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Barbiturate Metabolism in the Livers of Chronic Ethanol-Fed Mice

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BARBITURATE METABOLISM IN THE LIVERS OF CHRONIC ETHANOL-FED MICE

by

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AUTOBIOGRAPHY

The author was born in Chicago, Illinois, on November 29, 1943. He has been a life long resident of Chicago. His grammar school education began at Our Lady of Grace and was completed at St. Cornelius due to his family moving to another part of the city.

He attended Weber High School where he received honors all four years and had perfect attendance. He was a member of the concert band and was a letter winner in football and track.

After completing high school he immediately pursued his life-long ambition of being a dentist by enrolling in pre-dental at Loyola University in Chicago. After completion of two years of pre-dental training he was fortunate enough to be accepted into Loyola University Dental School.

He successfully completed dental school and received his D.D.S. in June of 1967. Thereafter he began his graduate studies in Oral Biology at Loyola University.

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DEDICATION

To my dear wife, Diane, for her constant understanding and help.

To my parents for their love and guidance through life.

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CHAPTER I

INTRODUCTION

A. Introductory remarks:

There are a number of ways in which drugs can effect one another. They can enhance each other, prolong each other's effect, or inhibit each other. The factors which determine how one drug will effect another are many and varied, such as the nature of the drugs, their sites of action, their pathway of excretion and their chemical reactivity.

It is of a practical significance for anyone who prescribes or deals with the use of drugs to be aware of some of the causes and consequences of drug interaction.

There are many factors which are of importance in obtaining a proper perspective of drug metabolism. Among these factors are: absorption, metabolism, tissue binding properties, pretreatment with other

drugs, nutrional and hormonal states.

It is also important to know where in the body and in what type of systems that drugs are metabolized. The duration and intensity of action of drugs in the body is largely determined by the speed with which they are metabolized by enzymes in liver microsomes, and by the rates of excretion through various pathways.

B. Statement of the problem.

This study was designed to investigate the effects of chronic ethanol intake upon the rate of barbiturate metabolism by the liver of white mice.

CHAPTER II

REVIEW OF LITERATURE

There are a number of ways that the chronic administration of one drug can effect the results obtained from the use of another drug. The use of one drug can enhance, prolong, or inhibit the actions of another drug.

Drug metabolism is one of the factors which can affect the duration and intensity of drug action. The way in which a drug is metabolized can affect its action both qualitatively and quantitatively. It is important to remember that metabolism does not always cause inactivation, but can lead to a more active compound or one with essentially equal activity. Drugs can be metabolized in various tissues of the body. Which tissue is most important in metabolism depends upon the drug, and may be related to such things as the lipid and water solubility of the drug. An important site of metabolism for many drugs is the liver, but the hepatic cell has several locations of systems capable

of catalyzing drug metabolism. One area in the hepatic cell where several drug metabolizing systems seem to be concentrated is the endoplasmic reticulum. Drug metabolizing enzymes located there can be isolated from liver homogenates in the microsomal fraction. (Fouts, 1964)

Burns (1964) states that the chronic administration of one drug reduces the pharmacologic activity of another by stimulating its metabolic inactivation. Drugs exert this action by increasing the amount of drug metabolizing enzymes present in liver microsomes. It is thought that such "enzyme induction" can account for tolerance. The stimulatory effect of chronic barbiturate administration upon barbiturate metabolizing enzymes offers an explanation for tolerance and cross tolerance of the drugs. Phenobarbital or barbital increase the rate of metabolism of a wide variety of drugs. To predict the effect of a given dose of a drug, it is important to know whether the patient had been previously exposed to materials which

affect drug metabolism. He summarises that the chronic administration of certain drugs can cause an increase in metabolism of that drug.

Fouts (1964) has shown that the duration and intensity of drug action can be affected by 1.) drug metabolism, 2.) rate of absorption, 3.) distribution and tissue binding and 4.) excretion of the drug. As the rate of metabolism of a drug changes so does the duration or intensity of action of this drug change.

As a general class of drugs, inhibitors of drug metabolism are among the oldest drugs used, e.g. the anticholinesterases. However, inhibitors of the drug metabolizing enzymes in hepatic-microsomes are relatively new discoveries. Fouts (1964) generalizes that the inhibition of these microsomal enzymes is associated with rather marked effects on the duration of action of a variety of drugs has been confirmed and reported by many laboratories, this one included. In most of the cases studied by Fourts, he showed that inhibition

is associated with prolonging the action of several drugs. Such "enhanced" drug effects have been seen in both animals and humans and can be life threatening. Such "toxicity" is an extension of the therapeutic effects of most drugs. For example, hexobarbital usually produces hypnosis and some respiratory depression. In conditions in which the metabolism of hexobarbital is interfered with, the action of the barbiturate can be prolonged and enhanced causing a longer lasting hypnosis and a more pronounced depression of respiration than normal - in other words, the same effects one would get by giving several times the "usual" dose of hexobarbital.

Inhibitors can work in a number of ways. Netter (1962) explains that:

A.) The inhibitor can combine directly with the enzyme at the active center or at a point other than the active center.

