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THE METABOLIC AND PHYSIOLOGICAL EFFECTS OF SOME PHARMACOLOGICALLY ACTIVE SUBSTANCES ON THE COURSE OF THIOPENTAL ANAESTHESIA

BY

HANS KÜNDIG

THESIS SUBMITTED FOR THE DEGREE OF MASTER OF SCIENCE AT THE UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG, JUNE 1968.

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WITHOUT WHOSE PATIENCE, FORBEARANCE AND COMFORT THIS DISSERTATION COULD NOT HAVE BEEN COMPLETED

ACKNOWLEDGEMENTS

I am extremely grateful to the University of the Witwatersrand for having made it possible for me to submit this dissertation for the degree of Master of Science.

Professor P.R. Levy and Professor J.H. Gear constantly stimulated and advised and encouraged me during difficult times, and without their help the work could not have been completed. I am particularly grateful to Professor Levy and his department who have sponsored me as a candidate for a higher degree. Professor Levy has drawn my attention to the biochemical aspects of Pharmacology; my horizons have been broadened, and I shall continue in the future to apply the chemical approach to the best of my ability. Professor Gear's special contribution has been to curb my tendency to be distracted by irrelevant issues, and to stiffen my resolve when I was disheartened.

I am grateful to Dr. N. Savage for many fruitful discussions, and to Dr. N.W. Levin and Professor

R.W. Charlton for checking drafts. Professor Charlton has also been most helpful in advising me about grammar.

Miss L. Lucas and the staff of the Medical School Library, particularly Miss A. van Hoogstraten, Mrs. C. Botha, Miss M. Morris, Miss A Sacks and Mrs. A. Koen have been very kind and obliging in obtaining several hundred references from inter-library loans, as well as in re-checking the references quoted in this dissertation. I am also grateful to Miss D. Lee, Miss J. Timmerman and Miss J. Willis for the typing of the several drafts and the final stencil copies; to Mr. A. Shevitz of the Department of Medicine and Mr. M. Ulrich of the South African Institute for Medical Research for the photographs; and to Mr. H.C. McGarity of M.S.D., Dr. B.M. Burger of S.K.F. Laboratories, Mr. P.J. van Heerden and Mr. D.J. Potgieter of Abbott Laboratories and Mr. M. Holland and Mr. R. Smith of Ciba for the drugs used throughout this dissertation.

For the assistance given in the reproduction of diagrams and photographs onto stencils, I wish to thank Mr. J.B. Cooney and Mr. R. Phillips of Rank Xerox.

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PREFACE

It is scarcely possible to study any aspect of pharmacology without becoming aware of the interaction between drugs. A drug may modify the effect of a second drug in a number of different ways. Its action may be directly antagonistic or synergistic. It may potentiate or inhibit the metabolic manipulation of the second drug. It may accelerate or slow down its excretion. It may alter its transport across cellular membranes. Finally, its effect on the physiology of the body may indirectly alter the action of the second drug, e.g. through changes in the body temperature, the circulation or the comppsition of the body fluids. All these possible mechanisms of drug interaction have to be considered whenever two drugs are administered simultaneously. Furthermore, the effect of a drug administered even a considerable time previously may still be detectable when the second drug is given.

During a number of years of personal experience in various areas of experimental pharmacology the interaction between drugs has made a deep impression on the present author. One aspect of the subject which has been of particular interest is the effect of a large variety of drugs on the duration

and depth of anaesthesia. This type of drug interaction is of importance in those pharmacological methods involving the use of intact animals under anaesthesia. Anaesthesia produced with any type of anaesthetic agent may be affected by concurrent drug administration.

In spite of a considerable amount of investigation, understanding of many aspects of this subject appears incomplete at the present time. The present study is an attempt to gain further insight into some of these problems.

