrington and Raventos[83] and Peterson et al[112], Helldorf[114] states that no close parallelism exists between clinical effect and concentration of barbiturate in the blood. In partial agreement with Helldorf are Maynert and Van Dyke[74] who state "except for barbital which probably undergoes no degradation in the body, the relationship of estimated blood level to pharmacological effect has to be accepted with caution since most methods do not convincingly distinguish between the administered barbiturate and the metabolically altered derivatives which, at present, appear also to be barbiturates. Even if levels in the blood were accurately determined, their correlation with pharmacological

response is best for short or very short acting drugs and poorest for a long acting barbiturate such as barbital."

Dille et al[82] lends support to this latter contention since they have shown that after giving sodium barbital by mouth to a series of patients, the blood content fell from 39 to 25 milligrams per cent without obtaining any anesthesia; however, when anesthesia did occur in this series of observations a concentration of only 12 to 15 milligrams per cent was registered. In another case which they report it was found that a blood level of 8.4 milligrams per cent was present during coma due to overdosage with barbital, while the post-coma level was 7.6 milligrams per cent. Thus, such findings were interpreted as having no correlation. It is to be noted that Dille and his co-workers employed the colorimetric method of Koppanyi[35] which has been subject to much criticism regarding its validity as a quantitative method of barbiturate determination.

Taylor and Richards[80] have tabulated all the available data pertaining to the awakening barbiturate-blood level in animals. From these data the material pertinent to pentobarbital usage in dogs has been extracted and appears in Table II (figure 5). In this same article there appears data regarding the time-rate of barbiturate removal in experimental animals; this data was also tabulated and is presented in Table III (figure 5).

From the vast amount of experimental evidence collected by Taylor and Richards[80], they concluded that the plasma level at the time of awakening for the short acting barbituric acid derivatives was quite constant for each animal group. They also felt that the

TABLE II

Barbiturate Plasma Level at Awakening - Single Dose
(adapted from Richards and Taylor[80]).

Barbiturate	Species	Dose mgm./Kg.	Route	No. of Subjects	Plasma Level at Awakening microgm./ml.
Pento-	Dog	20	I.V.	6	15 ± 2.0
barbital	Dog	25	I.V.	6	17 ± 2.0

TABLE III

Rate of Removal of Barbiturates from Bodies of Different Species
(adapted from Richards and Taylor[80]).

Barbiturate	Species	No. of Subjects	Dose mgm./Kg.	Rate % last/hr.
Pento-	Dog	6	25	15
barbital	Dog	2	30	31.5
	Dog	4	?	15

FIGURE 5

plasma value found at the time of awakening was not dependent upon the dosage of barbiturate administered. This was probably the most important conclusion drawn by these authors.

Butler[115], on the other hand, felt that the depth of anesthesia was more closely correlated with the concentration and rate of penetration of the barbiturate into the central nervous system. Furthermore, Brodie[116] demonstrated that this correlation was influenced by previous sensitivity to the drug and physical factors such as the water/lipoid coefficient. Lamson, Grieg, and Robbins[117], have shown that glucose infusion and other factors related to carbohydrate metabolism will render a person more susceptible to the anesthetic actions of the barbiturates. Other factors which affect such a correlation are as follows: temperature, potentiating drugs, metabolic inhibitors, age, weight and sex. These and other factors concerned with barbiturate concentration in the central nervous system are excellently covered in the review article by Richards and Taylor[80].

D. METABOLISM OF THE BARBITURATES.

Along with numerous researches dealing with the distribution and concentration of the barbiturates in various body tissues, attention has been given to the manner in which these drugs are detoxified and excreted from the body. For this report, due to the great wealth of material which has been accumulated during the past twenty years dealing with the modes of metabolism and the various metabolites of the barbiturates, only that information pertaining to pentobarbital will be included. For more comprehensive surveys of this subject the reader is

referred to the excellent reviews by Maynert and Van Dyke[74], Raventos [43], and Richards & Taylor[80].

According to Maynert and Van Dyke[74], the body possesses two methods for removing the barbiturates: (1) Destruction or chemical alteration, principally in the liver, and (2) Excretion through the kidneys. Raventos[43] further attempts to classify the chemical reactions which may occur in the metabolism of barbiturates as follows:

a = oxidation of radicals in position 5 with the formation of keto, hydroxy and carboxy barbituric acid,

b = loss of N-alkyl radicals,

c = desulphuration of thiobarbiturates,

and d = hydrolytic opening of the barbiturate ring.

Of the above, a. represents the most important of the processes for barbiturate inactivation.

That very little unchanged pentobarbital is excreted in the urine has been demonstrated by Herwick[118, 119], Shonle et al[120], Brundage and Gruber[121], and Van Dyke et al[122]. However, the contention of Shonle and his co-workers that destruction of pentobarbital occurred primarily by hydrolytic cleavage was shown to be incorrect by Van Dyke, Scudi and Tabern[122]. These authors found that less than 8 per cent of the N^{15} isotope administered as nitrogen tagged pentobarbital was excreted as ammonia and urea.

The first metabolite of pentobarbital was isolated by Maynert and Van Dyke[123]. After extensive chemical purification and physical

analysis, it was concluded that the isolated compound was 5-ethyl, 5(3-hydroxy-1-methyl-butyl) barbituric acid (figure 6). This metabolite was without hypnotic powers when injected into mice. Barris and Magoun[124] reported the presence of a reducing substance in urine following pentobarbital injection; however, identification was not completed. Roth and his co-workers[125], using filter-paper chromatography, located five radioactive metabolites after administering pentobarbital tagged with carbon-14. None of the metabolites so isolated represented urea. These results were substantiated by Kahn[126].

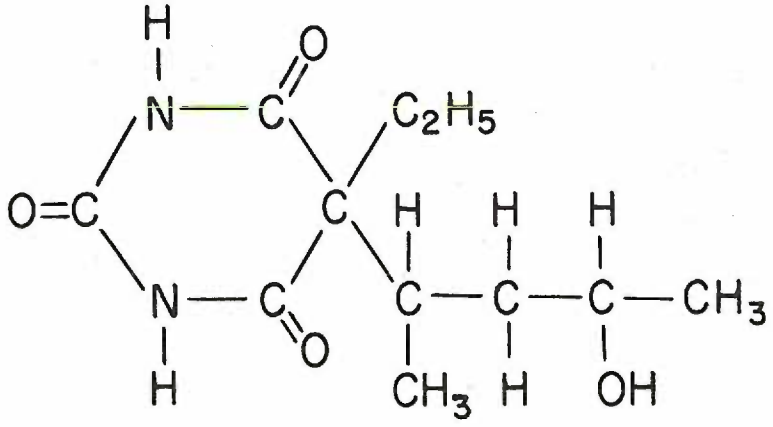
The work of Maynert and Van Dyke[123] was further pursued by Maynert and Dawson[127] who, after giving pentobarbital to dogs by the oral route, were able to isolate two urinary metabolites. Both were found to be the same hydroxy metabolite as previously reported by Maynert and Van Dyke[123]; however, each metabolite showed different optical rotation. Of the total administered pentobarbital, these authors were able to recover 45 per cent as the afore mentioned urinary metabolite, 33 to 36 per cent as the dextro-hydroxy metabolite and 15 per cent as the levo-hydroxy metabolite.

Brodie and his colleagues[85], in a human study using orally administered pentobarbital, were able to isolate a urinary metabolite identical with the hydroxy derivative of Maynert and Dawson. However, this metabolite accounted for only 15 per cent of the total administered dose.

Algeri and McBay[56] were able to demonstrate three zones of apparent pentobarbital metabolite following paper chromatographic

Figure 6

A pictorial representation of the structural formula of 5-ethyl-5(3-hydroxy-1-methyl-butyl) barbituric acid, the urinary metabolite of pentobarbital isolated by Maynert and Van Dyke[123].


H
N
O
C
C2H5
O=C
C
H
H
H
N
C
O
C
C
C
CH3
H
CH3
H
OH

separation of the urine of dogs given pentobarbital. The Rf* zones were located at 0.46, 0.35, and 0.08. They concluded that Rf 0.46 represented the diastereoisomers reported by Maynert and Dawson[127]. The Rf 0.08 zone was felt to represent a different metabolite of pentobarbital, while no conclusions regarding the Rf 0.35 zone were possible.

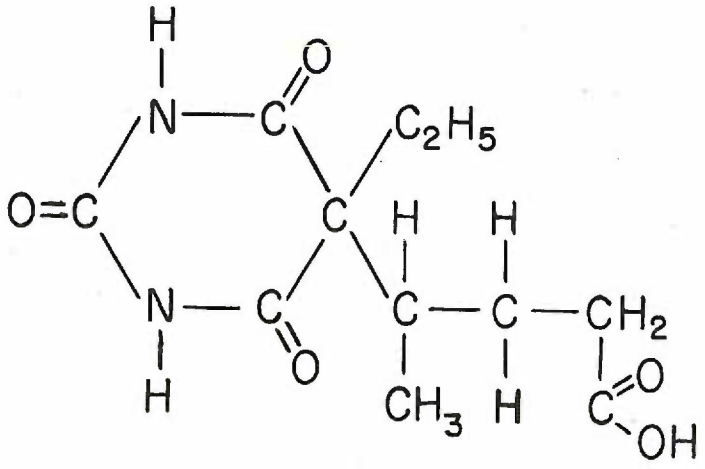
This work has been further investigated by Titus and Weiss[128]. In their work paper chromatographic separation of C-14 tagged pentobarbital breakdown products revealed nine urinary metabolites. Two of these metabolites were the diastereoisomers reported by Maynert and Dawson[127]. A third was isolated and found to be 5-ethyl-5 (1-methyl-3-carboxy-propyl) barbituric acid (figure 7) and probably corresponds to the ether-extractable acidic metabolite of Rf 0.08 which Algeri and McBay[56] reported.

Recently Winters et al[129] have shown that minced rat liver can, in vitro, desulfurate thiopental to produce pentobarbital. While in vitro metabolism of pentobarbital has been demonstrated using liver, skeletal muscle, brain, intestine and kidney[78].

*Rf - is the mathematic representation of the location of the unknown compound on the chromatogram and is compiled by dividing the movement of the zone by the movement of the solvent surface, both expressed in centimeters.

Figure 7

A pictorial representation of the structural formula of 5-ethyl-5-(1-methyl-3-carboxy propyl) barbituric acid, the urinary metabolite of pentobarbital isolated by Titus and Weiss[128].


H
N
C
O
C2H5
O=C
C
H
H
N
C
O
C
C
CH2
H
CH3
H
C
O
OH

PURPOSE OF PRESENT INVESTIGATION

The present study was undertaken in an attempt to correlate the distribution of pentobarbital in selected tissues of dogs with the amount present in the whole blood samples. Furthermore, the distribution within the component parts of whole blood was investigated since much of the present literature reports the barbiturate concentration in serum[15, 16, 17, 18, 130, 131, 132] rather than the whole blood content.

The availability of comparative information is meager[25, 80, 133], yet there is an ever-increasing volume of reports regarding blood-barbiturate concentrations sufficient to be lethal[20, 21, 22, 25, 133]. It was, therefore, the hope of this writer that a correlation might exist between the concentration of barbiturate in blood and the amount present in other body tissues or fluids. If such were true it would allow one to utilize the data relating to lethal barbiturate blood concentrations when only the tissue content was available.

A further extension of this work incorporated the principle set forth by Sunshine and Hackett[133] in their article on "half-life" of phenobarbital blood levels. This was an attempt to correlate clinical symptoms, phenobarbital blood levels, and rate of metabolism into one complete concept. It was found that a reduction of half of the phenobarbital blood concentration would occur in a 3-day period. This occurred regardless of the 3-day sampling period chosen after giving the drug. The writer has therefore attempted to determine how rapidly

pentobarbital and its metabolites were lost from the animals whole blood as measured by ultraviolet spectrophotometry.

METHODS AND MATERIALS

A. Animals.

The selection of animals was carried out in such a manner as to obtain near equal sex distribution. The animals' weights ranged from 7.0 kilograms to 29.0 kilograms, the majority being between 10 to 20 kilograms. The strain of dog used for these studies was not considered as important since past experience failed to disclose any marked variation with regard to response to the barbiturates. A few of the animals included in this series were used and studied in conjunction with the group in our department performing surgically induced cardiac valvular lesions. These particular animals all received anesthetic doses of pentobarbital by the intraperitoneal route. The data obtained from these animals was used only for determining drug recovery and estimating the blood-barbiturate anesthetic concentration.

The total number of dogs involved in the project was 38. This includes 6 animals which were used in cooperation with the afore mentioned workers. All of the data computed from the entire series of animals is included in Tables IV-A and IV-B. Not all animals were used to gain the same data, and some dogs were used more than once to determine if any change in response to pentobarbital had occurred. The complete sex and weight distribution of these dogs is shown in Table V.

B. Drug Administration.

The barbiturate selected for study was pentobarbital sodium. The preparation used was dispensed in 100 ml. vials with a concentration of 50 mgm./ml. and contained in a buffered solution adequate to allow

either intravenous or intraperitoneal application.* Also, a powdered preparation was obtained for oral administration. The intraperitoneal route was used in most cases, since anesthesia was induced almost as rapidly as by the intravenous route and drug administration was much easier with the former route. Three animals were selected for the oral route, the powder being mixed in hamburger and fed in the morning while in the fasting state.

Animals which received pentobarbital by the intravenous and intraperitoneal route were available for sampling 15 to 20 minutes after injection. Between the time of anesthesia induction and the first sampling, the animals were placed on the animal board and a femoral cut-down was performed. The femoral vein was isolated and cannulated with polyethylene tubing connected to an 18 gauge needle. The needle, in turn, was attached to a 3-way syringe outlet, all metal parts having been pre-treated with silicon to prevent any hemolysis. The animal's tongue was withdrawn to maintain an adequate airway.

C. Specimen Procedure and Preparation.

The selection of samples was handled in such a way as to give as much comparative information as possible. In addition, a source previously unreported, the bone marrow, was utilized. The introduction of bone marrow as a tissue source was an attempt to overcome a

*Abbott Laboratories preparation of Nembutal(R) was used in all experiments. An analysis of a lot specimen of this preparation was performed and demonstrated a concentration of 49.2 mgm./ml.

deficiency noted while performing toxicological analyses in medico-legal cases where the suspected tissues were found some days to weeks after death and were unsuitable for routine analysis. Under these conditions bone marrow seemed to offer the best source for drug analysis, if such was present, since it was relatively unaffected by the autolysis which occurs following death. Also, marrow should not be subject to the degenerative effects of micro-organisms, macro-organisms or the elements.

The following specimens were included in this survey: whole blood, plasma, erythrocytes, serum, clots, cerebrospinal fluid, liver, stomach contents and bone marrow. The work on clotted blood offers a particularily interesting facet of investigation in that such a specimen can be screened for the presence of drug, even after the administration of ultra-violet interfering substances such as embalming fluid.

1. Blood and its Component Parts.

a. Whole Blood.

Samples were drawn at either half-hourly or hourly intervals following the initial barbiturate injection. The amount withdrawn was 10 mls. except in the cases where numerous serial samples were to be drawn and then the amount was reduced to 5 mls.

A 10 ml. syringe was attached to the three-way system previously described and 10 mls. of venous blood was allowed to gradually fill the syringe, a minimum of pressure being applied to further reduce the chance of hemolysis. The blood so obtained was then transferred to an oxalated test tube to serve as the whole

blood specimen for use in the barbiturate extraction procedure. Two mls. of whole blood were set aside to be used for analysis, the remaining 8 mls. placed in a Wintrobe hematocrit tube and centrifuged at 4200 rpm. for twenty minutes. At the end of the first twenty-minute period, the respective red cell and plasma volumes were recorded. The tube was again centrifuged for twenty minutes, this procedure being repeated until the packed red cell volume remained constant. Once this value became stable the venous hematocrit was recorded for the particular sample being investigated.

b. Plasma.

Two mls. of plasma, for analysis, were obtained by removing the plasma layer with a micropipette after completion of the hematocrit determination. The red cell specimen was then procured by reintroducing the micropipette at the phase layer to remove any remaining plasma. This procedure usually resulted in the sacrifice of a small portion of the red cell layer in order to be confident that all the plasma had been removed.

c. Erythrocytes.

The usual amount of red blood cells selected for analysis was 2 mls. When the cells were analysed before and after washing the sample amount was reduced to 1 ml. due to the volumetric limitations. Red cell washing was carried out in the hematocrit tube, after removing the first 1 ml. sample.