B.) The inhibitor decreases the co-enzyme concentration by interfering with its bio-

synthesis or causing its depletion.

C.) The inhibitor suppresses metabolism in general by interfering with protein synthesis.

D.) The inhibitor changes the permeability characteristics of biological membranes.

The results of stimulating the rate of metabolism of a given drug are quite dependent upon how fast the drug is being metabolized even before stimulation, whether the metabolism is an inactivation or an activation. Fouts (1964) states that most compounds that stimulate the hepatic microsomal metabolism of drugs seen to act indirectly: 1.) the stimulation is slow to appear and usually is not seen for at least twelve hours after the stimulator is administered, 2.) does not occur when stimulator and enzymes are merely mixed together "in vitro", but is seen only when the stimulator is administered to a living animal or human, and 3.) is fairly long lasting with "abnormally" high enzyme activity that continues for several days. The

most widely accepted theory is that enhanced drug metabolism is a result of the presence of more drug metabolizing enzyme protein. The stimulators seem to increase the amount of these enzymes in the liver, either by stimulating their synthesis or by inhibiting their destruction or, possibly, by both mechanisms.

Burns' (1962) investigations reveal that considerable species differences have been reported for the metabolism of barbiturates. The mouse metabolizes hexobarbital twenty times faster than man. Burns concludes that the activity of liver microsomes affect the metabolization of drugs.

Burns et al (1963) showed that the pretreatment of rats with barbital, aminopyrine, phenadrine or phenobarbital did increase the activity of the liver microsomes to metabolize hexobarbital and this increased enzyme activity paralleled a shorter duration of hexobarbital sleeping time. The pretreatment of rats with drugs that induce the synthesis of drug

metabolizing enzymes also stimulates the metabolism of various naturally occuring compounds. Evidence suggests that drug administration increases enzyme activity by inducing the synthesis of more enzyme protein.

Hubbard (1949) discusses the mechanism of tolerance to thiopental in mice and summarizes it in the following:

A.) Mice develope a tolerance to the daily administration of 50 mgm./kgm. of thiopental. This tolerance is maximal in 5 to 6 days and amounts to a decrease in sleeping time of about 50%.

B.) The tolerant animals awaken at higher tissue levels than do controls. The degree of tolerance as determined by per cent reduction in sleeping time is significantly correlated with the tissue levels at awakening.

C.) The tolerance mechanism appears to be one of adaptation to higher thiopental tissue levels and not to either an increased rate of excretion or destruction.

Conney (1967) states that the duration and intensity of action of many drugs are largely determined by the speed at which they are metabolized in the body by enzymes in liver microsomes. The activities of these enzymes can be altered by dietary and nutritional factors, hormonal changes in the body, and the ingestion of foreign chemicals. Studies during the past decade have shown that the activities of drug metabolizing enzymes in liver microsomes are markedly increased when animals are treated with various hormones, drugs, insecticides and carcinogens. This increase in activity appears to represent an increased concentration of enzyme proteins and is referred to as "enzyme induction". The induction of liver microsomal enzymes is important pharmacologically, for it leads to an

accelerated biotransformation of drugs in vivo and so alters the duration and intensity of drug action in animals and man.

Brody (1955) shows that under normal circumstances the oxidative metabolism of living cells and of certain isolated systems leads to synthesis of compounds containing high energy phosphate bonds. These compounds are known to be essential for such functions as growth, muscle contraction, etc.. A number of substances are able to alter this relationship by depressing the formation of high-energy bonds without depressing simultaneously the oxygen consumption of the system. This dissociation of oxidation and phosphorylation is termed "uncoupling". Mitochondria isolated from a variety of sources appear to posses identical biochemical properties. The original studies were carried out on mitochondria from rat and rabbit liver and kidney. However, mitochondria from heart muscle, striated muscle, and spleen seem to contain the same enzyme

complement.

Since we are dealing with the liver a brief description of its anatomy and function is appropriate.

Hugill (1950) states that the liver size, double blood supply, and two types of functional cells with a remarkable power to regenerate are the main characteristics pertinent of its function. The per cent of blood contributed by the hepatic artery was found to range from 12-30 with an oxygen saturation of 85% under a pressure of 90-100 mm. of mercury. According to most investigations it contributes the major part of the oxygen supply. Ligation of the hepatic artery causes necrosis of the liver cells and is incompatible with life. The major blood supply to the liver is through the portal vein, which carries 40-75% of the blood at an O_2 saturation of 30% and a pressure of 10-20 mm. of mercury. Ligation of the main portal vein is incompatible with life, owing to shock and shunting of a large amount of blood in the intestinal

tract out of general circulation.

The substance of the liver is composed of histologic units called lobules, held together by areolar tissue. The hepatic artery and portal vein, upon entering the liver substance, branch into the interlobular vessels at the periphery of the lobules and these in turn give rise to the capillary - like vessels, the hepatic sinusoids.

The sinusoids (Bloom and Fawcett, 1950) are larger than capillaries and are different from them in that their lining cells are immediately associated with the epitheleal cells of the parenchyma with no intervening tissue.