ABBREVIATIONS

The following abbreviations are commonly employed in learned journals, and will be used throughout this dissertation:

ATP	=	ADENOSINE TRIPHOSPHATE
C.N.S.	=	CENTRAL NERVOUS SYSTEM
5-HIAA	=	5-HYDROXYINDOLACETIC ACID
5-HT	=	5-HYDROXYTRYPTAMINE, SEROTONIN.
5-HTP	=	5-HYDROXYTRYPTOPHAN
LD_{50}	=	GRAPHICALLY CALCULATED DOSE WHERE 50% OF THE ANIMALS DIED.
a-MD	=	ALPHA-METHYLDOPA (α-METHYL-3,4-DIHYDROXYPHENYL-ALANINE)
TLC	=	THIN LAYER CHROMATOGRAPHY

DEFINITIONS

SEDATION

This is a state of reduced activity, but nevertheless of awareness of the surroundings. The animal, when disturbed, will immediately shift its resting place. There are various degrees of sedation:-

a) light sedation which can only be detected by means of an activity-measuring apparatus such as the actograph.

b) moderate sedation which can be easily observed, but during which the animals can be disturbed by noise and touch.

c) marked sedation or hypnosis (sleep). The animals maintain their righting reflexes but are ataxic and inactive unless subjected to painful stimuli. Noise alone does not arouse them.

ANAESTHESIA

No stimuli will arouse the animals. There is a complete loss of righting reflexes. The first sign of recovery is that the animals regain their righting reflexes.

SLEEPING TIME (or Anaesthetic time)

The term 'sleeping time' will frequently be used in this dissertation. It specifies the time which elapses between the loss and the return of the righting reflexes. The duration of post-anaesthetic ataxia and sedation are not included when assessing the sleeping time.

LOSS OF RIGHTING REFLEXES

This indicates that the animals may be placed on their backs and remain in this unnatural position without any movement of the limbs.

Partial loss of righting reflex specifies that the animals will remain in the position as described above, but exhibit spontaneous movements of the limbs and usually turn onto their sides.

CHALLENGE

The verb 'to challenge' is a highly specific term which is in common usage in some scientific journals (Pinakatt and Richardson, 1964; Roth and Tabachnick, 1965; Miller, 1966). 'Challenging' in this dissertation refers to the effects on the whole organism of a standard dose of substance 'A' after

pretreatment with a carefully defined dose of substance 'B'. It has a stronger meaning than the 'testing' stimulus used by neurophysiologists since it refers to the intact animal rather than to a system, organ or tissue.

1.00 INTRODUCTION

1.10 HISTORY OF BARBITURATE ANAESTHESIA:

Barbituric acid (malonylurea) was first synthesised in 1863/64 by von Baeyer. In 1882 Conrad and Gutzheit prepared the first barbituric acid derivatives by a different method, namely 5,5-diethyl-barbituric acid (Barbital), yet more than twenty years passed before its potent hypnotic action was described by E. Fischer and von Mering (1903). Since that time many hundreds of barbituric acid derivatives have been synthesised.

Before 1935 only a few 2-thiobarbiturates were known. They were mainly used as intermediates for the preparation of their corresponding oxybarbiturates (Miller, Much and Crossley, 1935). Tabern and Volwiler (1935) were among the first to be associated with reports on the pharmacological action of 2-thiobarbiturates. It was demonstrated that the drugs produced a profound anaesthesia of short duration, when injected intravenously in the form of their sodium salts (Miller *et al*, 1936). Thiopental was the first of the 2-thiobarbiturates which was found to be suitable for clinical use

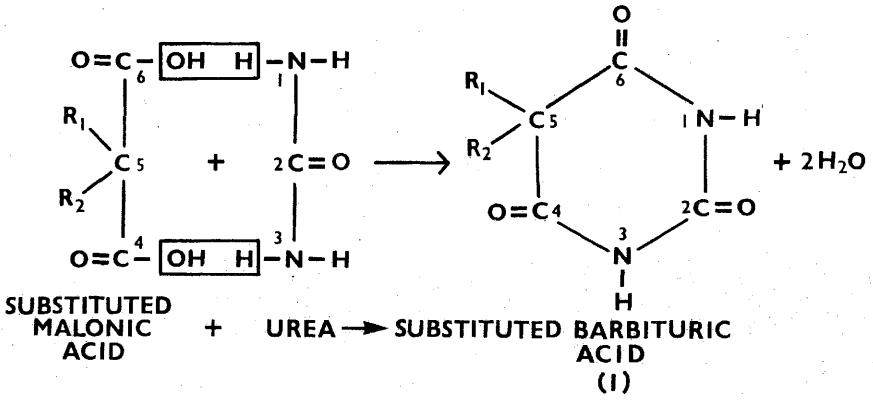
(Lundy, 1935; Volwiler and Tabern, 1939). Many different thiobarbiturates have since been prepared and introduced into the clinical field, but disappeared after periods of popularity (Carrington and Raventós, 1946; Walter, Goodson and Fosbinder, 1945; Dornette and Tuohy, 1951; Irwin et al, 1956). Today only thiopental still enjoys widespread use.