The volume of physiologic saline used for cellular washing was twice the packed red blood cell volume. Washings were

repeated three times and carried out by slowly rotating the hematocrit tube between the palms of the hands. After two minutes of rotation, the tube was again centrifuged for fifteen minutes. The resultant upper aqueous phase was then withdrawn by the micropipette and deposited in a clean test tube.

Since later determinations showed that the first washing was strongly positive for barbiturates and the third containing only a trace, all three washings were pooled to obtain the average amount of barbiturate per ml. of washing. The pooling was taken into account when calculating the amount of barbiturates in these washings. The remaining washed erythrocytes were also analyzed.

d. Serum and Clotted Blood.

Another blood component selected for investigation was the sera from a blood clot as well as the clot itself. In those animals receiving fatal doses of barbiturate, clots were removed from the ventricular cavity of the heart and analyzed. The serum was obtained by allowing 10 mls. of venous blood to clot. The barbiturate was then extracted from the serum in the same manner as plasma or whole blood. When the clot was used for analysis it was broken up with a glass stirring rod and a volume of 2 to 4 mls. of macerated clot used for extraction. When the clot could not be completely disintegrated by simple mechanical means, the entire clot was then placed in a graduate cylinder containing fifty to one hundred milliliters of chloroform, depending upon clot size, and the clot's volume obtained by measuring the displacement of the chloroform. The clot and chloroform solution were

transferred to a beaker, warmed slightly, and stirred until even distribution of the clot throughout the chloroform was obtained. The chloroform layer was permitted to settle out and removed for analysis. The remaining fibrin material was then washed several times with two or three 25 ml. volumes of chloroform to remove any residual drug. Since chloroform was used both as the initial extracting agent and for washing, the chloroform gain from the entire procedure was pooled and reduced, by evaporation, to a 25 or 50 ml. volume. The remaining steps of analysis (Table VI) were then carried out on this chloroform fraction.

2. Cerebrospinal Fluid.

In the experiments terminated by the dog's death, cerebrospinal fluid was obtained and analyzed. Cistern puncture, at the base of the occiput, was the technique employed in collecting cerebrospinal fluid samples. By such a maneuver it was possible to gain access to the cisterna magna and, by attaching a syringe to the puncture needle, to withdraw a sufficient amount of cerebrospinal fluid for analysis of its barbiturate content.

3. Liver.

Most of the compiled data regarding the concentration of barbiturate in the body has been based upon analyses using portions of liver tissue. Also, liver tissue represents the specimen most commonly used in toxicological analysis. Hence, in order to correlate the amount of barbiturate found in the blood with the data offered in past experimental research, it was necessary to include analysis of liver specimens. The liver from a deceased dog was removed, in toto,

and weighed. Two or three representative sections of approximately 20 grams each were removed. The individual sample was then trimmed until it's weight was exactly 20 grams. Homogenation of the specimen in a Waring blender, a process which required from fifteen to twenty minutes was then performed. If a semi-liquid consistence could not be obtained, the material was treated with 40 mls. of physiologic saline.

Two mls. of undiluted homogenate was selected for sampling. In those instances where the material was not semi-liquid and dilution was necessary the liver homogenate was washed three times with 40 mls. of physiological saline. Each washing was then separated from the liver tissue by centrifugation. The resultant washings were pooled for analysis. The decision to wash only three times was based on the data collected previously which demonstrated that the fourth washing, in most cases, failed to contain detectible amounts of drug. From the pooled liver washings 6 mls. of extract was used as the specimen size.

An alternate procedure used to test the validity and accuracy of the above method of liver extraction was microhomogenation of small amounts of liver tissue. The resultant sample was analyzed and compared with the recovery of barbiturate obtained by the Waring blender method. While there was slightly greater recovery of barbiturate by the microhomogenation method the difference was not sufficient to warrant the use of this more tedious procedure.

With liver derived from human sources, and used for toxicological analysis, the presence of fat in the liver homog nates often

makes extraction difficult. The presence of fat constitutes a serious problem during extraction since the barbiturates are quite soluble in lipoid material. No such interference was detected in the liver samples taken from the animals.

4. Bone Marrow.

So far as can be determined bone marrow has not been previously used as a speciman for toxicological analysis. Thus, this tissue is being reported for the first time as a speciman source for such investigation. The reason for the selection of bone marrow as a speciman source was because of two cases where it was necessary to perform toxicological analysis on decomposed bodies. In both cases the body had been exposed to the elements with bacterial, autolytic and insect decomposition taking place. The problem was to detect drug in decomposed tissue. It was suggested that bone marrow might be the ideal source since it was reasonably well protected from such forms of decomposition. However, since no previous work had been reported concerning the presence of drug within marrow, the idea was temporarily abandoned.

With the advent of the present project, it was decided to include marrow as one of the specimens to be investigated. Initially it was hoped that several dogs could be fatally poisoned with pentobarbital and then exposed to the elements for six weeks to three months and then analyzed for barbiturate. However, due to the inability to locate a suitable place for such exposure, the idea was modified to include only that bone marrow which was available following acute poisoning of the animal.

Several methods for obtaining bone marrow samples were tried. The first utilized a 4 x 1 cm. piece of sternum and/or rib being macerated with a steel pestle and mortar and then extracted with 25 to 50 mls. of chloroform. Due to the semi-liquid nature of the marrow, such a procedure was found to be technically impractical. The second procedure attempted was removal of a femur from 2 dogs, one of which weighed 12 kilograms and the other 18 kilograms. Both ends of the femur were sawed off leaving a mid-shaft portion of approximately 7 to 8 cm. in length. Using a thirty milliliter syringe, fitted with a 15-G needle, the entire bone marrow cavity was then flushed with physiologic saline. The latter method proved to be satisfactory; however, quantitation by this crude procedure was only grossly possible and, at present, would not appear applicable for medico-legal purposes.

The marrow extract so obtained was then further extracted with chloroform and analyzed. Obtaining suitable extracts from bone marrow presented the same problem of high lipoid content as that encountered with the extraction of liver tissue in human beings. In this study the problem was solved by repeated chloroform washings of such fat.

5. Gastric Contents.

The gastric contents of 2 dogs given sodium pentobarbital orally and 5 receiving the drug intraperitoneally or intravenously were collected and analyzed. Specimens were withdrawn by stomach tube at periods of one, two, and three hours (Table IV-B) after drug administration. In those cases where the dog died, the gastric

contents were procured at autopsy. Analyses of the gastric contents obtained from the two orally fed animals were inconsistent probably because the drug was mixed with hamburger and the material retained for some time in the dog's stomach. This made analyses and estimation of the precise amount of drug retained in the stomach difficult. In fact, only 2 of the 10 animals receiving oral pentobarbital obtained a sufficient barbiturate concentration to cause anesthesia. Another reason for these inconsistent results may be that not all of the gastric content, procured from the anesthetized animal by stomach tube, was withdrawn.

D. Method Used For Extraction of Pentobarbital from Specimens.

Since main interest centered in recovering and establishing quantitative amounts of pentobarbital in the various samples, the chromatographic separation method was not utilized in the animal data which compose the results of this study. While the possibility of incorporating the combined chromatographic-spectrophotometric method of Algeri and McBay[51] was considered, this was not feasible due to the specialized apparatus required.

The considerations involved in the selection of the method of extraction utilized are enumerated below:

(1) The Brackett and Bradford[57] procedure was representative of the most widely accepted method for barbiturate determination.

(2) The Department of Pharmacology has had approximately six years of experience with this particular method.

(3) Very precise quantitation of the amount of barbiturate

present in biological material can be gained by this procedure.

(4) Much of the published data utilized in comparison with the present results were obtained using similar spectrophotometric procedures.

(5) The extraction procedure used represented a clinically applicable method. If the experimental data so collected were to serve a useful purpose, it had to be correlated with, and correspond to, the clinical observations obtained in humans.

(6) The procedure selected incorporated all of the important aspects of barbiturate extraction which have appeared in the research and clinical literature[58-60, 114].

As pointed out above the basic scheme of extraction used was that described by Brackett and Bradford[57]. Certain modifications in the procedure were introduced as pointed out later. The Brackett and Bradford extraction procedure, in its entirety, provides three fractions for final analysis, the acid, basic and neutral. However, since the barbiturates are extracted and retained in the acid fraction only, it was not necessary to carry out steps to obtain the two other fractions.

The reagents used, apparatus required and procedure followed in this modified Brackett and Bradford extraction procedure are described in Table VI.

E. Spectrophotometric Analysis of Extracted Samples.

The Beckman Model D.U. Spectrophotometer was used for analysis of the extracts. The ultraviolet accessory set of this instrument

provides 1 cm. quartz cells which are matched as to their transmission qualities. Suitable correction curves using distilled water for the determinations were calculated. This correction was then utilized when computing the amount of drug present.

The actual technic used in operating this instrument is as follows: Before commencing the spectrophotometer analysis, the sensitivity switch of the instrument was rotated three turns counter-clockwise from the clockwise stop and retained in this position throughout the remainder of the analysis. The samples to be analyzed were then placed in individual cuvette containers and inserted into the apparatus. The "dark" current dial then being zeroed to the null point.* Following this the wave length dial was set as 220 millimicrons and the blank cuvette then centered in the light path with the shutter switch positioned to "on". The slit adjustment was then used to re-zero the indicator to the null point. After correcting for the reagent density, by the above procedure, the cuvette containing the unknown sample was alined in the light path and the density dial used to re-zero the indicator to the null point. The density of the unknown specimen was then recorded. The data obtained for each specified wave length was then transferred to graph paper so that a curve could be plotted. The initial reading was taken at 220

*The dark current dial is used to balance out the current passing through the phototube and other circuit components when the phototube is not exposed to light. Zeroing to the null point is done to insure accuracy of measurements taken after phototube is exposed to light pathway.

millimicrons and each subsequent readings at increments of 4 millimicron until 236 millimicrons was reached. From this point readings were taken at each millimicron until 244 millimicrons was reached. Beyond 244 millimicrons readings were again taken at each 4 millimicron increment up to 260 millimicrons and then every 5 millimicron increment until a reading of 300 millimicrons was reached. The initial reading of 220 millimicrons was selected because readings below this wave length are of doubtful reliability.

The unknown samples so analyzed had a pH of 9.5. Once the readings of these samples, at a pH of 9.5, had been recorded, the blank and the unknown samples were then treated with sufficient 0.5N HCl (usually 0.15 ml.) to obtain a pH of 1.5. The fractions with the adjusted pH of 1.5 were then replaced in the spectrophotometer and the density at 240 millimicrons was recorded. The reading so obtained represented the background interference of all substances other than barbiturates[59], and was used in the calculation of barbiturate content.

F. Methods Employed in Calculating Results.

Since each sample varied with dosage of barbiturate used and time taken, a common denominator was necessary in order to allow both comparison and statistical analysis of the various results. To obtain such a standard value it was decided that the drug level of the material to which the comparison was being made should be considered as 100 per cent. The comparison could then be computed according to the following master formulae:

X = material being compared.

Y = material to which comparison is being made.

$$\% \text{ in } X = \frac{\text{pentobarbital concentration in } X}{\text{pentobarbital concentration in } Y}$$

A more detailed explanation of each particular computation used for the various specimens analyzed in this study follows:

1. Calculation of Whole Blood - Plasma - Erythrocyte Relationship.

To obtain the per cent of pentobarbital in the plasma as compared to whole blood, the following formula was used:

$$\% \text{ in Plasma} = \frac{\text{Plasma concentration/ml. x Plasma Hematocrit}}{\text{Whole Blood concentration/ml.}} \times 100$$

Similarly, to compute the per cent pentobarbital in the erythrocyte fraction as compared to whole blood, the following formula was employed:

$$\% \text{ in RBC} = \frac{\text{RBC concentration/ml. x RBC Hct.}}{\text{Whole Blood concentration/ml.}} \times 100$$

However, in order to obtain a 100 per cent total from the plasma percentage plus the erythrocyte percentage, it was necessary to correct the per cent values for each fraction so that they add up to 100 per cent by using the following relationship:

$$\frac{\text{\% in Plasma}}{\text{Sum of \% in Plasma and RBC}} = \frac{\text{Corrected \% in Plasma}}{100\%}$$

The corrected per cent in plasma is then subtracted from 100 per cent to gain the corrected percentage in the erythrocyte.

The theoretical whole blood pentobarbital concentration was computed by using the following formula:

$$\text{Conct. pentobarb. in whole blood} = \frac{\text{plasma conct.}}{\text{ml.}} \times \text{plasma Hct.} + \frac{\text{RBC Conct.}}{\text{ml.}} \times \text{RBC Hct.}$$

This value was then compared with the calculated whole blood level by using the Dixon Sign Test.*

The results of these calculations are recorded in Table VIII-A.

When statistically analyzing the per cent of pentobarbital present in plasma as compared to erythrocyte content, the actual calculated percentages were utilized, rather than the corrected percentages.

2. Calculation of whole blood - serum relationship:

The raw data expresses the amount of pentobarbital detected as gamma/milliliter (u Gm./ml.). In order to determine if a relationship exists between these two, it was felt that the whole blood

*Dixon Sign Test for paired observations:

H: $X = X^1$

$(X_1 - X^1)\ (X_2 - X_2^1) \ldots X_n - X_n^1$

X = computed whole blood levels.
X^1 = theoretical whole blood levels.
n = # of paired observations.
r = X of times the less frequent sign occurs.

concentration should be considered as representing the 100 per cent value. The serum percentage was then computed using the following formula:

$$\% \text{ in serum} = \frac{\text{pentobarbital concentration in serum (uGm/ml)}}{\text{pentobarbital concentration in w. blood (uGm/ml)}} \times 100$$

The results are tabulated in Table IX-B.

3. Calculation of whole blood - clot relationship:

As with previous calculations, where the data compiled were to be correlated with whole blood results, a percentage value was computed by considering the whole blood value as equal to 100 per cent. Calculation of the percentage relationship between clot and whole blood pentobarbital concentration employed the following formula:

$$\% \text{ in clot} = \frac{\text{pentobarbital concentration in clot}}{\text{pentobarbital concentration in w. blood}} \times 100$$

The results are tabulated in Table IX-C.

4. Comparison of the barbiturate content of washed erythrocytes and saline washings to the amount present in unwashed red blood cells:

The per cent comparison was calculated in the same way as for previous specimens, except here the pentobarbital content of the unwashed erythrocyte sample was assigned the 100 per cent value. The following formulae were utilized:

$$\% \text{ in washed cells} = \frac{\text{pentobarb. conc't. in wash cells}}{\text{pentobarb. conc't in RBC}} \times 100$$

$$\% \text{ in saline washings} = \frac{\text{pentobarb. conc't in sal. wash} \times 3}{\text{pentobarbital concentration in RBC}} \times 100$$

Since washing resulted in a dilution of three times the original specimen amount (the raw data being expressed as a u Gm./ml. of saline washing) the concentration so computed was multiplied by 3 in order to obtain an equivalent basis for comparison.

The results of these calculations are presented in Table IX-D.

5. Comparison of liver, cerebrospinal fluid, and bone marrow pentobarbital concentration to that present in whole blood:

Here again the barbiturate content of whole blood was chosen to represent 100 per cent. Comparative percentage values for liver, cerebrospinal fluid and bone marrow were then computed in accordance with the master formula discussed at the beginning of this section.

6. Computation of Pentobarbital Loss Due to Partitioning of Specimen Samples.

a. Whole blood concentration versus plasma and erythrocyte content.

Recovery data was computed to evaluate the amount of extracted pentobarbital lost by separating the whole blood into its major components, plasma and erythrocytes. The actual calculation involved the following:

$$\frac{\text{Theoretical W. Blood /ml.}}{\text{Calculated W. Blood /ml.}} \times 100 = \% \text{ recovery}$$

The results for these determinations are tabulated in Table IX-A.

b. Washed red cells plus saline washing.

Similarly the recovery for washed red cells plus saline washings, when compared to unprocessed red cells, was computed by using the same technique:

$$\frac{\text{Saline washings /ml.} + \text{Washed red cells /ml.}}{\text{Unwashed red cells /ml.}} \times 100 = \% \text{ recovery}$$

The results are presented in Table IX-D.

7. Time rate of pentobarbital disappearance from whole blood.

To obtain a base line for comparison, due to individual animal variation, the whole blood pentobarbital concentration present in the initial sample was assumed to be 100 per cent. The other sample percentages were then computed according to the following scheme:

X = W. Blood sample concentration to be determined.