The lining of the sinusoids consists of a very thin layer of cells and differs from a typical endothelium in that cell boundaries do not blacken with silver nitrate and the component cells are of two types. One is a typical endothelial cell whose cytoplasm extends as a thin film in the wall of the

sinusoid. It has a small compact nucleus that stains so darkly that structural details cannot be made out in it. The other is a fixed marcophage, the phagocytic stillate cell of von Kupffer. This is a larger type of cell with a large oval nucleus and a small, prominent nucleolus. The cytoplasm may extend into well-defined processes which give it a stillate appearance and it frequently contains granules of pigment, engulfed erythrocytes in various stages of disintegration, or iron containing granules. It may also sometimes contain droplets of fatty material. These cells phagocytize particulate materials injected into the blood stream.

The liver cells (Bloom and Fawcett, 1962) are polygonal in shape, with six or more surfaces. The surfaces are of three sorts: exposed to the subsinusoidal space, to the lumen of the bile canaliculus and to the adjacent liver cell. The cytoplasm of the liver cell presents an extremely variable appearance which reflects to some extent the functional state of the cell.

The principal source of variation is the content of stored material, principally glycogen and fat. In preparation of the usual histological sections both fat and glycogen have been removed, but the former presence of glycogen is indicated by irregular empty spaces, and that of fat by round vacuoles. By appropriate methods both fat and glycogen may be preserved and stained. The content of these materials in the liver may vary extensively with the diet or with the state of digestion. The amount of individual cells varies accordingly.

Since the main site of the metabolism of short acting barbiturates is the liver, it is important to know what part of the liver is actually responsible for it.

Fouts (1962) states that the endoplasmic reticulum is the in vivo structure which breaks up during homogenization to yield the fragments called microsomes.

Palade (1956) shows that most microsomes are

morphologically identical with the rough surfaced elements of the endoplasmic reticulum of hepatic cells. They appear as isolated, membrane bound vesicles, tubules and cisternae which contain an appearently homogenous material of noticeable density and bear small dense particles (100-150A) attached to their outer aspect. In solutions of various osmolar concentrations they behave like osmometers. Findings suggest that they derive from the endoplasmic reticulum by a generalized pinching off process rather than by mechanical fragmentation. The microsome fractions contain in addition relatively few vesicles free of attached particles probably dervied from the smooth surfaced parts of the endoplasmic reticula. Findings suggest that the microsomal RNA is associated with the small particles whereas most of the protein and nearly all of the phospholipids, hemochromogen, and DPNH cytochrome C reductase activity are associated with the membrane or content of the microsome.

Gram et al (1967) have separated the endoplasmic reticulum into a smooth and rough fragments and found that the smooth metabolized at a rate of five times faster. The data presented in their paper indicated that several hepatic-drug-metabolizing enzymes are predominately localized in the smooth-surfaced microsomal fraction in both rats and rabbits. This was performed on normal control animals.

Gram (1967) also showed the pretreatment of rats with phenobarbital markedly increased the activities of several hepatic-drug-metabolizing enzymes in smooth-surfaced microsomes relative to rough-surfaced microsomes. It would be interesting to see how certain drugs, mainly barbiturates, are metabolized in normal livers as compared to livers which have been under the influence of various drugs, namely alcohol. This is exactly why this experiment has been undertaken.

In reviewing all related literature as far back as 1946, I have been unable to find any discussion

directly related to this topic.

In this experiment I decided to use Pentothal since it is well known, and used daily, and about which a vast amount of literature has been previously written. The generic name is thiopental, trade name is Pentothal, and the chemical name is thiobarbituric acid (5-ethyl-5-1-methylbutyl).

The basic methods for analysing barbiturates as stated by Maynert (1949) are 1.) gravimetric, 2.) colorimetric, 3.) ultraviolet spectrophometric, 4.) isotopic, and 5.) pharmacological.

Richards (1956) grades these and other methods according to their specificity in the following chart:

METHOD	ESTIMATED LIMIT OF DETERMINATION (mg.)	SPECIFICITY
Galvimetric	10	Fair
Colorimetric	.05	Metabolites may interfer
Paper Chromatographic	.05	Good
Ultraviolet	.01	Metabolites may interfer
Infra Red	1-0.1	Good
Radioactive	.001	Metabolites may interfer

Hellmann (1943) was the first to describe a

spectrophotometric method for the estimation of thiopental in blood. His procedure utilized a characteristic absorption band at 288 mu. in ether solution.

Jailer and Goldbaum (1946) modified the method for thiopental by employing chloroform for the extraction and by making the measurement of the ultraviolet absorption in chloroform or preferably in sodium hydroxide solution.

Subsequently, Walker and Goldbaum (1948) described fairly similar procedures for the determination of 5, 5 disubstitued barbituric acids.

Gould (1949) and co-workers have reported the use of contimuous extraction with ether instead of multiple extraction with chloroform.

In 1952 Goldbaum described a variation of his earlier method of the ultraviolet spectrophotometric procedure for determination and differentiation of barbiturates.

In 1952 Shideman developed a method of analysing the metabolism of barbiturates which is worthwhile describing. His experiment was initiated in an attempt to increase the activity of a cell free homogenized preparation of liver by fortification with various substracts and cofactors.