Ultra short-acting oxybarbiturates are also used for the induction of anaesthesia. Oxybarbiturates suitable for use as anaesthetic agents have a high lipid:water partition coefficient. The oxybarbiturate which is used most frequently for the induction of anaesthesia is methohexital (Weyl, Unal and Alper, 1958; Taylor and Stoelting, 1960). No non-barbiturate induction anaesthetic compound with advantages over thiobarbiturates for general use has yet been described.

Hydroxydione, a synthetic steroid hormone (Selye, 1941; P'an et al, 1955; Laubach, P'an and Rudel, 1955) and gamma-hydroxybutyrate (Laboriet et al, 1961; Blumenfeld, Sumtay and Harmel, 1962) both have certain drawbacks as practical anaesthetics,

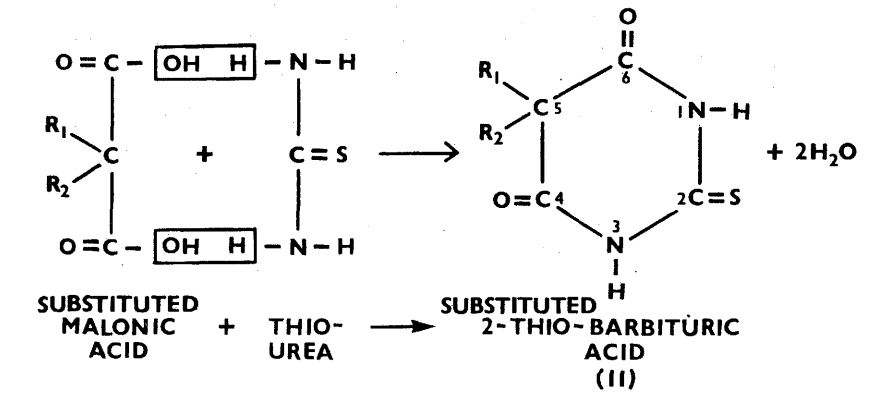
while Propanidid, a recently introduced eugenol derivative is too short-acting to become widely used (Cole, 1965; Zindler, 1965). Thiopental remains the intravenous anaesthetic of choice.

FIGURE 1



Simplified chemical reaction in the synthesis of substitued oxybarbituric acid derivatives.

FIGURE 2



Simplified chemical reaction in the synthesis of substituted thiobarbituric acid derivatives.

1.20 CHEMISTRY OF THE BARBITURATES

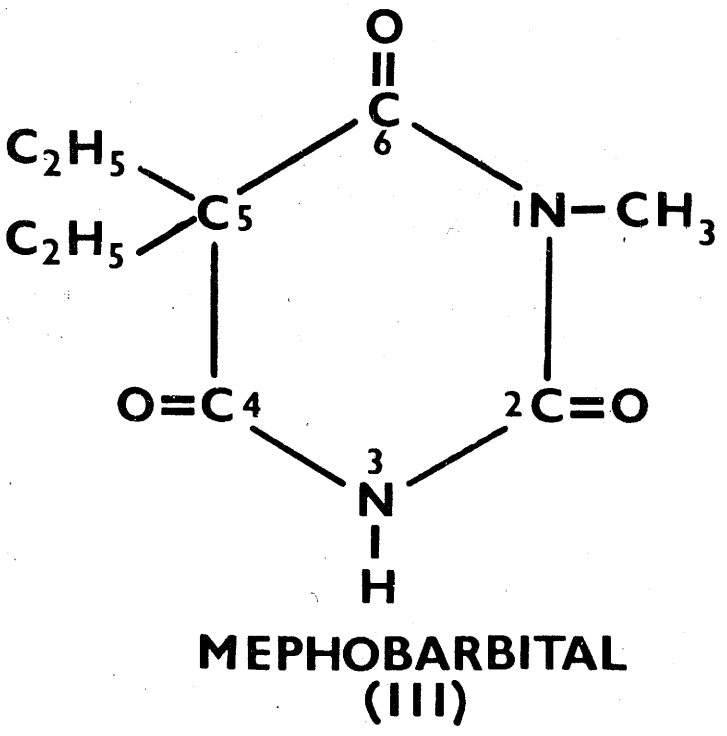
SYNTHESIS

The most important method for the synthesis of barbituric acid and its derivatives (including the thiobarbiturates) is based on the original method of E. Fischer and Dilthey (1904). Diethyl malonate and its alkyl and aryl-substitution products are condensed in the presence of sodium ethylate with urea or thiourea to give oxybarbiturates and thiobarbiturates respectively (Fig. 1 and 2).