Y = Initial W. Blood concentration (at 45 minute sampling time).

$\frac{X}{Y} \times 100 = \%$

The data obtained by these computations were then recorded in Table X.

RESULTS

In this research a total of 40 observations were made on 38 animals with two dogs (No. 7 and No. 20) each subjected to two separate analyses (Table IV-A and IV-B). Thirty-two animals were used exclusively for this project, while analyses were performed on six dogs used primarily by another research group in this department attempting to induce cardiac lesions in the animals. On these six dogs the only studies performed were those dealing with red cell-plasma pentobarbital distribution and per cent recovery. The limitations imposed on the latter animal group were necessary because the initial anesthetic dose of pentobarbital required supplementation during the sampling period, due to the prolonged nature of the surgery being attempted.

The analytical results of the entire series were divided into related groups with many animals being utilized for more than one purpose. As previously stated, the initial goal of this work was to correlate barbiturate blood concentration with tissue concentration. However, as the experiment proceeded, many other valuable observations were gained. In order to adequately present each of these observations they have been divided into six categories as follows:

A. Barbiturate whole blood concentration present at time of arousal:

These observations were obtained using 21 animals, all but 3 being anesthesized for periods longer than 3 hours (180 minutes) The end point selected for arousal was the first sign of awareness of the

animal to its surroundings. Initially it was hoped that recovery of the deep tendon reflexes could be employed as one of the criteria for arousal, but this response was too varied to be used as a suitable index. Analysis of the blood at arousal time gave maximal post-anesthetic levels since the measurement was performed on blood drawn at the time when the animal first demonstrates objective signs of emerging from the effects of the anesthesia. Moreover, awakening time was opportune for sampling since the dogs were not subject to the peripheral sensory stimulation accompanying instrumentation as they had been previously prepared and maintained in a quiet environment throughout the entire period of sampling. The blood-barbiturate concentration for each animal at the time of arousal is recorded in Table VII.

The average awakening pentobarbital concentration was 16.43 ± 2.3 gamma per milliliter. However, since the route of drug administration varied, a further presentation of the results has been made according to whether the drug was given intraperitoneally, intravenously or orally. Statistical analysis of the drug concentrations for these three groups showed those dogs receiving intraperitoneal pentobarbital to have an awakening pentobarbital blood concentration of 17.4 ± 1.6 gamma per milliliter, while those given the drug intravenously awoke at an average value of 15.7 ± 2.8 gamma per milliliter. The three animals given oral pentobarbital awoke, on the average, at a blood barbiturate concentration of 13.6 ± 0.6 gamma per milliliter.

B. Correlation between injected dose of pentobarbital and the whole blood barbiturate concentration:

The injected dose of pentobarbital ranged from 25 milligrams per kilogram to 95 milligrams per kilogram with a range of ± 2.5 milligrams per kilogram allowed at each dosage selected for evaluation (Table VIII-A). Samples were taken at 30 minute intervals with the first sample procured 30 minutes after injection. The time of the final sample was dependent upon either the length of anesthesia or when the animal expired.

The failure to obtain complete serial data for all of the 16 animals in this particular set of observations was due to: (1) the variation of individual animal response for the same time-dosage interval; (2) improperly timed sampling on the part of the observer; and, (3) inability to give fixed and definite anesthetic producing amounts of pentobarbital to all dogs each time since the drug was administered in a milligram per kilogram dosage.

From the data accumulated on this particular subject (Table VIII-A), it is obvious that the only two dosage ranges where an adequate number of observations were available for statistical evaluation were the 30.0 ± 2.5 mgm./kg. and the 37.5 ± 2.5 mgm./kg. dosages. The remainder of the values are included for completeness only. No inference can be made from the remainder of these observations since they do not represent a large enough group to be experimentally valid.

The correlation between injected dose and pentobarbital blood concentration, over a specific time interval, for the animals which received either 30 ± 2.5 mgm. of pentobarbital per kilogram body weight or 37.5 ± 2.5 mgm. of pentobarbital per kilogram body weight are tabulated in table VIII-B. All the pentobarbital concentrations which

correspond to the time and dosage range desired have been included and do not necessarily represent the serial samples for one particular animal. Only those animals which received one dose of pentobarbital for anesthesia were included thus eliminating from consideration any animal which required additional doses to maintain anesthesia. Definite correlation between dosage and blood concentration was not obtained and in all probability these results merely represent variations in individual animal response and metabolism of the pentobarbital.

C. Distribution of Pentobarbital in various tissue and body fluids:

In this study comparison was made between the quantity of barbiturate found in the tissue to that present in whole blood except in cases where blood was not applicable and then the appropriate body fluid or tissue was substituted. The following relationships were studied:

1. Whole blood versus plasma-red cell distribution.

This data was compiled in an attempt to show equal distribution between the red cell mass and plasma. In Table IX-A the findings related to this particular distribution are tabulated. When the Dixon Sign test (figure 8) was applied to the paired observations, i.e. the measured and theoretical whole blood levels, and the null hypothesis applied, it was found that there was a probability of greater than 0.25 that these findings came from the same population. Such a test was valid since the only assumption used in computing theoretical whole blood concentration was that the barbiturate distribution between plasma and red blood cells would be modified by the hematocrit value. Once the theoretical whole blood value had

FIGURE 8

Dixon Sign Test:

X = computed whole blood barbiturate levels.

X^1 = theoretical whole blood barbiturate level.

n = number of paired observations.

r = number of times the less frequent sign occurs.

From Table VIII-A:

n = 144 paired observations.

r = 69 (-)

From "Introduction to Statistical Analysis" by W. J. Dixon and F. J. Massey, the following formula is taken which allows one to estimate the "r" value for a given "n" value, when "n" is greater than 90. The answer so obtained is the nearest interger less than the one gained when the formula is solved.

$(n-1)/2 = k\sqrt{n+1}$ when k = 0.5752 for .25

$143/2 = 0.5752\sqrt{145}$

$71.5 = 0.5752\ (12.04)$

$71.5 = 6.94 = 63.56$

r = 63 when n = 144

Therefore, the probability is greater than .25 that these data come from the same population.

been computed, with due consideration given to the hematocrit reading, comparison could be made to determine whether or not the theoretical and calculated whole blood concentrations were of the same population. If they were then equal drug distribution must exist between the red cell and plasma since the only allowance for variation in distribution considered was the hematocrit reading.

The net difference between the 143 comparisons performed on the calculated whole blood concentration versus theoretical whole blood concentration was +25.23 micrograms per milliliter.

2. Whole blood versus serum.

Nineteen observations were included in this series with 13 animals being sampled. Table IX-B tabulates the individual observations. When comparison to whole blood, obtained at the same sampling time, was performed it became apparent that serum contained, on the average, approximately four-fifths as much pentobarbital (82.6 + 24.6%), milliliter for milliliter, as did the whole blood.

In four dogs (Dogs No. 9, 10, 23 and 26) more than one serum determination was possible. When the sera from dogs No. 23 and No. 26 were evaluated no correlation of the serial samples could be obtained. However, the results of the determinations performed on dogs No. 9 and No. 10 showed reasonable correlation with almost equal distribution. The interval of time between the initial dosage and sampling did not seem to influence the correlation.

The statistical 95 per cent range of comparative pentobarbital percentage present in serum for the entire group was from 34.4 to 130.8 with the group average being 82.6 per cent.

This series was further divided into two rather distinct groupings. The first contained 10 animals and demonstrated an average value of 99.5 per cent with a standard deviation of ± 6.2 per cent. The second was composed of 7 animals showing an average of 53.9 per cent with a standard deviation of ± 9.7 per cent.

3. Whole blood versus clot.

Nine animals were included in this group with twelve samples procured. The average concentration of pentobarbital present in the clots was 92.1 per cent of that found in whole blood sample drawn simultaneously (Table IX-C). The standard deviation of this group was ± 12.0 per cent. It is interesting to note that except for animals No. 21 and No. 30 (samples 3 and 4), the results were grouped in two localized areas. Four samples were in the 83 to 89 per cent range and five samples were in the 97 to 109 per cent range.

4. Erythrocyte, washed cell-saline washing comparison:

A total of 15 animals were included with all but 3 being sampled more than one time. Of the 39 samples acquired, all demonstrated detectible pentobarbital in the unwashed erythrocytes and the saline washings of the red blood cells. However, after washing, only 9 of the 39 erythrocyte samples were positive for pentobarbital, with the other 30 failing to show any barbiturate present. The amount of pentobarbital present in the saline washings was 84.2 per cent ± 31.6 per cent of that present in the unwashed blood cells.

Per cent recovery, based on the total percentage of pentobarbital found to be present in both washed red blood cells and saline washings, when compared to the amount recovered in the unwashed red

blood cells, gave an average of 99.3 per cent. The 95% range of recovery values extended from 79.8 to 115.8 per cent, the standard deviation being ± 9.2 per cent.

5. Comparison of whole blood, liver, cerebrospinal fluid, and bone marrow:

The individual results are tabulated in Table IX-E. From the results obtained a correlation between whole blood barbiturate content and the compared tissue values was found to exist, provided at least fifteen minutes was allowed between drug administration and sampling so that adequate tissue distribution could occur. The per cent of barbiturate concentration in the bone marrow was uniformly low and exceeded 10 per cent in only 2 of the 16 animals (Dogs No. 22 and No. 25) sampled. In all cases the per cent liver barbiturate concentration was higher than the per cent present in cerebrospinal fluid.

A total of 16 animals were sampled at autopsy with 20 liver samples, 17 cerebrospinal fluid samples, and 21 bone marrow samples obtained. Comparison of these three specimens to whole blood concentration will be considered individually:

a. Whole blood versus liver pentobarbital content:

On the average 90.5 per cent as much pentobarbital was present in 1 ml. of liver homogenate as in a comparable milliliter of whole blood. The 20 liver samples had a standard deviation of ± 8.0 per cent with a statistical 95 per cent range of 74.8 to 106.2 per cent.

b. Whole blood versus cerebrospinal fluid pentobarbital content:

Of the 17 samples obtained, it was computed that, on the average, only 64.2 per cent as much pentobarbital was present in 1 ml. of cerebrospinal fluid as was present in 1 ml. of whole blood sampled at the same time. The standard deviation was ± 4.9 per cent with a statistical 95 per cent range of 54.6 to 73.8 per cent.

c. Whole blood versus bone marrow pentobarbital content:

This group comprised 21 analyses. Statistical analysis of the results showed that only 6.1 per cent as much pentobarbital was present in 1 ml. of marrow extract as was present in 1 ml. of whole blood sampled simultaneously. The procedure used to obtain the marrow has already been outlined; however, 3 ml. was selected as the sample amount for analysis since each specimen of femur was flushed three times with 10 ml. of physiologic saline. From the 30 ml. of pooled marrow flushings, a 3 ml. sample was analyzed, i.e. 1 ml. for each of the three flushings. The final calculation then reduced the amount of pentobarbital present to that found in a 1 ml. sample of pooled marrow extract.

The standard deviation for pooled marrow extracts was ± 2.6 per cent with a statistical 95 per cent range of 1.0 to 11.2 per cent.

D. Per cent recovery:

Recovery data for pentobarbital was obtained for two different groups of observations, the first being the calculated whole blood level versus theoretical whole blood level. The average recovery

value for this comparison was 99.3 per cent, with a standard deviation of ± 10.8 per cent. The statistical ninety-five per cent range was 78.1 to 120.5 per cent.

The second group of recovery values were those of the washed erythrocytes and saline washings as compared to the amount present in the unwashed red cells. The results of the comparison demonstrated an average recovery of 97.8 per cent. The standard deviation was ± 9.2 per cent with a statistical ninety-five per cent range of 79.8 to 115.8 per cent.

E. Time-rate of pentobarbital removal from the blood.

Seven animals were selected which fulfilled the following criteria: (1) the anesthetic dose of pentobarbital was between 30 and 40 mgm./kg. body weight and given parenterally; and, (2) that the first blood sample had been drawn 45 minutes after pentobarbital administration, and further that at least two additional samples had been taken over the next two hour and fifteen minute period. Twenty-nine observations were carried out on these 7 animals, or an average of 4.14 observations per animal.

The graph which accompanies Table X was plotted using average pentobarbital percentage and statistical 95 per cent range for each sampling period. It is interesting to note that a steady decline in blood barbiturate concentration occurred, with a 50 per cent value being registered at approximately 100 minutes after initial sampling. The statistical 95 per cent range for the various averages overlaped only at the 150-180 minute sampling range, and then by only 2.2 per cent.

DISCUSSION

This investigation was undertaken to provide more information and data dealing with the distribution of one of the most widely used barbiturates, pentobarbital, in the body tissues after administration by several routes to dogs. It was also hoped that information could be provided with respect to any correlation existing between the concentrations of pentobarbital found in the blood and the various tissues examined. Other than a few reports dealing with the blood concentration of barbiturates in fatally poisoned humans[20, 21, 22, 25, 106, 133], and a still smaller group of publications[80, 85, 86, 133] which attempted to correlate blood barbiturate concentration with either tissue values or clinical response, no information of this type exists. Some work regarding correlation between Pentothal(R) blood concentration and tissue distribution has been performed[64, 72, 75, 80, 84]. However, such information was of little value when considering pentobarbital distribution since the two drugs are markedly different, not only in their rapidity and duration of action, but also in their fate and detoxification within the body.

The need for correlative data regarding barbiturate blood concentrations and the concentrations in several tissues such as the liver and brain determined at various intervals after administration is great. This information could serve as a helpful diagnostic and prognostic criterion for the clinician to establish the severity and duration of barbiturate poisoning and to follow the response to

treatment. It would also be of value to the toxicologist since it would allow usage of established lethal blood barbiturate concentrations[15-18, 130-132] when only the tissue values were available.

The phase of this research discussed first was the attempt to establish pentobarbital blood concentrations present at the time of the dog's arousal. Anesthesia, in this group of animals, was produced by giving pentobarbital in several dose ranges by either the oral, intravenous or intraperitoneal route.

This study was deemed of primary importance due to conflicting reports regarding the existence of such a correlation. As had been previously mentioned Carrington and Raventos[83], and Peterson et al[112] maintained that the correlation between blood barbiturate concentration and awakening time was present for both ultra-short and short-acting barbiturates. On the other hand Helldorf[114], and to a lesser extent Maynert and Van Dyke[74], felt that such a correlation did not exist. However, impressions appeared to be the sole support of the latter contention.

A. Correlation of arousal time pentobarbital blood concentrations.

Before discussing the results of this study and attempting to establish correlative data, several factors influencing the interpretation of these observations must be considered.

First, these blood levels represented the measured pentobarbital content at the time of awakening, a value which included both unmetabolized and metabolized drug.

Secondly, and probably of greater importance was the fact that preparations for obtaining blood samples intravenously were made

during the stage of deep anesthesia. Thus, when the dog regained consciousness much of the peripheral and visceral sensory stimulation accompanying intravenous puncture was lacking.

The composite pentobarbital concentration present in whole blood at time of arousal, computed to be 16.4 ± 2.3 gammas per milliliter, falls between the plasma values of 15 ± 2.0 gamma per milliliter and 17 ± 2.0 gamma per milliliter reported by Taylor et al[88] using 12 dogs given intravenous pentobarbital.

Since only 5 of the 21 animals reported in Table VII received pentobarbital intravenously, the animals were considered in three separate groups, according to route of drug administration.

The average concentration in whole blood for the group receiving pentobarbital by the intravenous route was 15.7 ± 2.8 micrograms per milliliter. This corresponded to the lesser figure just shown for Taylor and his co-workers[88]. The average of the group receiving the drug by intraperitoneal route was comparable to the greater concentration found by Taylor and co-workers.

The finding of 13.6 ± .6 micrograms per milliliter of pentobarbital in the blood of animals given the drug orally implied that a more prolonged effect was obtained, at least in animals, by this method of administration. Or, since a greater interval of time was necessary for absorption to occur, and since the drug entered the metabolic pathways at a somewhat reduced rate, the unmetabolized-metabolized pentobarbital ratio may not have been as great after oral administration as was present in animals who receive their drug by parenteral administration. The latter factor, if true, would allow a lower ultra-violet

measured pentobarbital blood concentration, i.e. pentobarbital plus its metabolites which show ultra-violet absorption, to be registered. However, the anesthetic potential (unmetabolized drug) of the pentobarbital concentration so measured would be equivalent to the higher values recorded in the injected group.