The components of the system for the in vitro metabolism of thiopental are:

1.5 ml. Buffer KH_2PO_4, 0.1M (pH 7.0)

1.2 ml. KCl, 0.02M

0.3 ml. $MgCl_2$ 0.1M

0.2 ml. H_2O

0.9 ml. Succinate (sodium salt) 0.2M

0.6 ml. Nicotinamide, 0.6M

0.3 ml. Adenosinetriphosphate .00565M

0.5 ml. Cytochrome C, 0.00026M

0.5 ml. Thiopental 0.0048M

3.0 ml. Rat liver homogenate, 16.7 and in ice-cold isotonic containing K_2CO_3. (0.00016M K_2CO_3 in 1.15% KCl)

Increasing the amount of liver in the incubating

medium resulted in an increase in the total amount of thiopental metabolized.

Shideman (1952) showed that the acetate and pyruvate did little to improve the metabolism of thiopental. Krebs cycle intermediates citrate, - ketoglutarate, succinate, fumarate, and malate markedly enhanced the reaction. In control experiments the system was fortified with substrate (- ketoglutarate) and the cofactors cytochrome C, Adenosinetriphosphate, nicotinamide and DPN. The exclusion of nicotinamide resulted in the most striking decrease in metabolism of thiopental. The extracted liver standing at 0°C, either in the form of a whole tissue or homogenate, resulted in some cases in a slightly more active, in others, less active one. The activity of the homogenate was usually diminished about 30% by standing at 25°C and almost completely inactivated by standing at 38°C for three hours.

The activities of the fractions (supernate and

particulate) were not altered appreciably by variation in certrifugation speed between 500-2400 r.p.m.. Calculation of activity on a tissue dry weight basis demonstrated the activity of the supernatant was generally within the range of that of the whole homogenate; the particulate matter was about two to four times as active.

Results of these studies by Shideman (1952) suggest that the metabolism of the thiopental probably is not accomplished by the direct action of an enzyme or enzymes, but rather by a series of enzymatic reactions from which sufficient energy is released to attack the compound. The evidence giving support to such an hypothesis is that while only slight activity is exhibited by the non-fortified homogenate pronounced activation is brought about by the addition of an intermediary metabolite such as, ketoglutarate, succinate, fumarate, malate and to a lesser degree citrate, and by the addition of the cofactors, adenosinetriphosphate, cytochrome C, and nicotinamede. The effect of these

intermediary metabolites of thiopental is in some manner dependend upon the Krebs tricarboxylic acid cycle.

Maher and Puckett (1955) used a method similar to Goldbaum's but their method solves the three major drawbacks of the previous methods i.e. distortion of the absorption curve due to variation in concentration, inability to identify the molecular species of barbiturate specifically, and the distortion of the absorption curve due to background interference in extracts from body tissues and fluids.

A variation of Broughton (1956) procedure was the method chosen for our experiment. Later I will discuss the changes and improvements that I made.

Damm and King (1965) describe some finer methods of analysis. They state that barbiturates are isolated from biological materials by solvent extraction. This is done at physiological or acid pH, with or without first precepitating proteins. Various solvents

have been used but chloroform and ether are most common. The resulting extract is concentrated and analyzed using a.) paper, thin layer, or gas chromatography, b.) ultravioled analysis of an NaOH extract of the solvent, c.) a mercury, copper, or colbalt salt of the barbiturate which is soluble in an organic solvent.

I chose to use the ultraviolet spectrophotometric method because of its high degree of accuracy and sensitivity as compared to the other methods, the ease at which to check and rerun experimental findings, and the limited amount of apparatus needed thus reducing error.

Maynert (1949) states that the factor limiting the sensitivity of the ultraviolet spectrophotometric procedures is the ratio of the amount of barbiturates to the amount of other tissue "chromogens" concomitantly extracted. Therefore, in my experiment all possible precautions were taken to keep this to a minimum.

In Broughton's (1956) experiment tissues were extracted by homogenizing a weighted (1-5 gm.) sample with $CHCl_3$. This in turn was then extracted with 0.45N-NaOH. The absorption spectrum of the barbiturate in the clear alkaline solution was measured at pH 13.4 and at pH 10 (approx.) from 228-265 mu. in 1 cm. silica cells, with a Unicam SP 500 spectrophotometer.

In my experiment I kept the level of ethanol fed to the mice (three per cent) to a minimum to mininize liver damage. I was concerned with a normal liver which has been on a chronic, low-level ethanol diet. However, it is important to realize the consequence and effect of a prolonged administration of alcohol on the liver.

Many experiments have been preformed with varying concentrations of alcohol which produced fatty livers.

Richter and Campbell (1968) have shown that the amount of alcohol consumption which rats drink in a self selection situation is directly dependent upon

the concentration of the solution offered. It is usually about ten per cent.

Decarli and Lieber (1967) have replaced carbohydrate with alcohol to the extent of 36% of the total calories. Rats fed with alcohol developed fatty liver which was evident both morphologically and upon chemical analysis. After twenty-four days of alcohol, hepatic triglycerides had increased on the average six fold and cholesterol esters five fold; compared with the control. These studies demonstrate that prolonged alcohol intake can produce a fatty liver even when given with a diet with a nutrionally adequate content of protein, vitamins and minerals and an amount of fat less than that of the average American diet.