It will be noted that substituted malonic esters give rise to 5 substituted barbiturates. These substances generally exhibit activities ranging from central convulsants through sedatives, hypnotics and even anaesthetics. Only the latter group will be considered in this dissertation.

Barbiturates possessing marked pharmacological effects have the following structual features in common. (i) Both hydrogen at position 5 (Fig.1(i) and 2(ii)) have to be substituted. This sub-

FIGURE 3



MEPHOBARBITAL
(III)

An example of a N-1 substituted barbituric acid derivative.

stitution can be two alkyl groups (e.g. 5,5-diethylbarbituric acid - Veronal) or one substituent may be alkyl and the other aryl (e.g. 5,ethyl-5-phenylbarbituric acid - Phenobarbital).

(ii) All active barbiturates (Fig. 2(ii)) have either oxygen or sulphur at C-2.

(iii) The hydrogen atoms on N-1 and N-3 may also be replaced by alkyl groups (e.g. 1-methyl-5-ethyl-5-phenylbarbituric acid - Mephobarbital). (Fig. 3).

All barbiturates are acidic, and form salts with alkali metals. These salts are far more soluble than the parent compounds, their aqueous solutions having a strongly alkaline reaction.

Barbiturates in the free acid form are usually very soluble in organic solvents such as chloroform, diethyl ether, benzene and alcohol, but are relatively insoluble in petroleum ether and water (Warren, 1943).

The degree of chemical stability of the individual barbituric acid derivatives varies with the position, shape, kind and number of the substituent

groups. Salts of barbituric acids rapidly hydrolyse in water to form alkaline solutions. Sodium salts are the commonly used compounds. Solutions of these have a pH between 9 and 10. Buffering has little practical value in stabilisation as has been demonstrated by Husa and Jatul (1944).

1.30 IDENTIFICATION AND QUANTITATIVE DETERMINATION OF BARBITURATES

Numerous tests and methods have been described in the past for the characterisation of individual barbituric acids. Such qualitative identifications and quantitative determinations of barbiturates are of importance in studying their distribution, fate and excretion. Unfortunately, few of the tests are specific, and with the generally used methods one can only examine a single compound at a time.

It is impossible to follow accurately a mixture of barbiturates in a biological system if the compounds are not isotopically labelled, e.g. with ^{14}C or ^{35}S, etc. For these reasons many of the barbiturates of clinical importance have been radioactively labelled for '*in vivo*' studies. Thiopental received some attention in this respect and these methods have been used by some investigators (Taylor, Richards and Tabern, 1952; Wase and Foster, 1956).

Paper chromatography and thin-layer chromatography may be of some value in this connection. They may help to differentiate qualitatively between certain barbituric acid derivatives, for example between thiobarbiturates and their oxy-congeners. The order of sensitivity of techniques described in earlier publications is, however, rather low. It was suggested that a minimum of 50 µg. was required for identification and quantitative determination (Grieg, 1952; Kjaer and Rubinstein, 1953; Paulus and Mallach, 1954; Deiniger, 1955 and others).

However, there are specific methods for qualitative determination, based upon the physical properties of the individual barbituric acids and their derivatives e.g. a precise melting-point determination carried out in combination with the measurement of the index of refraction of the melts (Brandstätter, 1951; Büchi and Perlia, 1954).

A micro-sublimation method followed by melting point and mixed melting point determinations are other possible methods for the identification of barbiturates (Turfitt, 1948).

An ultraviolet spectrophotometric method has been described and is probably the most extensively used technique for some of the barbituric acid derivatives (Goldbaum, 1948; 1952; Brodie et al, 1950; Brodie et al, 1953). The method is based on obtaining characteristic ratios from the absorption data determined at various wave lengths and with different pH values of the solution.

The specific absorption spectra of oxybarbituric acid derivatives have a maximum at 220 mμ in an acid media and 255 mμ in an alkaline solution, while thiobarbiturates exhibit these at 290 mμ in acid and 305 mμ in alkaline media (Goldbaum, 1948). The method is probably one of the best established and also seems to be one of the fastest techniques available at present, and has therefore been most widely used during the past twenty-five years (Elvidge, 1940; Stuckey, 1941; Born, 1949 and others). The disadvantage of this method is that barbituric acids and their corresponding metabolites with an un-

broken ring show the same spectra within the group.