The average values computed for the intravenous and intraperitoneal arousal levels demonstrated a difference which was present regardless of initial dosage. Whether or not this represented a true difference was subject to question since the number of animals in the two groups were not equal and therefore not comparable. However, since this difference was less than that following oral dosage a better correlation apparently existed between the concentrations of blood pentobarbital found following intravenous-intraperitoneal injection.

With respect to the influence of increasing the dosage of pentobarbital to the blood concentrations found at time of arousal, the only consistent animal response was prolongation of the duration of anesthesia. The possibility of increasing dosage producing high measured pentobarbital levels at time of arousal was also investigated, but no correlation was found to be present. This conclusion was the same as presented by Taylor and Richards[80].

The results gained from study of the arousal-pentobarbital blood concentrations were so encouraging that an extension to include the possibility of a correlation between injected dosage and the resultant barbiturate blood concentration at various time intervals was undertaken. Although Helldorf[114] stated that there was no close parallelism between clinical effect and concentration of barbiturate

in the blood, he admitted that the possibility of a more consistent animal response did exist. With this in mind, the results shown in Table VIII-A were procured.

The wide range of pentobarbital blood concentrates found to be present at any one sampling time (Table VIII-A) indicated that Helldorf's[114] clinical findings could be extended to include data obtained using dogs. It was obvious that variable other than dosage enter into determining what would be the amount of drug present in the whole blood following any particular injected dose.

One variable that could influence the correlation between dosage and barbiturate concentration was the rate of absorption from the peritoneal surface following intreperitoneal injection. However, the group of dogs given intravenous administration showed an almost equal depression of values indicating that absorption played little, if any, part in the failure to obtain a correlation.

That the rate of absorption did not influence the dosage-blood concentration correlation was an important finding. In some pilot studies preceding this research, it was found that, on the average, it required 2 to 5 minutes to induce anesthesia in dogs when pentobarbital was administered intravenously. In the comparable group of dogs given pentobarbital intraperitoneally, it required approximately 20 minutes to gain the same effect. Since all other factors remained constant, it was felt that the fifteen minutes represented a difference in absorption.

In the same study it was evident that the animals which received intravenous pentobarbital awoke sooner than the comparable

intraperitoneal group, but no constant correlation could be recognized.

Another factor which could have accounted for an inability to correlate dosage with blood level was a cyclic fluctuation of blood-barbiturate concentration as observed by Anderson and Essex[89] in dogs given intravenous pentobarbital. While this observation has not been corroborated, it introduces an interesting concept.

The declining trend noted in the blood barbiturate concentration during the sampling period will be discussed when the time rate of barbiturate removal from the blood is considered.

In order to ascertain whether or not there existed an equal distribution of pentobarbital within the major whole blood components, i.e. plasma and erythrocytes, it was decided that the Dixon sign test would be valid for statistical analysis since the comparative information was derived from the same population. The null hypothesis, i.e. that both groups of observations came from the same population, was considered to be a valid approach since the computation of the amount present in the theoretical whole blood was based upon the assumption that a fifty-fifty distribution existed, with the only variable being the hematocrit reading.

At first it might appear that a comparison of plasma and red cell pentobarbital blood levels on a milliliter for milliliter basis would be more exact. However, it was obvious that statistical analysis was not applicable to these two materials since the end result would not take into consideration the whole blood value for which the comparison was intended.

A probability of greater than 0.25 was taken as evidence that

the initial hypothesis was true as stated. Due to the large sample size used, it could be concluded that the distribution within the red cell and plasma was one of equality. Furthermore, the hematocrit appeared to be the major distributing factor since its value determined the exact percentage-distribution present.

The consistence of hematocrit values during pentobarbital anesthesia has been well demonstrated by the work of Gilmore[134]. Since, in the present series, only slight fluctuations in hematocrit occurred with any given animal during a specific sampling time, this further indicated that the hematocrit was a rather consistent factor with regard to pentobarbital distribution in whole blood components.

The only available pentobarbital distribution data regarding plasma and red cells was published by Brodie et al[85], who reported a value of 34.4 milligrams per kilogram for plasma and 36.0 milligrams per kilogram for erythrocytes, which seemed to represent equal distribution. Yet, this report did not state the hematocrit value for the animal so analyzed. Because the hematocrit value was not stated, no conclusion could be derived regarding the question in point.

Goldbaum and Smith[95] concluded that a selective plasma-binding of barbiturate existed. However, they likewise did not include consideration of the hematocrit which, in the present study, appeared to be the primary factor in determining what the final percentage distribution will be.

However in 1948 Goldbaum[60] did show that the level of pentobarbital present in the plasma of rabbits was almost equivalent to

the level present in whole blood, an observation which has been substantiated in this series.

The failure of all previous observers to consider the hematocrit when computing barbiturate distribution within whole blood appears to be the basis for the erroneous conclusion that preferential plasma binding exists, at least in the case of pentobarbital sodium.

In the work of Fretwurst and Voss[90] it was concluded that in the human blood samples which they investigated, virtually none of the barbiturate was present in the erythrocytes. This was rather a surprising finding until one realizes that they "washed" all cells prior to analyzing an observation which will be discussed later in light of the present findings.

The value obtained in the comparison of serum-pentobarbital content to whole blood samples taken at the same time was difficult to understand since serum, per se, represents all the components of plasma except those utilized in "carrying out" clot formation. Yet the correlation for plasma-whole blood was much closer than that existing for serum-whole blood. The difference was especially prominent when one notes that the standard deviation in the serum series was 24.6 per cent with a statistical ninety-five per cent range of approximately a 100 percentage units.

Here again one can only theorize regarding the possible explanation for such a deviation. One possible explanation could be that certain components of serum enter into the clot formation which contains appreciable amounts of detectable barbiturate, and thus remove such from detection. However, analysis of the per cent values

gained show that of the 19 samples so investigated, 13 had values greater than the group average, 6 being greater than 100 per cent in value.

The possibility of serum trapping within the clot can be excluded as a variable since all samples were allowed to stand until clot retraction was complete before the serum samples were removed.

In conclusion one is left with an unexplanable variation in correlative whole blood-serum-pentobarbital levels since this relationship is apparently subject to variabilities which have escaped observation and understanding.

Later, an observation of some interest was made on this particular series of samples. It was noted that there were two rather distinct localizations of the values, except for the specimens from animals No. 28 and No. 20 (second run). Ten of the observations fell around the 100 per cent value, i.e. an average of 99.5 $\pm$ 6.2 per cent, while the remaining seven showed an average of 53.9 $\pm$ 9.7 per cent or almost one-half that of the first mentioned group. Careful recheck of the notes made during collection of these samples failed to show any reason for this finding. Such differences were suggestive of faulty extraction or failure to observe proper precautions during sample collection in the second group. However, such was not the case, for all samples were handled in the same way.

That we were measuring the same fluid would seem evident since no variable was selectively introduced in the second group; yet the results would seem to indicate that such exists. Whether this represents a variability in clot formation within this animal group is not known.

There is no available literature which reports whole blood-serum correlation, and thus it was impossible, at this time, to add further evidence regarding these observations.

A finding similar to that noted with the serum values was also found to be present in the clotted samples. The first localization of results included four samples and the second contained five. The remaining three specimens, i.e. dog No. 21 and dog No. 30 (samples 3 and 4) did not fall within these two groups. The first group had an average value of 84.2 ± 1.3 per cent, while the second group demonstrated an average value of 101 ± 3.4 per cent. The grouping noted in the clotted samples did not show the wide interval present in the observations on serum and probably represented an artifact rather than a true difference.

The average pentobarbital concentration in the animals where clotted blood was analyzed proved to be 92.1 ± 12.0 per cent as much as present in equivalent whole blood samples. Such a value shows a very good correlation between blood pentobarbital content and that present in the clot. As mentioned previously, the blood clot, as such, constitutes a particularly interesting sample source for toxicological analysis since it is less apt to be altered by ultra-violet interfering substances such as embalming fluid[135]. Also, it would appear to give values which are quite valid for medio-legal purposes.

The results obtained for the washed red blood cells and saline washings show excellent correlation. As mentioned in the section on Method, the washing of the red cell's was a delicate procedure. Due

to the possibility of traumatizing the red cells sufficiently to cause disruption, it was quite possible that inadequate washing could have accounted for the instances where pentobarbital was detected in the washed erythrocytes. In all other cases where red cells were washed, the lack of detectable pentobarbital allows one to reach the same conclusion as did Fretwurst and Voss[90], i.e., that erythrocytes contain little, if any, barbiturate after washing has been performed.

It was further felt that since washing removed the pentobarbital, one of two explanations were possible regarding the nature of the erythrocyte-pentobarbital combination. The first was that any barbiturate present was absorbed to the red cell surface without chemical binding. This probably represented a sample physical attraction since it could be removed by the process of washing.

The second was the possibility that the pentobarbital present in the unwashed, packed red cells actually represents trapped plasma. The latter was very unlikely since, for the procedure used for obtaining red cell-plasma separation, it had been demonstrated that only a small amount of plasma was retained in the packed cells[136].

The correlation between whole blood levels and liver levels was apparently independent of sampling time, provided at least fifteen to thirty minutes was allowed for complete tissue distribution to occur. However, the findings of 90.5 $\pm$ 8.0 per cent as much pentobarbital in the liver as in the whole blood were far different from the 190 per cent value as computed from Brodie *et al*[85] results (Table I, fig. 4). Much of this difference was based upon the units used in reporting pentobarbital, i.e. Brodie's being reported in

milligrams per kilogram tissue, whereas the present work was reported in gamma per milliliter of liver homogenate.

The reason for selecting such a unit in the present study was because liver analysis was performed after preparation of a semi-liquid homogenate. Homogenation was considered necessary to insure that adequate cellular destruction had occurred so that complete pentobarbital extraction could be obtained. A review of the experimental notes taken during the extraction of the liver samples showed that 20 grams of liver, when minced according to the procedure described under the section on Method, yielded a volume of from 19 to 23 milliliters. Thus, it was apparent that a milliliter of extract was approximately equal to one gram of liver tissue.

In the cases where a semi-fluid homogenate could not be procured, and an additional solvent was necessary (Dogs No. 22 and No. 23), no marked variation from the undiluted samples could be demonstrated.

From the results of the 19 animals included in this series, it appeared that liver values for pentobarbital were approximately 90 per cent that of whole blood values. Furthermore, such a relationship was linear and persisted, at least for nine hours, after introduction of the barbiturate into the animal body.

Maynert and Van Dyke[74] believed that the conflicting reports regarding barbiturate concentration in the cerebrospinal fluid were due to variations in the initial barbiturate dose and sampling time. In order to alleviate this situation, it was felt that a comparison to whole blood concentrations might remove these variables. This contention was strengthened by the work of Brodie and co-workers[84],

who demonstrated that a direct proportionality existed between plasma water (plasma ultrafiltrate) and the cerebrospinal fluid-barbiturate level, provided at least thirty minutes was allowed for the drug to cross the blood-brain barrier and reach the cistern. Furthermore, Louis[100] concluded that not only was there a direct proportionality between plasma-barbiturate level and cerebrospinal fluid-barbiturate level, but that the cerebrospinal fluid concentration and the amount present in plasma ultrafiltrate were quantitatively equal.

Statistical analysis of the comparative barbiturate concentrations present in seventeen plasma-cerebrospinal fluid determinations made by Lous resulted in 52.9 ± 4.2 per cent as much barbiturate found in cerebrospinal fluid as in serum. These data were obtained using an ultra-violet technique and were far more accurate than the colorimetric method employed by Fretwurst and Voss[90], who found no appreciable difference in barbiturate concentration in plasma and cerebrospinal fluid once equilibrium had been reached.

The present results revealed 64.2 ± 4.9 per cent as much pentobarbital present in the cerebrospinal fluid as was found in equivalent whole blood samples. This was higher than the value reported by Brodie et al of 52.9 per cent for one animal (Table I, fig. 4), but once again comparison was not possible due to the discrepancy between the two sample sizes. The report of Kozelka and Tatum[97] appeared to be in direct contradiction to the data obtained in our series. They detected less than 0.1 milligrams of phenobarbital in 15 milliliters of cerebrospinal fluid, i.e., less than 6.66 micrograms per milliliter, when sampled eight hours after intravenous injection into the animals.

However, corresponding blood phenobarbital levels are not reported, so again no legitimate comparison could be made. Also, they utilized a colorimetric means of quantitation which lacked accuracy in the lower range of barbiturate concentration.

The comparison of cerebrospinal fluid barbiturate content to that present in whole blood also, like comparative liver-whole blood values, showed an excellent relationship which was present regardless of the experimental sampling time. These findings were important because of Lous',[100] conclusion that the barbiturate content of the cerebrospinal fluid represented the ultrafiltrate of the plasma. Furthermore, as previously pointed out, Taylor[96] has shown that the ultrafiltrate contains the physiologically active barbiturate (unmetabolized drug). Since this was the case, and a linear relationship existed between whole blood and cerebrospinal fluid pentobarbital concentrations, as demonstrated by the present results, one could conclude that ultra-violet analysis for barbiturates was a reliable means of studying clinical response to these drugs. This is contrary to the contention of Maynert and Van Dyke[74].

From the above findings one could further show that the total barbiturate versus unmetabolized barbiturate (as measured in the cerebrospinal fluid) concentration represented a constant, direct, linear proportion, at least over the time duration of the present experiments. A finding similar to this having been reported by Kahn[86] in his work with radioactive pentobarbital in rats.

In pooled bone marrow extract, the amount of pentobarbital present was only 6.1 per cent of that found in whole blood samples taken at

the same time. These samples showed very little depression, i.e. a standard deviation of only 2.6 per cent. Therefore, although the values were low, their consistency was so uniform as to allow one to regard such values as valid.

As outlined in the section on method, it was first felt that performing quantitative determinations for pentobarbital in bone marrow would give variable results. Yet, the data for these determinations, when compared to whole blood levels, appeared to give reliable results. The correlation demonstrated was sufficient to warrant further investigation. However, the practical application of this information must await modification of the extraction method used so that a more exact quantitation with duplication of results may be obtained.

Unlike liver and cerebrospinal fluid, bone marrow did not require a period of time for the initial distribution of pentobarbital. This was well shown in the case of Dog. No. 27 where the bone marrow specimen was obtained within 15 minutes after pentobarbital injection (Table IX-E). Further, the existence of a direct proportionality between blood and bone marrow seemed evident since the percentage values showed little deviation over the variety of sampling times selected.

The data obtained on the gastric contents following intravenous or intraperitoneal injection of pentobarbital were included to determine if any detectable amount of drug diffused, by retrogression, across the gastric mucosa. In those animals given barbiturate via the oral route pentobarbital determinations were done later to determine

how much absorption of the drug had taken place. The failure to detect any pentobarbital in the animals given the drug parenterally indicated that no reverse transfer occurred across the gastric mucosa.

The recovery data on whole blood was investigated to show that the method employed was valid for experimental purposes. The average of 99.3 per cent with a standard deviation of ± 10.8 per cent showed very good recovery from biological specimens in which more than one manipulation was necessary. These results also substantiated the previous finding of equality of pentobarbital distribution within plasma and erythrocytes.

In this work emphasis was placed on recovery from biological material as the criteria for the validity of the method. One reason for this was that much of the previously published recovery data, based on similar ultraviolet methods, were derived by adding a specific amount of barbiturate to a sample not having biological activity. This procedure appeared misleading and inaccurate because the drugs which the toxicologist and clinical pathologist are called upon to detect have undergone certain metabolic changes as the result of such activity. Therefore, the only recovery data included in this report was that obtained from biologically active tissue.

Of even greater significance, and very gratifying to us, was the recovery following washing of the erythrocytes which amounted to 97.8 ± 9.2 per cent of the pentobarbital present in the unwashed cells.

The time required for reduction of the whole blood barbiturate concentration to one-half (50%) its initial *in vivo* value was analyzed

in light of the fact that the initial sample were withdrawn 45 minutes after the introduction of pentobarbital into the animal's system. The 45 minute interval was allowed since, on the average, about 30 minutes was required for the drug to become distributed throughout the body. A similar finding has been reported by Richards and Taylor in their recent review of barbiturate distribution and metabolism(80).

The graph (Table X) which resulted from these data showed an almost linear decline over the sampling period. This was especially evident during the first sixty-minute sampling period. The remaining 60 minutes of the curve demonstrated a tendency toward a parabolic shape, a finding similar to that demonstrated by Kahn(86) and Brodie et al(85).