Many other experimenters have found the same results. Liber et al (1965) found that both in man and rats ethanol ingestion produces fatty livers, despite maintenance of adequate diets. Accumulation of fat in the liver can be produced by the consumation

of relatively moderate amounts of alcohol, either in addition to or as isocaloric substitution for carbohydrates in an otherwise adequate diet.

Liber (1965) noticed that rats, under normal feeding, would not consume alcohol in amounts greater than 20% of normal carbohydrate intake. This amount was too low to show significant changes. To increase this, the prolonged intakes of substantial amounts of alcohol was achieved by overcoming the natural aversion of rats for alcohol through incorporation of the ethanol in a completely liquid diet with the exclusion of any other source of food. In this manner they were able to substitute 36% of the total calories with alcohol. They concluded that alcohol does not support growth as well as carbohydrates. It was noted that in the alcohol fed mice the total body weight was less but the liver weight increased from fat. It was also concluded that the capacity of alcohol for producing a fatty liver is greater than that of fat itself.

Other investigations by Dajani et al (1961) showed that during prolonged alcohol treatment liver fatty infiltration could occur despite adequate food consumption. Moreover, hepatic lipid synthesis appears to be very much influenced by the metabolic reactions which ethanol undergoes in the liver. The increased levels of both ADH (alcohol dehydrogenase) and ACDH (acetaldehyde dehydrogenase) and the decreased activity of the citric acid cycle during chronic ethanol ingestion appear to play a role in the process of fatty infiltration. Withdrawal of ethanol before the occurrence of advance liver damage appears to cause a reversion of liver cells to near normal.

Iseri et al (1966) showed that livers of rats maintained on a completely synthetic, nutritionally adequate liquid diet containing ethanol showed lipid accumulation in the centrilobular cells with marked changes in the endoplasmic reticulum.

Rubin (1967) showed that alcohol induced fat

accumulation in the liver, resulting from changes in fat metabolism, may lead to alterations of hepatic cellular organelles, and that the escape of lipid from liver cells has been suggested as the cause of hepatic fibroses.

Rubin (1967) also has shown that the hepatic changes induced by isocaloric substitution of alcohol for carbohydrates in an otherwise complete diet include 1.) fatty metamorphosis, 2.) enlargement and disfiguration of mitochondria, 3.) vacuolization and increase in endoplasmic reticulum, 4.) increased in free polyribosomes and 5.) changes in bile structure. Rubin concluded that alcohol exerts a direct toxic action on the liver which precedes or is concomitant with fatty metamorphosis.

Lieber (1966) states that ethanol, which is readily absorbed from the G.I. tract, is oxidized at 90-98%. Trace amounts of ethanol may also be synthesized endogenously. It is generally accepted that the metabolic pathway for disposal of alcohol

involves oxidation of acetaldehyde followed by conversion to acetyl coenzyme A. The initial oxidation of ethanol to acetaldehyde is catalyzed by a zinic-containing enzyme alcohol dehydrogenase (ADH), which has been isolated in pure form from the soluble fraction of the liver cells; other tissues oxidize only small amounts of alcohol although more recently it has been pointed out that the gastroiatestinal tract may play a larger part than has been suspected.

Although in my experiment all the mice are receiving an identical diet with the exception of ethanol placed in the drinking water of half, we should be aware of that fact that various nutritional factors affect drug metabolism.

Dixon (1960) showed that starvation depresses hepatic-microsomal-drug-metabolism both as measured "in vitro" and "in vivo". This depression is believed to be caused by an actual loss of enzyme protein in the microsome. Oxidative pathways are affected more than reductive pathways and indeed starvation may

result in an activation or reduction of nitro and azo groups. Therefore, the nutritional status of the animal prior to drug administration becomes important as a factor in the response obtained.

Smith and Newman (1959) have shown that liver slices from fasting rats metabolize alcohol about one half as rapidly as do slices from fed rats. This inhibition has been shown not to be due to a deficiency in alcohol dehydrogenase or the DPN in the liver, nor to a metabolic block in the oxidation of the intermediaries acetaldehyde or acetate. Agents which reoxidize DPNA such as ferrecyonide and methylene blue, have been shown to increase the rate of alcohol metabolism by liver slices from fasting but not from fed rats, pyruvate and alamine raise the rate in both.

Booker et al (1952) have demonstrated that high protein and high carbohydrate diets increase the dosage requirement of thiopental for the maintenance of surgical anesthesia. They concluded that high protein and high carbohydrate diets improved hepatic

function; thus permitting more rapid transformation of thiopental. Herman and Wood (1952) have noted an enhanced effect of thiopental in lean rats as compated to those animals fed a fat supplemented diet.

CHAPTER III

EXPERIMENTAL APPROACH

In this experiment I used ninety male CFI mice. The mice were placed fifteen to a cage and separated into two groups. One group received normal drinking water while the other group received an ethanol-supplemented supply of drinking water. The amount of ethanol was nine per cent by weight. Except for their drinking water the two groups of mice were fed identical diets of standard laboratory chow. The food and drinking supply was constantly available for the mice, and since the number of mice per cage was kept to a minimum, there was no problem of some mice being crowded out of food.

In determining the concentration of Pentothal, I used a spectrophotofluorometer made by the American Instrument Company, catalog number 4-8202.

My initial task was to determine what wavelengths are the most accurate for a particular dilution of

Pentothal.

The manufacturers manual for the spectrophotofluorometer stated the following:

	MAXIMUM ACTIVATION	MAXIMUM FLOURESCENCE	
PENTOTHAL	315 um.	530 um.	pH 13

The ultimate sensitivity of the appartus for barbiturate analysis was stated at .1 microgram per liter.

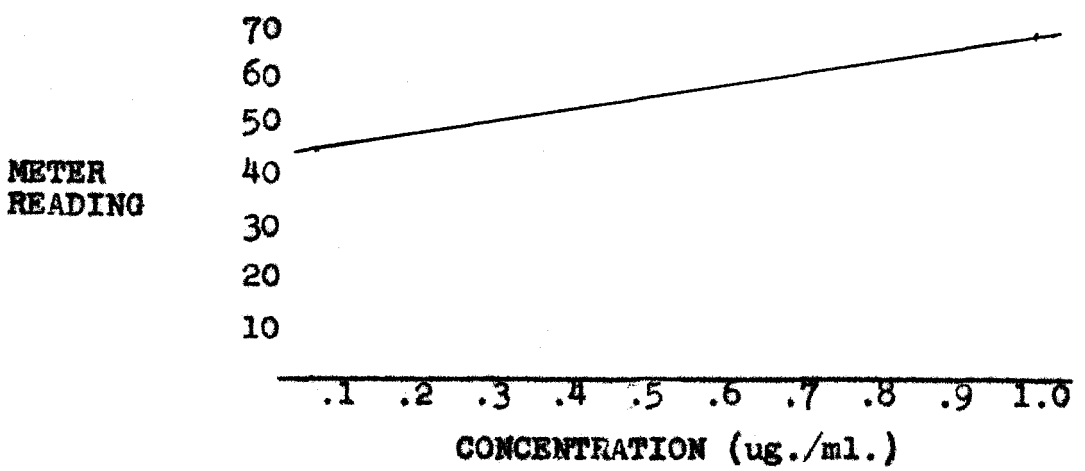
In order to adopt it to my particular situation a blank solution of .1N NaOH at a pH of 13 was placed into the spectrophotofluorometer. The maximum activation was determined to be 260 um. and the maximum flourescence or emission to be 510 um.

The meter of the spectrophotofluorometer is calibrated into a series of numbers ranging from one to fifty. These numbers represent linear scale values. The meaning of these numbers must be correlated for the particular type of experiment employing the spectrophotofluorometer.

Initially the following one milliliter solutions of Pentothal was mixed with two milliliters of sodium hydroxide (necessary to obtain at pH of 13): 0.3 mg./ml.; 0.03 mg./ml.; 0.003 mg./ml.; and 0.0003 mg./ml..

It was determined that the most accurate readings could be obtained between .1 ug./ml. and 1 ug./ml.. The following results were determined.

CONCENTRATION	METER MAGNIFICATION	NUMERICAL READING
0.1 ug./ml.	0.03	42
0.2 ug./ml.	0.03	38
0.3 ug./ml.	0.03	47
0.4 ug./ml.	0.03	49
0.6 ug./ml.	0.03	58
0.8 ug./ml.	0.03	62
1.0 ug./ml.	0.03	68



The latter results show the linear value of the meter reading related to the concentration of Pentothal present. The numerical reading is expressed in units were one unit equals x amount of Pentothal. The greater the number of units the greater the amount of Pentothal present.

In order to keep my readings consistant and maintain a peak barbiturate fluorescence, all determinations were performed at a pH of 13. A 0.1N solution of NaOH was used to adjust the pH of the final solution.

The mice were three weeks old at the time of arrival. Half of them were immediately placed on a nine per cent solution by weight of ethanol. After one week the concentration of ethanol was reduced to three per cent by weight. This was done to reduce the possibility of liver damage.

When the mice were seven weeks old, a group of normal and ethanol fed mice were sacrificed. The

ethanol fed mice had been receiving ethanol supplemented drinking water for four weeks. When the mice were eleven weeks old another group of normal and of ethanol fed mice were sacrified. At this time the ethanol fed mice had been receiving ethanol supplemented drinking water for eight weeks.

After this latter group of mice were sacrificed the drinking water of the ethanol fed mice was changed to plain water, not containing ethanol.

When the mice were fifteen weeks old both groups, those on normal drinking water and those who had initially been on the ethanol-supplemented diet were sacrificed.

The following is a detailed description of the refined methods that I used in each experiment.

On November 29, 1968 five of the twelve ethanol fed mice were weighed. Their average weight was 32.4 grams.

At that time the mice were seven weeks old and

had been on an ethanol-drinking solution for four weeks.