Although it was realized that such data represented both unmetabolized and metabolized pentobarbital, it has been shown(86, 80, 43) that in blood and most animal tissues the majority of the measured whole blood pentobarbital content represented unmetabolized drug. In urine, the reverse holds true. In the article by Kahn(86), where the metabolic and unchanged pentobarbital were compared, the rate of decline of both showed the exponential shape so characteristic of drug destruction within the body. Furthermore, both were found to be "roughly parallel throughout the time observed." It was this latter finding which validated the results computed from the observations made during this study regarding trends in drug distribution. Also Kahn found a 50 per cent reduction in both total measured C_{14} activity (pentobarbital + metabolites) and unchanged drug occurred at the same time, i.e. 140 minutes after initial sampling period, lending further support to the

validity of the results of this research.

The difference between the results Kahn reported and those incorporated in this paper was probably due to selection of sampling time. Kahn took four samples over a four hundred and eighty minute period, while the four samples used in this study were taken over only a one hundred and eighty minute period.

Brodie's[85] work was much more difficult to evaluate since he reported the decline in blood pentobarbital on an hourly basis. Also, his failure to allow for the distribution of the drug prior to obtaining his initial sample introduced a variable which was absent in both Kahn's results and those reported here.

To compare these results to those of Taylor *et al*[85] and Axelrod[88], who reported their blood level decline as a rate per hour, was impossible. As had been shown by Kahn, Brodie *et al* and the author, the loss of pentobarbital from the blood was not truly linear, but rather exponential. Therefore, it was not possible to state the rate of pentobarbital removal from the blood as so much per hour, but, rather in the present study as a "half-life" value, as has been done.

Summary and Conclusion

The experiments reported in this manuscript were undertaken to ascertain whether or not any correlation existed between the amount of pentobarbital present in the whole blood and the amount found in other organs and body fluid of the same animal. The stimulus for such an investigation was the paucity of correlative evidence present in the literature. Also, a consideration of great importance was that such data would be of value in toxicological analysis and in gauging the response to treatment in cases of barbiturate poisoning.

The procedure used in preparation and procurement of animal specimens was as follows. A dog was given sufficient pentobarbital, either by intravenous or intraperitoneal injection, to gain anesthesia. Next the femoral vein was exposed so that serial blood samples could be obtained. In cases where a fatal outcome was desired the dog received sufficient pentobarbital to cause death. In the latter cases, the liver, femur and cerebrospinal fluid were obtained for analysis in addition to whole blood.

Pentobarbital was extracted from the biological material by altering the pH and using chloroform as the solvent. The chloroform was then treated with sodium hydroxide-boric acid buffer to recover the pentobarbital. The resultant solution was analyzed in the ultraviolet spectrophotometer with quantitation obtained by application of the Bouguer-Beer law.

A total of 38 dogs were included. Not all the animals were

included in every observation made; however, an adequate number were present in each category to allow some conclusion to be drawn.

The results of an attempt to correlate whole blood pentobarbital content with that present in various dog tissues and fluid demonstrated excellent correlation for clot, liver, cerebrospinal fluid and bone marrow; however, serum failed to show a consistent relationship.

With respect to the amount of pentobarbital found in whole blood at the time of animal awakening, it was noted that a much lower value was present in the dogs given oral medication than those receiving it by parenteral routes. Furthermore, it was found that the awakening pentobarbital blood level is not influenced by increasing the dosage.

The distribution between the plasma and the erythrocytes was found to be equal and the major factor which determined the barbiturate distribution in whole blood was the hematocrit value.

Furthermore, to demonstrate the validity of ultraviolet spectrophotometry as an instrument in clinical diagnosis it was demonstrated that the amount of pentobarbital measured in the whole blood was directly proportional to the amount of physiological active barbiturate present in the blood. It was also shown that this relationship was parallel and did not vary over a prolonged sampling period.

Finally, the rate of disappearance of pentobarbital from the blood was tabulated, the resultant graph showing the exponential curve so characteristic of drug excretion in the body.

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APPENDIX

Legend of Abbreviations Used in Following Tables

	Abbreviation	Meaning
1.	W.B. or W.Blood	Whole Blood (with anticoagulant added)
2.	B.M. or B.Marrow	Bone Marrow
3.	B.	Blood
4.	CSF	Cerebrospinal Fluid
5.	L. or Liv.	Liver
6.	P. A.	Post-anesthetic
7.	S. or Ser.	Serum
8.	C.	Clot
9.	R.B.C.	Red Blood Cell
10.	W.C. or Wash. cells	Washed cells (red blood cells)
11.	S.W. or Sal. Wash.	Saline Washings from red blood cells
12.	Hct.	Hematocrit
13.	ext.	Extract
14.	term. or T.	Terminal or at time of death
15.	I.P.	Intraperitoneal
16.	I.V.	Intravenous
17.	Spec.	Spectrophotometer with Ultraviolet (U.V.) attachment
18.	Max.	Maximum - referring to in the ultraviolet spectra

	Abbreviation	Meaning
19.	R.O.A.	Route of Administration
20.	M.	Male
21.	F.	Female
22.	P.	Plasma
23.	C.	Cell (red blood cell)
24.	Gm.	Grams
25.	ml.*	Milliliter
26.	mgm. or mg.	milligram
27.	Kg.	Kilogram
28.	cc.*	cubic centimeter
29.	γ	gamma (a gamma is equal to a microgram uGm.)
30.	mμ	millimicron
31.	Nem.	Nembutal (same as Pentobarbital)
32.	Pentobarb. or Pento	Pentobarbital
33.	Sam. No.	Sample number
34.	Min.	minutes
35.	Barbs.	Barbiturates (refers to them as a class of drug)
36.	T.D.	Total dose

*milliliters and cubic centimeters are used interchangeably and are assumed to be equal. ml. = cc.

	Abbreviation	Meaning
37.	Sampling time	Refers to the time, in minutes or hours, interval between the time the drug was administered and the time the sample was taken for analyzation.
38.	Amt.	Amount
39.	hrs.	hours
40.	Dose of Drug	Actual amount of drug the animals have received during the experiment from the initial dose to the time of sampling inclusive.
41.	Time interval in minutes	The time period which has elapsed from the initial dose of the drug until the particular sample in question was drawn.
42.	Total amount of drug given during time interval	Refers to the total quantity of drug given to the animal previous to withdrawal of the sample in question. This includes the initial dose of drug.
43.	Type of Specimen	Refers to the nature of the biological sample which has been analyzed for drug content.
44.	Dog No.	This is a number assigned to each animal used in this particular series of experiments on drug distribution, recovery, etc.
45.	——— level in γ/ml. (unqualified)	Refers to the calculated drug as obtained from procedure used (Brackett and Bradford).
46.	B. & B. Method	Brackett and Bradford method for drug analysis in biological material (1952).

Abbreviation	Meaning
47. Theoretical level of drug in γ/ml.	Refers to the drug level which should be found due to finding in a group of components of a particular biological material, i.e., the plasma or red cell drug level/ml. should equal the level found in whole blood, if the drug is equally distributed between the two components of whole blood. Conversely, the multiplication of the red cell, plasma, and whole blood levels by the hematocrit, in %, should give equal results assuming equal distribution of the drug.
48. Amount of Drug in P or C portion of W. Blood γ/ml. of W. Blood	See the explanation of 47 under i.e.
49. Plasma and Red Cell breakdown γ/ml. W.B.	Same item as 48. Only the name was changed, the results are gained in the same manner as is outlined in 47.
50. Theoretical value for W.C. & S.W. γ/ml. R.B.C. - or - Theoretical recovery value for S.W. γ/ml. R.B.C.	Prior to 8-15-55. The amount of saline washings gained where 3 times as much as the red cells that were washed. The theoretical value is computed on the basis of the amount of drug that was present on the red cell prior to washing, subtracted from the amount of drug found to be present after washing. The amount so computed should be equal to the amount found per ml. of saline washings multiplied by 3 (this is done to equalibrate the saline washings to red cells used). The new procedure for washing red cells will use 6 times, rather than 3 times, the amount of cells washed; thus the figure which is gained on a ml. basis will have to be multiplied by 6 in order to evaluate it in light of the theoretical recovery value. The value of saline washings should represent the amount of drug

Abbreviation	Meaning
	adsorbed to the surface of the red cell, since care is taken not to rupture the cell membrane while it is being washed. The value of washed cells should represent either the amount of drug which remains adsorbed to the red cell (incomplete removal during washing), or a quantity of drug which is contained within the red cell itself, and is only released when the cells are hemolyzed (as they are during the extraction phase of the analysis, where they are shaken in a chloroform solution with a pH of 4.5).
51. pesos or "O"	Oral route of administration.

TABLE IV-A.

Composite records of the pentobarbital content in the whole blood, plasma and erythrocytes of dogs.

Dog No.	Sample No.	Dose of Pentobarb. mgm/Kg.	Route	Sample Time	W.Blood Level	Plasma Level γ/ml.	R.B.C. Level γ/ml.	Hematocrit Plasma	Hematocrit R.B.C.
1	1	35.20	I.V.	105	48.25	39.65	44.86	61.9	38.1
	2	35.20	I.V.	150	35.50	36.73	28.98	60.4	39.6
	3	35.20	I.V.	180	28.36	25.98	27.32	61.5	38.5
	4	35.20	I.V.	210	20.76	19.98	20.70	59.8	40.2
	5PA	35.20	I.V.	255	15.36	13.92	12.65		
2	1	30.30	I.P.	60	35.52	29.87	34.50	51.7	49.3
	2PA	30.30	I.P.	180	15.76	18.92	16.56	51.7	49.3
3	1	27.30	I.V.	90	25.42	26.86	31.20	54.2	45.8
	2	27.30	I.V.	150	20.20	19.86	21.32	54.0	46.0
	3PA	27.30	I.V.	210	10.90	13.20	12.10	55.0	45.0
4	1	37.10	I.P.	45	65.82	54.80	59.27	59.7	40.3
	2	37.10	I.P.	90	48.90	50.20	45.45	59.0	41.0
	3	37.10	I.P.	120	39.91	41.08	36.56	58.6	41.4
	4A	37.10	I.P.	150	30.61	28.76	31.56	59.1	40.9
	4V	37.10	I.P.	150	29.55	32.66	30.92	61.3	38.7
	5	37.10	I.P.	180	23.76	22.45	21.81	59.2	40.8
	6PA	37.10	I.P.	255	18.76	17.96	19.02	60.0	40.0
5	1	80.00	I.P.	30	142.00	139.90	142.60	49.3	50.7
	2T	80.00	I.P.	55	78.96	81.32	76.66	52.6	47.4
6	1	32.5	I.P.	45	42.78	39.42	41.68	68.7	31.3
	2	32.5	I.P.	90	34.19	35.42	33.27	59.2	40.8
	3	32.5	I.P.	120	26.54	27.00	25.09	64.5	35.5
	4	32.5	I.P.	150	20.18	20.94	19.00	69.0	31.0
	5PA	32.5	I.P.	180	16.56	15.48	15.72	62.1	37.9
7	1	30.00	I.P.	45	64.50	66.51	60.95	42.0	58.0
	2	30.00	I.P.	90	44.81	40.48	38.37	44.9	55.1
	3	30.00	I.P.	180	22.91	28.62	24.06	40.0	60.0
	4PA	30.00	I.P.	235	19.36	20.54	15.73	48.1	51.9
8	1	47.08	I.P.	360	71.65	72.86	69.35	52.7	47.3
	2T	47.08	I.P.	380	65.72	73.55	68.91	55.1	44.9
9	1	42.00	I.V.	180	34.60	35.20	35.72	54.6	45.4
	2	42.00	I.V.	210	29.83	30.20	31.00	54.6	45.4

(Table IV-A)

Whole blood, plasma, erythrocyte concentration of pentobarbital.

Dog No.	Sample No.	Dose of Pentobarb. mgm/Kg.	Route	Sample Time	W.Blood Level	Plasma Level γ/ml.	R.B.C. Level γ/ml.	Hematocrit Plasma	Hematocrit R.B.C.
10	1	39.32	I.V.	150	31.00	29.90	32.04	48.2	51.8
	2	39.32	I.V.	210	24.90	25.36	25.08	48.7	51.3
11	1	36.73	I.V.	150	40.00	37.00	39.05	37.3	62.7
12	1	35.40	I.V.	240	27.60	30.20	30.75	54.6	45.4
	2	35.40	I.V.	300	23.86	24.20	22.90	55.0	45.0
	3PA	35.40	I.V.	360	17.54	18.08	17.96	55.0	45.0
13	1	31.60	I.P.	30	36.20	32.00	35.40	66.6	33.4
	2	35.50	I.P.	110	54.50	46.22	51.96	66.0	34.0
	3T	51.86	I.P.	165	82.36	86.45	76.32	66.0	34.0
14	1	32.00	I.V.	30	27.10	29.40	29.40	64.0	36.0
	2	32.00	I.V.	60	30.22	32.10	31.11	64.5	35.5
	3	32.00	I.V.	120	22.19	23.04	22.59	61.9	38.1
	4PA	32.00	I.V.	165	17.63	15.86	16.32	60	40.0
15	1	32.75	I.V.	45	21.35	32.20	24.80	52.9	47.1
	2	43.65	I.V.	240	29.75	30.95	25.10	51.0	49.0
	3PA	43.65	I.V.	310	17.09	15.29	19.86	51.0	49.0
16	1	31.20	I.P.	35	42.35	45.10	lost	48.6	51.4
	2	31.20	I.P.	90	26.63	29.50	21.90	49.0	51.0
	3	47.60	I.P.	280	24.25	23.45	30.75	52.3	47.7
	4PA	47.60	I.P.	325	19.87	20.01	18.56	51.0	49.0
17	1	24.60	I.V.	25	29.55	41.50	24.80	69.0	31.0
	2	41.00	I.V.	125	24.35	25.10	17.70	53.0	47.0
18	1	45.20	I.P.	70	143.00	171.00	151.80	49.7	50.3
	2	45.20	I.P.	150	56.97	54.29	58.82	52.6	47.3
	3	45.20	I.P.	210	41.40	39.87	37.65	52.0	48.0
	4	45.20	I.P.	255	35.42	31.30	32.86	54.1	45.9
	5	45.20	I.P.	330	24.73	25.03	22.98	55.8	44.2
	6PA	45.20	I.P.	390	17.56	19.22	17.40	52.7	47.3
19	1	48.56	I.P.	60	18.00	15.88	16.10	65.1	34.9
	2	53.08	I.P.	120	27.90	22.42	19.70	65.0	35.0
	3	53.08	I.P.	180	21.45	20.50	21.46	64.2	35.8
	4	53.08	I.P.	240	22.55	21.10	23.59	63.8	36.2
	5T	53.08	I.P.	355	22.65	23.65	21.88	64.8	35.2

(Table IV-A)

Whole blood, plasma, erythrocyte concentration of pentobarbital.

Dog No.	Sample No.	Dose of Pentobarb. mgm/kg.	Route	Sample Time	W.Blood Level	Plasma Level γ/ml.	R.B.C. Level γ/ml.	Hematocrit Plasma	Hematocrit R.B.C.
20	1	42.40	"O"	360	15.90	17.05	21.45	41.3	58.7
	2PA	42.40	"O"	420	13.25	12.91	14.61	42.0	58.0
21	1	47.65	I.P.	90	99.00	122.20	107.10	55.6	44.4
	2	47.65	I.P.	105	78.42	82.09	74.86	55.0	45.0
	3	47.65	I.P.	135	45.76	46.20	42.98	54.9	45.1
	4	47.65	I.P.	165	37.56	38.80	35.74	55.0	45.0
	5	47.65	I.P.	195	30.86	29.50	30.30	56.7	43.3
	6	47.65	I.P.	225	24.86	24.91	22.54	54.8	45.2
	7PA	47.65	I.P.	265	17.56	18.02	16.56	55.2	44.8
25	1	27.10	I.V.	60	31.40	25.60	29.40	64.2	35.8
	2	30.97	I.V.	90	59.90	54.90	65.30	59.1	40.9
	3PA	30.97	I.V.	115	34.67	35.40	32.60	61.9	38.1
20	1	67.80	"O"	125	19.10	18.65	14.00	41.9	58.1
	2	67.80	"O"	185	23.55	27.60	22.15	42.0	58.0
	3PA	67.80	"O"	480	13.30	11.08	19.85	42.0	58.0
22	1	36.00	I.P.	95	15.92	15.85	16.32	62.8	37.2
	2	36.00	I.P.	190	39.20	36.20	36.80	66.0	34.0
	3T	45.00	I.P.	350	34.95	38.97	33.60	64.9	35.1
7	1	28.60	I.P.	60	19.66	21.47	25.57	58.4	41.6
	2	31.46	I.P.	180	42.70	19.40	32.35	59.0	41.0
	3T	32.89	I.P.	240	48.62	45.36	39.47	58.0	42.0
23	1	54.60	I.V.	195	15.48	16.40	18.58	68.4	31.6
	2	54.60	I.V.	255	13.68	21.80	11.45	65.7	34.3
	3	57.70	I.V.	315	33.60	27.25	17.75	66.0	34.0
	4T	68.05	I.V.	590	115.90	113.30	111.80	69.1	30.9
26	1	30.00	I.P.	70	41.56	45.80	46.32	51.0	49.0
	2	30.00	I.P.	80	43.00	45.50	47.60	52.7	47.3
	3PA	30.00	I.P.	200	18.10	18.65	16.70	50.4	49.6
27	1	60.00	I.P.	90	35.30	38.30	32.50	60.0	40.0
	2	60.00	I.P.	150	34.95	34.55	29.20	61.9	38.1
	3	60.00	I.P.	210	33.00	37.40	38.90	54.0	46.0
	4	60.00	I.P.	270	37.40	29.00	33.95	60.0	40.0
	5	60.00	I.P.	330	25.80	23.95	22.75	58.2	41.8

(Table IV-A)

Whole blood, plasma, erythrocyte concentration of pentobarbital.

Dog No.	Sample No.	Dose of Pentobarb. mgm/kg.	Route	Sample Time	W.Blood Level	Plasma Level γ/ml.	R.B.C. Level γ/ml.	Hematocrit Plasma	Hematocrit R.B.C.
28	1T-A	120.00	I.P.	15	93.60	92.50	88.30	59.0	41.0
	1T-V	120.00	I.P.	15	47.00	46.95	56.60	58.7	41.3
29	1	32.00	I.P.	45	34.86	34.55	29.20	60.4	39.6
	2	32.00	I.P.	90	25.80	23.95	22.75	52.7	47.3
	3	32.00	I.P.	180	20.64	19.89	20.01	58.4	41.6
	4PA	32.00	I.P.	180	14.89	15.21	14.75	60.0	40.0
	5T	32.00	I.P.	245	10.04	11.01	10.00	59.8	40.2
30	1	36.00	I.P.	45	52.49	60.83	58.20	49.7	50.3
	2	36.00	I.P.	90	40.90	33.24	30.65	48.5	51.5
	3	36.00	I.P.	150	27.92	29.48	27.21	52.9	47.1
	4	36.00	I.P.	180	20.90	21.61	19.42	50.0	50.0
	5PA	36.00	I.P.	225	17.35	15.42	15.98	47.2	52.8
31	1	74.25	I.P.	30	102.40	92.83	107.20	60.5	39.5
	2T	74.25	I.P.	60	89.26	94.82	85.90	57.3	42.7
32	1	80.25	I.P.	35	111.50	119.30	115.60	55.1	44.9
	2	80.25	I.P.	60	99.26	101.40	93.26	55.1	44.9
	3T	80.25	I.P.	100	80.25	82.56	81.80	52.7	47.3
33	1	80.00	I.P.	25	79.36	80.80	69.32	63.5	36.5
	2	80.00	I.P.	60	91.56	89.30	90.31	65.2	34.8
	3T	80.00	I.P.	80	79.42	81.45	83.64	60.9	39.1
34	1	96.00	I.P.	20	109.25	122.60	116.30	54.0	46.0
	2T	96.00	I.P.	45	94.36	99.80	92.31	54.0	46.0
35	1	35.00	I.P.	45	42.63	38.27	40.40	58.4	41.6
	2	35.00	I.P.	90	32.56	29.45	31.38	60.2	39.8
	3PA	35.00	I.P.	180	17.24	16.85	20.92	58.6	41.4
	4T	65.00	I.P.	300	82.56	96.05	79.05	54.5	45.5
36	1	62.30	"O"	150	19.82	18.29	20.15	62.1	37.9
	2	62.30	"O"	210	25.36	26.20	27.00	60.5	39.5
	3	62.30	"O"	270	24.60	25.90	22.84	68.2	31.8
	4	62.30	"O"	330	20.75	18.34	16.55	69.0	31.0
	5PA	62.30	"O"	410	14.26	12.92	13.32	64.7	35.3
37	1	30.00	I.P.	60	27.90	29.35	30.20	56.6	43.4
	2	30.00	I.P.	105	24.50	22.96	24.05	56.4	43.6
	3PA	30.00	I.P.	165	18.25	17.30	15.27	55.0	45.0

(Table IV-A)

Whole blood, plasma, erythrocyte concentration of pentobarbital.

Dog No.	Sample No.	Dose of Pentobarb. mgm/kg.	Route	Sample Time	W.Blood Level	Plasma Level γ/ml.	R.B.C. Level γ/ml.	Hematocrit Plasma	Hematocrit R.B.C.
38	1	35.00	I.P.	45	44.54	40.96	41.40	59.1	40.9
	2	35.00	I.P.	90	35.16	36.20	34.40	59.0	41.0
	3	35.00	I.P.	120	26.50	24.32	27.90	59.0	41.0
	4	35.00	I.P.	150	21.75	20.84	23.60	57.8	42.2
	5	35.00	I.P.	180	17.24	18.57	17.75	60.0	40.0
	6PA	35.00	I.P.	215	14.36	15.60	13.95	58.3	41.7

TABLE IV-B

Composite Record of the Pentobarbital Concentration in the Various Tissues and Body Fluids of Dogs.

Dog No.	Sample No.	Washed R.B.C. γ/ml.	Saline Wash. γ/ml.	C.S.F. Level γ/ml.	Bone Marrow Level γ/ml.	Liver Level γ/ml.	Serum Level γ/ml.	Clot Level γ/ml.	Gastric Content γ/ml.
1	1								0.00
	2								0.00
	3								0.00
	4								0.00
	5PA								0.00
2	1								0.00
	2PA								0.00
3	1								0.00
	2								0.00
	3PA								
4	1								0.00
	2								0.00
	3								
	4A								
	4V								
	5								
	6PA								
5	1								
	2T			54.40	1.86	76.24			
6	1								
	2								
	3								
	4								
	5PA								
7	1								
	2								
	3								
	4PA								
8	1								
	2T			42.93	4.27	61.99			
9	1						34.00		
	2						27.70		

(TABLE IV-B)

Various Tissue and Body Fluid Concentration of Pentobarbital

Dog No.	Sam. No.	Washed R.B.C. γ/ml.	Sal. Wash. γ/ml.	C.S.F. Level γ/ml.	Bone Marrow Level γ/ml.	Liver Level γ/ml.	Serum Level γ/ml.	Clot Level γ/ml.	Gastric Content γ/ml.
10	1						34.30		
	2						25.21		
11	1						37.25		
12	1						21.86		
	2								
	3PA								
13	1								
	2								
	3T			54.56	2.86	59.85			
14	1								
	2								
	3								
	4PA								
15	1								
	2								
	3PA								
16	1		39.07						
	2		24.24						
	3		28.32						
	4PA		19.14						
17	1								
	2	1.90	15.63						
18	1	1.02	138.76						
	2		52.12						
	3		40.92						
	4		30.50						
	5		22.52						
	6PA								
19	1		14.36						
	2								
	3								
	4		21.97						
	5T		18.76	14.20	1.93	26.74			

(TABLE IV-B)

Various Tissue and Body Fluid Concentration of Pentobarbital

Dog No.	Sam. No.	Washed R.B.C. γ/ml.	Sal. Wash. γ/ml.	C.S.F. Level γ/ml.	Bone Marrow Level γ/ml.	Liver Level γ/ml.	Serum Level γ/ml.	Clot Level γ/ml.	Gastric Content
20	1	2.08	19.32				54.40	66.30	
	2PA		13.28						
21	1		110.54						
	2								
	3								
	4								
	5		32.52						
	6		19.16						
	7PA		15.32						
25	1		25.60						
	2	1.04	59.27						
	3PA	3.70	30.24	17.24	4.40	29.40			
20	1		16.78						38.72
(2nd	2								2.86
run)	3PA	1.69	20.43				10.56	11.40	
22	1	2.86	12.84						
	2						24.70	33.14	
	3T			23.42	3.65	29.85			
7	1		22.64						
(2nd	2		31.85						
run)	3T		37.91	27.52	2.09	41.56	23.75	40.38	
	4T				3.68				
23	1		14.92						
	2		13.00				8.31	11.41	
	3		14.84						
	4T		106.88	76.54	2.70	94.86	50.10		
26	1								
	2								
	3PA		19.35					40.79	
27	1								
	2								
	3								
	4								
	5								

(TABLE IV-B)

Various Tissue and Body Fluid Concentration of Pentobarbital

Dog No.	Sam. No.	Washed R.B.C. γ/ml.	Sal. Wash. γ/ml.	C.S.F. Level γ/ml.	Bone Marrow Level γ/ml.	Liver Level γ/ml.	Serum Level γ/ml.	Clot Level γ/ml.	Gastric Content
24	1		45.15						
	2		33.07						
	3								
	4		8.02						
	5								
	6								
	7T		29.75	21.50	1.23	32.00	33.65	31.26	
	8T				1.00	27.15			
28	1T-A	1.13	91.73	12.15	4.00	14.32	110.60		
	1T-V		54.60					93.10	
29	1		29.45						
	2		22.65						
	3		20.50						
	4PA	1.06	15.25						
	5T			6.25	.76	9.86			
30	1						54.91	54.20	
	2						38.64	42.94	
	3						18.27	30.48	
	4						21.54	18.62	
	5PA								
31	1		101.05						
	2T			56.20	5.73	75.24			
32	1								
	2								
	3T			52.86	3.26	72.86			
33	1								
	2								
	3T			55.46	6.20	62.81			
	4T			53.20	5.06	64.54			
34	1								
	2T			57.25	6.49	98.66			
	3T				6.96	90.58			

(TABLE IV-B)

Various Tissue and Body Fluid Concentration of Pentobarbital

Dog No.	Sam. No.	Washed R.B.C. γ/ml.	Sal. Wash. γ/ml.	C.S.F. Level γ/ml.	Bone Marrow Level γ/ml.	Liver Level γ/ml.	Serum Level γ/ml.	Clot Level γ/ml.	Gastric Content
35	1								
	2								
	3PA								
	4T			56.22	4.63	71.56			
	5T				5.01	79.36			
36	1								82.73
	2								0.91
	3								0.00
	4								
	5PA								
37	1								
	2								
	3PA								
38	1								
	2								
	3								
	4								
	5								
	6PA								

TABLE V

Animal Sex-Weight Distribution

Animal Number	Sex	Weight in Kilograms
1	Female	14.20
2	Female	8.25
3	Male	15.60
4	Female	10.50
5	Male	18.60
6	Male	24.30
7	Male	21.00
8	Male	13.40
9	Male	10.00
10	Male	12.95
11	Female	9.80
12	Male	11.00
13	Female	7.60
14	Male	17.80
15	Female	11.00
16	Female	7.70
17	Female	12.20
18	Female	14.00
19	Male	6.80
20	Male	29.50
21	Female	19.50
20-2*	Male	29.50
22	Male	10.00
7-2*	Male	21.00
23	Male	14.50
24	Female	10.90
25	Male	15.50
26	Male	14.00
27	Male	9.00
28	Male	9.00
29	Female	13.45
30	Male	21.70

* -2, this refers to the second set of samples which were drawn from an animal previously sampled.

TABLE V

Animal Sex-Weight Distribution (Con't)

Animal Number	Sex	Weight in Kilograms
31	Female	10.50
32	Male	18.70
33	Female	15.50
34	Female	12.50
35	Female	16.30
36	Male	27.30
37	Female	7.50
38	Female	14.70
	Average	14.70
	Standard Deviation	± 5.94
	95% Range	3.06 to 26.34

TABLE VI

REAGENTS, APPARATUS AND PROCEDURE USED IN EXTRACTION.

(1) Reagents:

a. Saturated potassium dehydrogen phosphate. - Analytical reagent grade dissolved in distilled water until saturation occurs.

b. Chloroform - Reagent grade, not further purified.

c. Sodium hydroxide solution 0.064N. - Made by dilution of standardized 0.2 N sodium hydroxide with distilled water.

d. Boric acid solution 0.1M. - This solution is made such that when the 0.1M Boric acid is added to an equal volume of 0.064N sodium hydroxide, the pH of the resulting solution os 9.4 $\pm$ 0.1.

(2) Apparatus:

a. 60 ml. separatory funnel.

b. 10 ml. and 25 ml. g.s. graduate cylinder.

c. Small funnel.

d. Whatman No. 40 filter paper - 9.0 cm.

e. 10 cc. syringe.

f. 15-G Spinal needle - square tipped.

g. 20 ml. centrifuge tubes and 50 ml. potato tubes.

h. Beckman Spectrophotometer Model D.U. with ultra-violet attachment.

(3) Extraction Procedure:

a. A 2 ml. sample volume was used for extraction.

(TABLE VI)

b. Add sufficient saturated potassium dihydrogen phosphate solution to adjust the pH to between 4.5 and 5.0. (This usually required 1 ml./ml. of whole blood, plasma, etc.)

c. Next is added 2 ml. of the particular specimen to be analyzed.

d. Shake vigorously for one minute, then allow phases to separate.

e. Draw off chloroform layer through a No. 40 Whatman filter into a 25 ml. g.s. graduate cylinder. Record the chloroform volume (V). This represents unknown acid fractions.

f. Add a volume of chloroform, (equal to Vi, to another Second 25 ml. g.s. graduate cylinder. This represents the "blank" acid fraction.

g. To both cylinders, the unknown and the blank, add 2.5 ml. of 0.064 N-NaOH.

h. Shake both cylinders for one minute, then allow to stand for approximately ten to fifteen minutes so phases will separate.

i. With 10 cc. syringe, fitted with 15-G needle, the upper eight to nine ml. of the two cylinders is removed and each portion, after instilling in proper tubes, is placed in centrifuge and rotated at 3000 rpm for ten minutes. (This gives a much sharper line of demarcation between phases, than that obtained using Brackett and Bradford's original procedure.)

j. Transfer 2.0 to 2.4 ml. of upper aqueous phase (sodium hydroxide

(TABLE VI)

extract) from both unknown and blank, to a clean 10 mg. g.s. graduate cylinder.

k. To both cylinders is added a volume of 0.1M Boric Acid equal to that of Sodium hydroxide extract and they are mixed.

l. The extracts are now transferred to the $1cm^2$ Beckman silicon cuvettes and are now ready for ultraviolet analysis.

*In their early work using this method Brackett and Bradford felt that 1 ml. of specimen was sufficient; however, after repeated comparison of analysis and numerous pilot runs, which varied only in the sample volume used, it was found that for practical purposes, a 2 ml. sample size resulted in best percentage recovery. Hence, in all extractions performed, unless otherwise specified, a 2 ml. sample volume was used.

TABLE VII

Whole Blood Pentobarbital Concentration at Time of Arousal

A. Intraperitoneal Route of Administration:

Animal Number	Dose of Pentobarbital	Pentobarbital Concentration	Statistical Analysis		
			Average	Standard Deviation	95% Range
	mgm./Kg.	γ/ml.	γ/ml.	γ/ml.	γ/ml.
2	30.30	15.76			
4	37.10	18.76			
6	32.50	16.56			
7	30.00	19.36			
16	47.60	19.87			
18	45.20	17.56			
21	47.65	17.56			
26	30.00	18.10			
29	32.00	14.89			
30	36.00	17.35			
35	35.00	17.24			
37	30.00	18.25			
38	35.00	14.36	17.40	± 1.6	14.17-20.55

B. Intravenous Route of Administration:

Animal Number	Dose of Pentobarbital	Pentobarbital Concentration	Statistical Analysis		
			Average	Standard Deviation	95% Range
	mgm./Kg.	γ/ml.	γ/ml.	γ/ml.	γ/ml.
1	35.20	15.36			
3	27.30	10.90			
12	35.40	17.54			
14	32.00	17.63			
15	43.65	17.09	15.70	± 2.8	10.13-21.27

(TABLE VII)

Whole Blood Pentobarbital Concentration at Time of Arousal (Con't)

C. Oral Route of Administration:

Animal Number	Dose of Pentobarbital mgm./Kg.	Pentobarbital Concentration γ/ml.	Statistical Analysis		
			Average γ/ml.	Standard Deviation γ/ml.	95% Range
20	42.40	13.25			
20-2	67.80	13.30			
36	62.30	14.26	13.60	± 0.6	12.48-14.72

D. Entire Series of Animals:

Animal Number	Dose of Pentobarbital mgm./Kg.	Pentobarbital Concentration γ/ml.	Statistical Analysis		
			Average γ/ml.	Standard Deviation γ/ml.	95% Range
			16.43	± 2.3	11.99-20.87

TABLE VIII-A

The Relationship Between Pentobarbital Dosage and Whole Blood Concentration at Various Time Intervals Following Parenteral Injection.