The twelve mice were sacrificed and their livers removed and kept cool. Slices of the liver were taken to be made into histologic slides in order to determine whether any pathological processes had occured.

The total weight of the extracted livers was 13 grams. A ten per cent homogenate, by weight, was made by adding 107 milliliters of saline to the livers and homogenizing them for five minutes in a blender.

Five milliliters of homogenate and one milliliter of Pentothal was added to each of seventeen test tubes and the contents were mixed. In order to establish a control group five milliliters of homogenate was was added to each of three test tubes containing one milliliter of saline. The contents were mixed.

A five milliliter sample of homogenate was placed in a weighing bottle. The homogenate in the

bottle was dried at 80°C and weighed one day later.

The twenty test tubes were placed in an incubator at 37°C. They were allowed to stand for five minutes in order to bring the chilled tubes up to a constant temperature of 37°C. The end of this initial warming up period was taken as zero time.

The tubes were removed at five minute intervals and one milliliter of cold ten per cent trichloroacetic acid was added to each tube. The contents were mixed, chilled, and centrifuged for five minutes.

One milliliter of supernatant was removed from each of the twenty test tubes and placed in another group of twenty test tubes correspondingly marked. Two milliliters of .1N NaOH were added to each of those test tubes in order to raise the pH to 13. That was the pH at which all reading were made.

Throughout the experiment all materials are kept cool with the exception of the time that the

test tubes spent in the incubator. The test tubes and materials were either kept in an ice water bath during use or placed in a refrigerator while not being used.

CHAPTER IV

PRESENTATION OF EXPERIMENTAL DATA

The following is a summary of the results obtained. Zero time is actually five minutes after the test tubes were placed into the incubator. This was done in order to have a starting temperature of 37°C. for all the test tubes.

A. ETHANOL FED MICE AGE 7 WEEKS

Average weight: 36 gms.
Weight of livers: 19.6 gms.
Dry weight: .251 mg./5ml.

0 MINUTES		5 MINUTES	
1. 6 units		5. 5½ units	
2. 6 units	6	6. 6 units	5.62
3. 6½ units	units	7. 5½ units	units
4. 5 units		8. 5½ units	

10 MINUTES		15 MINUTES	
9. 5 units		13. 5 units	
10. 5 units	5.37	14. 5 units	5.25
11. 5½ units	units	15. 5 units	units
12. 6 units		16. 6 units	

SALINE (0 MINUTES)

17.	7 units	
18.	7½ units	7.5
19.	8 units	units
20.	7½ units	

B. NORMAL MICE AGE 7 WEEKS

Average weight: 34.4 gms.
Weight of livers: 18.2 gms.
Dry weight: .213 mg./5ml.

0 MINUTES

1.	18 units	
2.	17½ units	
3.	16½ units	17.5
4.	18 units	units
5.	17½ units	

5 MINUTES

6.	16½ units	
7.	17 units	
8.	15 units	16.1
99.	16 units	units
10.	16 units	

10 MINUTES

11.	16 units	
12.	15 units	
13.	16 units	15.7
14.	16 units	units
15.	15½ units	

15 MINUTES

16.	17½ units	
17.	14½ units	
18.	16½ units	15.5
19.	16 units	units
20.	13 units	

C. ETHANOL FED MICE AGE 11 WEEKS ON THREE PER CENT ETHANOL FOR 7 WEEKS

Average weight: 34.4 gms.
Weight of livers: 25. gms.
Dry weight: .07 mg./5ml.

0 MINUTES

1.	18 units	
2.	17½ units	
3.	17 units	17.7
4.	17½ units	units
5.	18½ units	

0 MINUTES (SALINE)

21.	17 units	
22.	17 units	
23.	17½ units	17.7
24.	18½ units	units
25.	18½ units	

5 MINUTES

6.	17½ units	
7.	16½ units	
8.	16½ units	17
9.	16½ units	units
10.	18 units	

10 MINUTES

11.	15½ units	
12.	15 units	
13.	15½ units	15.5
14.	16 units	units
15.	15½ units	

15 MINUTES

16.	13½ units	
17.	13½ units	
18.	13½ units	13.4
19.	13 units	units
20.	13½ units	

D. NORMAL MICE AGE 11 WEEKS

Average weight: 36.4 gms.
Weight of livers: 23. gms.
Dry weight: .065 mg./5ml.

0 MINUTES

1.	36½ units	
2.	37½ units	
3.	36½ units	36.7
4.	36 units	units
5.	37 units	

0 MINUTES (SALINE)

21.	120 units	
22.	110 units	
23.	90 units	102
24.	90 units	units
25.	100 units	

5 MINUTES

6. 33 units
7. 30 units
8. 36 units
9. 33 units
10. 49

33 units

10 MINUTES

11. 32 units
12. 37 units
13. 32 units
14. 30 units
15. 29 units

30.7 units

15 MINUTES

16. 30 units
17. 28 units
18. 28 units
19. 32 units
20. 32 units

30 units

E. NORMAN MICE AGE 15 WEEKS

Average weight: 37.4 gms.
Weight of livers: 22.5 gms.
Dry weight: .111 mg./5ml.