Dosage Range mg./kg.	Time in Minutes after Pentobarbital Injection: At 30-15 min. µg./ml.	At 60 min. µg./ml.	At 90 min. µg./ml.	At 120 min. µg./ml.	At 150 min. µg./ml.	At 180 min. µg./ml.
25.0 2.5	29.55		24.92		19.37	
	42.78	31.40	25.42	19.66	20.20	15.76
	64.50	35.52	44.81	20.64	20.18	16.56
30.0 2.5	27.10	41.56	20.64	26.54		14.89
	45.35	27.90	34.19			22.91
	36.20	21.35	25.80			
	34.86					
	52.49	39.35	48.25	39.91	40.00	28.36
	42.63		32.56	26.50	35.50	17.24
37.5 2.5	65.82		35.16		29.55	29.65
	44.54		48.90		31.00	23.76
	46.60		40.90		27.92	20.90
					21.75	
60.0 2.5			35.30		34.95	
75.0 2.5	102.40		89.26			
	142.00	78.96	80.25			
80.0 2.5	111.50	99.26	79.42			
	79.36	91.56				
95.0 2.5	109.20	94.36				

* Whole blood levels are expressed in gammas/milliliter.

TABLE VIII-B

The Relationship Between Pentobarbital Dosage and Whole Blood Concentration at Various Time Intervals Following Parenteral Injection.

A. For the Dosage Range of 30.0 ± 2.5 mgm./kg.

	Time in Minutes					
	30±15 min. µg./ml.	60 min. µg./ml.	90 min. µg./ml.	120 min. µg./ml.	150 min. µg./ml.	180 min. µg./ml.
	42.78	31.40	25.42	19.66	20.20	15.76
	64.50	35.52	44.81	20.64	20.18	16.56
	27.10	41.56	20.64	26.54		14.89
	45.35	27.90	34.19			22.91
	36.20	21.35	25.80			
	34.86					
Average	41.30	31.60	30.20	22.30	20.20	17.60
Standard Deviation	12.70	7.60	9.50	3.70	0.00	3.60
95% Range	16.4-66.2	16.7-46.5	11.6-48.8	15.0-29.6	0.00	10.5-24.7

B. For the Dosage Range of 37.5 ± 2.5 mgm./Kg.

	30±15 min. µg./ml.	60 min. µg./ml.	90 min. µg./ml.	120 min. µg./ml.	150 min. µg./ml.	180 min. µg./ml.
	52.49	39.35	48.25	39.91	40.00	28.36
	42.63		32.56	26.50	35.50	17.24
	65.82		35.16		29.55	29.65
	44.54		48.90		31.00	23.76
	46.60		40.90		27.92	20.90
					21.75	
Average	50.40	39.35	41.20	33.20	31.00	24.00
Standard Deviation	9.40	0.00	7.40	9.50	6.30	5.20
95% Range	32.0-68.8	0.00	26.7-55.7	14.6-51.8	18.7-43.3	13.8-34.2

* Whole blood levels are expressed in gammas/milliliter.

DISTRIBUTION OF PENTOBARBITAL IN PLASMA AND ERYTHROCYTES AS COMPARED TO WHOLE BLOOD.

TABLE IX-A

Animal Number	Whole Blood Level γ/ml.	Plasma Level γ/ml.	R.B.C. Level γ/ml.	Hematocrit Plasma	Hematocrit R.B.C.	% Plasma Act.	% Plasma Cor.	% R.B.C. Act.	% R.B.C. Cor.	Theoretical Whole Blood level γ/ml.	% Pento. in Theo. W. Blood
1	48.25	39.65	44.86	61.9	38.1	50.9	59.0	35.4	41.0	41.63	86.3
	35.50	36.73	28.98	60.4	39.6	62.5	65.9	32.3	34.1	33.67	94.8
	38.36	25.98	27.32	61.5	38.5	41.7	60.3	27.4	39.7	26.50	69.1
	20.76	19.98	20.70	59.8	40.2	57.6	59.0	40.1	41.0	20.27	97.7
	15.36	13.92	12.65	59.8	40.2	54.2	62.1	33.1	45.8	13.41	87.3
2	35.52	29.87	34.50	51.7	49.3	43.5	47.6	47.9	52.4	32.45	91.4
	15.76	18.92	16.56	51.7	49.3	62.1	54.5	51.8	45.5	17.94	113.9
3	25.42	26.86	31.20	54.2	45.8	57.3	50.5	56.2	49.5	28.85	113.5
	20.20	19.86	21.32	54.0	46.0	53.1	52.2	48.6	47.8	20.53	101.7
	10.90	13.20	12.10	55.0	45.0	66.6	57.1	50.0	42.9	12.71	116.6
4	65.82	54.80	59.27	59.7	40.3	49.7	57.6	36.3	42.4	56.62	86.
	48.90	50.20	45.45	59.0	41.0	60.6	61.4	38.1	38.6	48.25	98.7
	39.91	41.08	36.56	58.6	41.4	60.3	61.7	37.9	38.3	39.21	98.2
	30.61	28.76	31.56	59.1	40.9	55.5	56.8	42.2	43.2	29.91	97.7
	29.55	32.66	30.92	61.3	38.7	67.8	62.6	40.5	37.4	31.99	108.3
	23.76	22.45	21.81	59.2	40.8	55.9	59.9	37.5	40.1	22.19	93.4
	18.76	17.96	19.02	60.0	40.0	57.5	58.6	40.6	41.4	18.39	98.1
5	142.00	139.90	142.60	49.3	50.7	48.6	48.8	50.9	51.2	141.27	99.5
	78.96	81.32	76.66	52.6	47.4	54.2	54.1	46.0	45.9	79.11	100.2

TABLE IX-A (Cont'd)

Animal Number	Whole Blood Level γ/ml.	Plasma Level γ/ml.	R.B.C. Level γ/ml.	Hematocrit Plasma	Hematocrit R.B.C.	% Plasma Act.	% Plasma Cor.	% R.B.C. Act.	% R.B.C. Cor.	Theoretical Whole Blood level γ/ml.	% Pento. in Theo. W. Blood
6	42.78	39.42	41.68	68.7	31.3	63.3	67.5	30.5	34.5	40.13	93.8
	34.19	35.42	33.27	59.2	40.8	61.3	60.7	39.7	39.3	34.54	101.0
	26.54	27.00	25.09	64.5	35.5	65.6	66.1	33.6	33.9	26.33	99.2
	20.18	20.94	19.00	69.0	31.0	71.6	71.0	29.2	29.0	20.34	100.8
	16.56	15.48	15.72	62.1	37.9	58.0	61.7	36.0	38.3	15.57	94
7	64.50	66.51	60.95	42.0	58.0	43.3	44.1	54.8	55.9	63.28	98.1
	44.81	40.48	38.37	44.9	55.1	40.6	46.2	47.2	53.8	39.32	87.8
	22.91	28.62	24.06	40.0	60.0	50.0	44.3	63.0	55.7	25.89	113.0
	19.36	20.54	15.73	48.1	51.9	51.0	54.7	42.2	45.3	18.04	93.2
8	71.65	72.86	69.35	52.7	47.3	53.6	53.9	45.8	46.1	71.20	99.4
	65.72	73.55	68.91	55.1	44.9	61.7	56.7	47.1	43.3	71.47	108.8
9	29.83	30.20	31.00	54.6	45.4	55.3	54.0	47.2	46.0	30.56	102.5
10	31.00	29.90	32.04	48.2	51.8	46.5	46.5	53.6	53.5	31.01	100.1
11	40.00	37.00	39.05	37.3	62.7	34.5	36.1	61.2	63.9	38.28	95.7
12	27.60	30.20	30.75	54.6	45.4	59.8	54.2	50.6	45.8	30.45	110.4
	23.86	24.20	22.90	55.0	45.0	55.8	56.4	43.2	43.6	23.62	99.0
	17.54	18.08	17.96	55.0	45.0	56.7	55.2	46.1	44.8	18.02	102.8
13	36.20	32.00	35.40	66.6	33.4	58.9	64.3	32.7	35.7	33.13	91.6
	54.50	46.22	51.96	66.0	34.0	56.0	63.4	32.4	36.6	48.18	88.4
	82.36	86.45	76.32	66.0	34.0	69.3	68.8	31.5	31.2	83.01	100.8

TABLE IX-A (Cont'd)

Animal Number	Whole Blood Level γ/ml.	Plasma Level γ/ml.	R.B.C. Level γ/ml.	Hematocrit Plasma	Hematocrit R.B.C.	% Plasma Act.	% Plasma Cor.	% R.B.C. Act.	% R.B.C. Cor.	Theoretical Whole Blood level γ/ml.	% Pento. in Theo. W. Blood
14	27.10	29.40	29.40	64.0	36.0	69.5	64.1	39.0	35.9	29.40	108.5
	30.22	32.10	31.11	64.5	35.5	68.5	65.2	36.5	34.8	31.75	105.0
	22.19	23.04	22.59	61.9	38.1	64.3	62.4	38.8	37.6	22.87	103.1
	17.63	15.86	16.32	60	40.0	54.0	53.7	46.5	46.3	17.46	100.5
15	21.35	32.20	24.80	52.9	47.1	79.8	59.3	54.7	40.7	28.71	134.5
	29.75	30.95	25.10	51.0	49.0	53.1	56.3	41.3	43.7	28.09	94.4
	17.09	15.29	19.86	51.0	49.0	45.6	44.5	56.9	55.5	17.53	102.5
16	42.35	45.10	lost	48.6	51.4						
	26.63	29.50	21.90	49.0	51.0	54.3	56.4	41.9	43.6	25.63	96.2
	24.25	23.45	30.75	52.3	47.7	50.6	45.5	60.5	54.5	26.93	111.1
	19.87	20.01	18.56	51.0	49.0	51.4	52.8	46.0	47.2	19.35	97.4
17	29.55	41.50	24.80	69.0	31.0	96.9	78.8	26.0	21.2	36.33	122.9
	24.35	25.10	17.70	53.0	47.0	54.6	61.4	34.3	38.6	21.64	88.9
18	143.00	171.00	151.80	49.7	50.3	59.4	52.7	53.4	47.3	161.35	112.8
	56.97	54.29	58.82	52.6	47.3	50.1	50.7	48.8	49.3	56.38	98.9
	41.40	39.87	37.65	52.0	48.0	50.1	53.5	43.6	46.5	38.80	93.7
	35.42	31.30	32.86	54.1	45.9	47.8	52.9	42.6	47.1	32.01	90.4
	24.73	25.03	22.98	55.8	44.2	56.5	57.9	41.1	42.1	24.13	97.6
	17.56	19.22	17.40	52.7	47.3	57.7	55.2	46.9	44.8	18.36	104.6
19	18.00	15.38	16.10	65.1	34.9	57.4	64.8	31.2	35.2	15.96	88.6
	27.90	22.42	19.70	55.0	35.0	52.2	67.9	24.7	32.1	21.47	76.9
	21.45	20.50	21.46	64.2	35.8	61.4	63.2	35.8	36.8	20.84	97.2
	22.55	21.10	23.59	63.8	36.2	59.7	61.2	37.9	38.8	22.00	97.6
	22.65	23.65	21.88	64.8	35.2	67.7	66.6	34.0	33.4	23.03	101.7

TABLE IX-A (Cont'd)

Animal Number	Whole Blood Level γ/ml.	Plasma Level γ/ml.	R.B.C. Level γ/ml.	Hematocrit Plasma	Hematocrit R.B.C.	% Plasma Act.	% Plasma Cor.	% R.B.C. Act.	% R.B.C. Cor.	Theoretical Whole Blood level γ/ml.	% Pento. in Theo. W. Blood
20	15.90	17.05	21.45	41.3	58.7	44.3	35.9	79.2	64.1	19.63	123.5
	13.25	12.91	14.61	42.0	58.0	40.9	39.0	63.9	61.0	13.89	104.8
21	99.00	122.20	107.10	55.6	44.4	68.6	58.8	48.0	41.2	115.49	116.6
	78.42	82.09	74.86	55.0	45.0	57.6	57.3	43.0	42.7	78.84	100.6
	45.76	46.20	42.98	54.9	45.1	55.4	56.6	42.4	43.4	44.74	97.8
	37.56	38.80	35.74	55.0	45.0	56.8	57.0	42.8	43.0	37.42	99.6
	30.86	29.50	30.30	56.7	43.3	54.2	56.0	42.5	44.0	29.85	96.7
	24.86	24.91	22.54	54.8	45.2	54.9	57.2	41.0	42.8	23.84	95.9
	17.56	18.02	16.56	55.2	44.8	56.7	57.3	42.3	42.7	17.37	99.0
25	31.40	25.60	29.40	64.2	35.8	52.4	61.0	33.5	39.0	26.97	85.9
	59.90	54.90	65.30	59.1	40.9	54.2	54.9	44.6	45.1	59.16	98.8
	34.67	35.40	32.60	61.9	38.1	63.2	63.8	35.8	36.2	34.33	99.0
20	19.10	18.65	14.00	41.9	58.1	40.9	49.0	42.6	51.0	15.94	83.5
	23.55	27.60	22.15	42.0	58.0	49.2	47.4	54.6	52.6	24.44	103.8
	13.30	11.08	19.85	42.0	58.0	35.0	28.8	86.5	71.2	16.16	121.5
22	15.92	15.85	16.32	62.8	37.2	62.5	62.1	38.1	37.9	16.02	100.6
	39.20	36.20	36.80	66.0	34.0	60.9	65.6	31.9	34.4	36.40	92.8
	34.95	38.97	33.60	64.9	35.1	72.4	68.2	33.7	31.8	37.08	106.1
7	19.66	21.47	25.57	58.4	41.6	63.8	54.1	54.1	45.9	23.18	117.9
	42.70	19.40	32.35	59.0	41.0	26.8	46.3	31.1	53.7	24.71	57.9
	48.62	45.36	39.47	58.0	42.0	54.1	61.3	34.1	38.7	42.89	88.2

TABLE IX-A (Cont'd)

Animal Number	Whole Blood Level γ/ml.	Plasma Level γ/ml.	R.B.C. Level γ/ml.	Hematocrit Plasma	Hematocrit R.B.C.	% Plasma Act.	% Plasma Cor.	% R.B.C. Act.	% R.B.C. Cor.	Theoretical Whole Blood level γ/ml.	% Pento. in Theo. W. Blood
23	15.48	16.40	18.58	68.4	31.6	72.5	65.7	37.9	34.3	17.09	110.4
	13.68	21.80	11.45	65.7	34.3	104.7	78.5	28.7	21.5	18.25	133.4
	33.60	27.25	17.75	66.0	34.0	53.5	74.8	18.0	35.2	24.03	71.5
	115.90	113.30	111.80	69.1	30.9	67.5	69.4	29.8	30.6	112.84	97.3
26	41.56	45.80	46.32	51.0	49.0	56.2	50.7	54.6	49.3	46.06	110.8
	43.00	45.50	47.60	52.7	47.3	55.8	51.6	52.3	48.4	46.49	108.1
	18.10	18.65	16.70	50.4	49.6	51.9	53.2	45.7	46.8	17.68	97.6
27	35.30	38.30	32.50	60.0	40.0	65.1	63.9	36.8	36.1	35.98	101.9
	34.95	34.55	29.20	61.9	38.1	61.2	65.8	31.8	34.2	32.52	93.0
	33.00	37.40	38.90	54.0	46.0	61.2	53.0	54.2	47.0	38.09	115.4
	37.40	29.00	33.95	60.0	40.0	46.5	56.2	36.3	43.8	30.98	82.8
	25.80	23.95	22.75	58.2	41.8	54.0	59.4	36.9	50.6	23.45	90.0
24	46.60	46.50	48.30	56.3	43.7	56.2	55.4	45.3	44.6	47.29	101.5
	39.35	36.00	32.55	56.3	43.7	51.5	58.8	36.1	41.2	34.49	87.6
	7.88	4.83	7.70	50.0	50.0	31.2	39.0	48.9	61.0	6.31	80.1
	20.50	16.55	16.97	52.5	47.5	42.2	51.9	39.1	48.1	16.75	81.3
	123.40	130.80	141.00	54.2	45.8	57.4	52.3	52.3	47.7	135.47	109.7
	32.20	36.80	33.90	55.0	45.0	62.9	57.0	47.4	43.0	35.50	110.3
28	93.60	92.50	88.30	59.0	41.0	58.3	60.1	38.7	39.9	90.78	97.0
	47.00	46.95	56.60	58.7	41.3	58.6	54.1	49.7	45.9	50.94	108.3

TABLE IX-A (Cont'd)

Animal Number	Whole Blood Level γ/ml.	Plasma Level γ/ml.	R.B.C. Level γ/ml.	Hematocrit Plasma	Hematocrit R.B.C.	% Plasma Act.	% Plasma Cor.	% R.B.C. Act.	% R.B.C. Cor.	Theoretical Whole Blood level γ/ml.	% Pento. in Theo. W. Blood
29	34.86	34.55	29.20	60.4	39.6	59.9	64.3	33.2	35.7	32.43	93.1
	25.80	23.95	22.75	52.7	47.3	48.9	53.9	41.8	46.1	23.38	90.7
	20.64	19.89	20.01	58.4	41.6	56.3	58.3	40.3	41.7	19.94	96.6
	14.89	15.21	14.75	60.0	40.0	61.3	60.8	39.6	39.2	15.03	100.9
	10.04	11.01	10.00	59.8	40.2	65.5	62.1	40.0	37.9	10.60	105.5
30	52.49	60.83	58.20	49.7	50.3	57.6	50.8	55.8	49.2	59.50	113.4
	40.90	33.24	30.65	48.5	51.5	39.4	50.5	38.6	49.5	31.90	78.0
	27.92	29.48	27.21	52.9	47.1	55.8	54.9	45.9	45.1	28.41	101.7
	20.90	21.61	19.42	50.0	50.0	51.7	52.6	46.5	47.4	20.52	98.2
	17.35	15.42	15.98	47.2	52.8	42.0	46.4	48.6	53.6	15.72	90.6
31	102.40	92.83	107.20	60.5	39.5	54.8	57.0	41.3	43.0	98.50	96.1
	89.26	94.82	85.90	57.3	42.7	60.9	59.7	41.1	40.3	91.01	102.
32	111.50	119.30	115.60	55.1	44.9	59.0	55.9	46.5	44.1	117.63	105.5
	99.26	101.40	93.26	55.1	44.9	56.3	57.2	42.2	42.8	97.74	98.5
	80.25	82.56	81.80	52.7	47.3	54.2	52.9	48.2	47.1	82.20	102.4
33	79.36	80.80	69.32	63.5	36.5	64.7	67.0	31.9	33.0	76.61	96.6
	91.56	89.30	90.31	65.2	34.8	63.5	64.9	34.3	35.1	89.65	97.8
	79.42	81.45	83.64	60.9	39.1	62.5	60.3	41.2	39.7	82.30	103.7
34	109.25	122.60	116.30	54.0	46.0	60.6	55.3	49.0	44.7	119.70	109.6
	94.36	99.80	92.31	54.0	46.0	57.1	55.9	45.0	44.1	96.35	102.1

TABLE IX-A (Cont'd)

Animal Number	Whole Blood Level γ/ml.	Plasma Level γ/ml.	R.B.C. Level γ/ml.	Hematocrit Plasma	Hematocrit R.B.C.	% Plasma Act.	% Plasma Cor.	% R.B.C. Act.	% R.B.C. Cor.	Theoretical Whole Blood level γ/ml.	% Pento. in Theo. W. Blood
35	42.63	38.27	40.40	58.4	41.6	52.4	57.1	39.4	42.9	39.16	91.8
	32.56	29.45	31.38	60.2	39.8	54.5	59.1	38.4	40.9	30.22	92.2
	17.24	16.85	20.92	58.6	41.4	57.3	53.5	50.2	46.5	18.53	107.2
	82.56	96.05	79.05	54.5	45.5	63.4	59.3	43.6	40.7	88.32	107.0
36	19.82	18.29	20.15	62.1	37.9	57.3	59.8	38.5	40.2	19.00	95.8
	25.36	26.20	27.00	60.5	39.5	62.5	59.8	42.1	40.2	26.52	104.6
	24.60	25.90	22.84	68.2	31.8	71.8	70.9	29.5	29.1	24.92	101.3
	20.75	18.34	16.55	69.0	31.0	61.0	71.2	24.7	28.8	17.78	85.7
	14.26	12.92	13.32	64.7	35.3	58.6	64.0	33.0	36.0	13.06	91.6
37	27.90	29.35	30.20	56.6	43.4	59.5	55.9	47.0	44.1	29.72	106.5
	24.50	22.96	24.05	56.4	43.6	52.9	55.3	42.8	44.7	23.44	95.7
	18.25	17.30	15.27	55.0	45.0	52.2	58.1	37.6	41.9	16.39	89.8
38	44.54	40.96	43.40	59.1	40.9	54.4	58.9	38.0	41.1	41.14	92.4
	35.16	36.20	34.40	59.0	41.0	60.8	60.3	40.1	39.7	35.46	100.9
	26.50	24.32	27.90	59.0	41.0	54.2	55.6	43.2	44.4	25.79	97.4
	21.75	20.84	23.60	57.8	42.2	55.4	54.7	45.8	45.3	22.01	101.2
	17.24	18.57	17.75	60.0	40.0	64.6	61.1	41.2	38.9	18.24	105.8
	14.36	15.60	13.95	58.3	41.7	63.3	61.0	40.5	39.0	14.91	103.8

Statistical Analysis:

Average - 99.3

Standard deviation - 10.8

95% Range - 78.1 - 120.5

TABLE IX-B

Comparison of Whole Blood-Serum Pentobarbital

Animal Number	Sample Number	Whole Blood Concentration µg./ml.	Serum Concentration µg./ml.	Per Cent Pentobarbital in Serum %
9	1	34.60	34.00	98.3
	2	29.83	27.70	93.5
10	1	31.00	34.30	110.6
	2	24.90	25.21	101.2
11	1	40.00	37.25	93.1
12	2	23.86	21.86	91.6
21	1	99.00	54.40	54.9
20-2	3	13.30	10.56	79.4
22	2	39.20	24.70	63.0
7-2	3	48.62	23.75	48.8
23	2	13.68	8.31	60.7
	4	115.90	50.10	43.2
26	1	41.56	17.05	41.0
24	7	32.20	33.65	104.5
28	1T-A	93.60	110.60	118.2
30	1	52.49	54.91	104.6
	2	40.90	38.64	94.5
	3	27.92	18.27	65.4
	4	21.61	21.54	103.1
Statistical Analysis:			Average	82.6
			Standard Deviation	24.6
			95% Range	34.4-130.8

TABLE IX-C

Comparison of Whole Blood-Clot Pentobarbital Concentration

Animal Number	Sample Number	Whole Blood Concentration µGm./ml.	Clot Concentration µGm./ml.	Per cent Pentobarbital present in clot. %
21	1	99.00	66.30	67.0
20-2	3	13.30	11.40	85.7
22	2	39.20	33.14	84.5
7-2	3	48.62	40.38	83.1
23	2	13.68	11.41	83.4
26	1	41.56	40.79	98.1
24	7	32.20	31.26	97.1
28	1	93.60	93.10	99.5
30	1	52.49	54.20	103.3
	2	40.90	42.94	105.0
	3	27.92	30.48	109.2
	4	20.90	18.62	89.1
			Average	- 92.1
Statistical Analysis:			Standard Deviation	-12.0
			95% Range	- 68.6 - 115.6

TABLE IX-D

Comparison of Erythrocyte Pentobarbital Content to that Present in the Washed Erythrocytes and the Saline Washings from these Erythrocytes.

Animal Number	Sample Number	% in R.B.C. %	% in Saline Washings %	% Recovery %
16	1	None	Lost	——
	2	None	110.7	110.7
	3	None	92.1	92.1
	4	None	103.1	103.1
17	2	6.2	88.3	94.5
18	1	0.7	91.4	92.1
	2	None	88.6	88.6
	3	None	108.7	108.7
	4	None	92.8	92.8
	5	None	98.0	98.0
19	1	None	89.2	89.2
	4	None	93.1	93.1
	5	None	85.7	85.7
20	1	9.7	90.1	99.8
	2	None	90.9	90.9
21	1	None	103.2	103.2
	5	None	107.3	107.3
	6	None	85.0	85.0
	7	None	92.5	92.5
25	1	None	87.1	87.1
	2	1.6	90.8	92.4
	3	11.3	92.8	104.1
20-2	1	None	119.9	119.9
	3	8.5	102.9	111.4
22	1	17.5	78.7	96.2
7-2	1	None	88.5	88.5
	2	None	98.5	98.5
	3	None	96.0	96.0

(TABLE IX-D)

Comparison of Erythrocyte Pentobarbital Content to that Present in the Washed Erythrocytes and the Saline Washings from these Erythrocytes

Animal Number	Sample Number	% in R.B.C. %	% in Saline Washings %	% Recovery %
23	1	None	80.3	80.3
	2	None	113.5	113.5
	3	None	83.6	83.6
	4	None	95.6	95.6
26	3	None	115.9	115.9
24	1	None	93.5	93.5
	2	None	101.6	101.6
	4	None	104.2	104.2
	7	None	87.8	87.8
28	1-A	1.3	103.9	105.2
	1-V	None	96.5	96.5
29	1	None	100.9	100.9
	2	None	99.6	99.6
	3	None	102.4	102.4
	4	7.2	103.4	110.6
31	1	None	94.3	94.3
Statistical Analysis:		Average	- 96.3	97.8
		Standard Deviation	- 9.6	9.2
		95% Range	- 77.5-115.1	79.8-115.8

TABLE IX-E

Comparison of Whole Blood Pentobarbital Content with that Present in Liver, Cerebrospinal Fluid, and Bone Marrow

Animal Number	Sample Number	Sampling Time in Minutes	Per Cent in Liver %	Per Cent in C.S.F. %	Per Cent in Bone Marrow %
5	2T	55	96.6	68.9	3.6
8	2T	380	94.3	65.3	6.5
13	3T	165	84.8	66.2	3.5
19	5T	355	104.8	62.7	8.5
25	3T	155	84.8	50.8	12.7
22	3T	350	85.4	67.0	10.4
7-2	3T	240	85.5	56.6	4.3
	4T	240			7.6
23	4T	590	81.8	66.0	1.8
24	7T	440	99.4	66.8	3.8
	8T	440	84.3		3.1
29	5T	245	98.2	62.3	7.6
31	2T	60	84.3	63.0	6.4
32	3T	100	90.8	65.9	4.1
	4T	100			4.3
33	3T	80	81.3	69.8	6.4
	4T	80	79.1	67.0	7.8
34	2T	45	104.1	60.7	6.9
	3T	45	96.3		7.4

(TABLE IX-E)

Comparison of Whole Blood Pentobarbital Content with that Present in Liver, Cerebrospinal Fluid, and Bone Marrow

Animal Number	Sample Number	Sampling Time in Minutes	Per Cent in Liver %	Per Cent in C.S.F. %	Per Cent in Bone Marrow %
35	4T	300	96.1	68.1	5.6
	5T	300	86.7		6.1
28*	1T-A	15	15.3	13.0	4.3
	Average		90.5	64.2	6.1
	Standard Deviation		8.0	4.9	2.6
	95% Range		74.8-106.2	54.6-73.8	1.0-11.2

* Dog #28 was not included in the statistical analysis since death occurred before adequate time for tissue distribution was possible.

TABLE X

Time-Rate of Pentobarbital Disappearance from Whole Blood

Animal Number	Dose of Drug mgm./Kg.	Time, in minutes, after pentobarbital injection. 45 %	90 %	120 %	150 %	180 %
4	37.10	100	74.3	60.6	44.9	36.1
6	32.60	100	80.0	62.0	47.2	38.7
7	30.00	100	69.5			35.5
29	32.00	100	74.0	59.2		42.7
30	36.00	100	77.9		53.2	39.8
38	35.00	100	79.0	59.5	48.8	39.4
35	35.00	100	76.4	___	___	40.4
Statistical Analysis:						
Average	34.00	100	75.9	60.3	48.5	38.9
Standard Deviation		0	3.6	1.3	3.5	2.5
95% Range		0	68.8-83.0	57.8-62.8	41.6-55.4	34.0-43.8

Figure 9.
(From TABLE X)

Graphic representation of the rate of disappearance of pentobarbital from the whole blood using the data supplied in Table X. The stippled area includes the statistical 95% range.

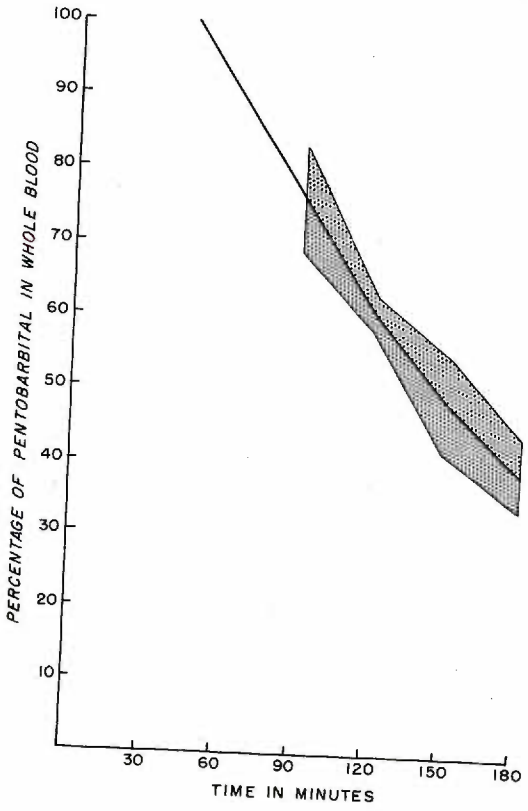
100
90
80
70
60
50
40
30
20
10
PERCENTAGE OF PENTOBARBITAL IN WHOLE BLOOD
30
60
90
120
150
180
TIME IN MINUTES

TABLE XI

Composite Statistical Values of included experimental results.

Specimen Number	Specimen Analyzed	Average	Standard Deviation	95 per cent Range
1.	Animal Weight in Kilograms	14.70	5.94	3.06-26.34
2.	Pentobarbital Arousal level in gamma/milliliter.	16.43	2.30	11.99-20.87
	a. Intravenous Group.	15.70	2.80	10.13-21.27
	b. Intraperitoneal Group.	17.40	1.60	14.17-20.55
	c. Oral Group.	13.60	0.60	12.48-14.72
3.	Comparison of Whole Blood-Serum concentration.*	82.6	24.6	34.4 -130.8
	a. Subgroup one.*	99.5	6.20	
	b. Subgroup two.*	53.9	9.7	
4.	Comparison of Whole Blood-Clot concentration.*	92.1	12.0	68.6 -115.6
	a. Subgroup one.*	84.2	1.3	
	b. Subgroup two.*	101.0	3.4	
5.	Comparison of Erythrocyte-Saline washings.*	96.3	9.6	77.5 -115.1
6.	Recovery following washing of Erythrocytes.*	97.8	9.2	79.8 -115.8
7.	Comparison of Whole Blood-Liver concentration.*	90.5	8.0	74.8 -106.2
8.	Comparison of Whole Blood-CSF concentration.*	64.2	4.9	54.6 - 73.8
9.	Comparison of Whole Blood-Bone Marrow concentration.*	6.1	2.6	1.0 - 11.2
10.	Time-rate of Pentobarbital disappearance from Whole Blood.			
	a. at 45 minutes.*	100.0	0.0	0.0 - 0.0
	b. at 90 minutes.*	75.9	3.6	68.8 - 83.0
	c. at 120 minutes.*	60.3	1.3	57.8 - 62.8
	d. at 150 minutes.*	48.5	3.5	41.6 - 55.4
	e. at 180 minutes.*	38.9	2.5	34.0 - 43.8

* Reported as percentage.