0 MINUTES

1. 41½ units
2. 36 units
3. 38 units
4. 38 units
5. 41 units

38 units

0 MINUTES (SALINE)

21. 72 units
22. 74 units
23. 62 units
24. 58 units
25. 58 units

64.8 units

5 MINUTES

6. 36 units
7. 30 units
8. 35½ units
9. 32 units
10. 32½ units

32.8 units

10 MINUTES

11. 29 units
12. 24½ units
13. 26½ units
14. 34 units
15. 28 units

29.4 units

15 MINUTES

16. 28 units
17. 24 units
18. 26 units 25.8
19. 26 units units
20. 25 units

F. MICE THREE WEEKS OF OF THREE PER CENT ETHANOL AGE 15 WEEKS

Average weight: 36.0 gms.
Weight of livers: 19.6 gms.
Dry weight: .110 mg./5ml.

0 TIME

1. 24½ units
2. 24½ units
3. 20½ units 21.9
4. 20 units units
5. 20 units

0 TIME (SALINE)

21. 24½ units
22. 24 units
23. 25½ units 26.6
24. 30 units units
25. 29 units

5 MINUTES

6. 18½ units
7. 18 units
8. 19½ units 19.2
9. 19½ units units
10. 20½ units

10 MINUTES

11. 17½ units
12. 16½ units
13. 17½ units 17.8
14. 20 units units
15. 17½ units

15 MINUTES

16. 16 units
17. 16 units
18. 15½ units 15.5
19. 15 units units
20. 15.6units

CHAPTER V

DISCUSSION

When the first group of normal mice were sacrified, their livers treated as outlined above and after adding the Pentothal and then NaOH, the reading on the spectrophotofluorometer was 17.5 units at "zero" time. Fifteen minutes later the reading was 15.5 units. Therefore, two units out of 17.5 units had disappeared, presumably by being metabolized. This shows that 11.4% of the Pentothal present at "Zero" minutes disappeared in a fifteen minute period.

Using this same type of reasoning the following results can be shown:

GROUP I AGE 7 WEEKS

	NORMAL		ETHANOL	
0 Minutes	17.5 units	11.4%	6 units	12.5%
15 Minutes	15.5 units		5.25 units	

GROUP II AGE 11 WEEKS

	NORMAL		ETHANOL	
0 Minutes	36.7 units	18.2%	17.7 units	24.3%
15 Minutes	30.0 units		13.4 units	

GROUP III AGE 15 WEEKS

	NORMAL		ETHANOL	
0 Minutes	38.0 units		21.9 units	
15 Minutes	25.8 units	32.1%	15.5 units	29.1%

By examining the results from the normal mice, it can be seen that there is a steady increase in the amount of Pentothal being metabolized as the mice grow older. This can be accounted for on the basis that, as the mice grow older, the liver matures and its efficiency to metabolize Pentothal seems to increase.

If we compare the per cent of Pentothal metabolized in the ethanol fed mice, it also shows a steady increase. In group one the livers of the ethanol fed mice metabolize a slightly higher per cent of Pentothal than do those of the normal mice. However, in group two, the livers of the ethanol fed mice show a very markedly higher per cent of Pentothal metabolized. These mice had been receiving ethanol supplemented drinking water for eight weeks.

The increased rate of metabolism of the livers of the ethanol fed mice can be accounted for by enzyme induction. This means that the chronic administration of ethanol there had induced an increase in the level of enzymes in the liver microsomes which increased the rate of metabolism of Pentothal.

In group three the previously ethanol fed mice had been returned to normal drinking water for four weeks. There was a moderate increase in the per cent of metabolism of Pentothal due to maturation of the liver. Since the administration of ethanol had been discontinued and not present to act as a stimulus for enzyme induction, the marked increase in the per cent of metabolism of Pentothal was not demonstrated.

CHAPTER VI

SUMMARY AND CONCLUSION

Two groups of mice were fed identical diets with the exception that on group received an ethanol supplemented drinking solution. The environment of both groups of mice was the same and constant. The two groups of mice were sacrified, twelve at a time at one month intervals over a three month period. After the second group of mice was sacrified, those mice receiving ethanol supplemented drinking water were switched to normal drinking water. I was investigating the effect of chronic ethanol intake upon the rate of barbiturate metabolism by the liver. The barbiturate that I used was Pentothal. Its rate of metabolism in the various groups of mice was compared.

This experiment shows that as the mice mature the rate of metabolism of Pentothal in the liver increases. It also shows that the mice receiving ethanol supplemented drinking water metabolize Pentothal at a greater rate than the mice receiving normal

drinking water. The increase in rate of metabolism of Pentothal is directly proportional to the length of time that the mice are receiving ethanol supplemented drinking water. When the mice are taken off the ethanol solution and returned to normal drinking water there is a noticable decrease in the rate of metabolism of Pentothal. This decrease in metabolism is due to a lack of enzyme induction previously stimulated due to the chronic administration of ethanol.

CHAPTER VII

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APPROVAL SHEET

The thesis submitted by Dr. Thomas S. Golec has been read and approved by four members of the Department of Oral Biology.

The final copies have been examined by the Director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 26, 1969
Date

Signature of Adviser