Infra-red absorption spectra have also been used in the identification of various barbiturates. Characteristic absorption bands can be obtained when the barbiturates are mulled in Nujol (Canback, 1956; Ribley et al, 1951; Umberger and Adams, 1952). The spectra of some of the investigated barbiturates contain the C-H stretching band between 3 and 3.6u and the C-O band between 5.6 and 6μ and the C-H bending bands between 7 and 7.8μ. Price and his co-workers (1954) have pointed out that there is apparently a relationship between the infra-red absorption spectra and the pharmacological action. Slightly higher band frequencies are found in those barbiturates which have a prolonged depressant action. This indicates slightly lower polarities and greater fat solubilities.

A technique infrequently applied to identify barbiturates is polarography. Zuman (1954) used this method to determine several 2-thiobarbiturates (see also Brezina and Zuman, 1958). A dropping mercury electrode is usually used, but rotating platinum

electrodes have also been employed. The supporting electrode is most commonly a 0.05M solution of borax.

The most recent addition to the methods for identification and quantitative determination of barbiturates is the gas-liquid chromatographic technique. Very small amounts (less than 1 mcg) in mixtures can be detected readily in a relatively short time (Brochmann-Hanssen and Svendsen, 1961; Parker and Kirk, 1961; Reith et al, 1965; Anders, 1966).

1.40 GENERAL PHARMACOLOGY OF BARBITURATES.

THE MECHAMISM OF DEPRESSANT ACTION.

Barbiturates (excluding the few with convulsant action) can be used to provide a degree of depressant action from mild sedation to deep surgical anaesthesia by varying the dosage. The precise mechanism or mechanisms by which the barbiturates produce their depressant effect is still unknown (Butler, 1950; Cohen and Dripps, 1965).

H. Meyer (1899 and 1901) and Overton (1901) were the first to suggest that the potency of a depressant drug is in direct proportion to its partition coefficient between lipid and water. Applied to living organisms, this would suggest a partition between cell lipids and the body fluid consisting of lymph, blood and extravascular fluids.

K.H. Meyer and Hemmi (1935) have restated the above hypothesis and suggested that a depressant action appears if a chemically indifferent substance has penetrated into the cell lipids to

reach a definite molar concentration. This modification of the earlier theory seems to be more satisfactory in that it reconciles certain inconsistencies previously not understood.

It has been shown that the higher the lipid solubility of a barbiturate, the more readily will it pass through biological membranes (such as blood-brain and other tissue barriers) and the faster will be the onset of the action (Butler, 1942; Mayer, Maickel and Brodie, 1960).

Thiobarbiturates generally, and thiopental specifically have high lipoid solubility (Mark et al, 1958) and it is believed that the short duration of action of thiobarbiturates is due to their redistribution (Brodie, 1952) and not to their rapid detoxication as formerly suggested.

The concentration of thiopental in the plasma decreases rapidly within minutes of the injection as the drug passes through cell membranes into the tissues. Initially it was thought that the body fat depots were the major site of localisation (Brodie et al, 1950), but it has been shown that

other organs and tissues such as skeletal muscle, liver and skin are more important (Goldstein and Aronow, 1960; Block and Ebigt, 1960b and 1961). The poorer blood supply of adipose tissue may account for this. The result of the rapid decline in plasma concentration is that thiopental moves out of the central nervous system, and when the anaesthetic threshold is reached consciousness returns. Although the initial rate of decrease in the plasma concentration is rapid, as equilibration with the tissue concentrations is approached, the fall is much slower. A second and subsequent doses of thiopental will therefore produce a more marked and prolonged anaesthetic effect. Not only will a higher initial plasma concentration result, but the rate of decrease in plasma concentration will be slower, because of the thiopental already present in the tissues.

The mechanism of action of barbiturate anaesthesia may possibly be related to its effects on cellular oxidative phosphorylation. For example, it has been observed (Hulme and Krantz, 1956) that there

is a correlation between the hypnotic activity of a given barbiturate and its ability to uncouple oxidative phosphorylation. It appears that this hypothesis does not hold for all barbiturates and probably depends on the method applied as well as the tissue from which the mitochondrial preparation has been obtained. Brain mitochondrial preparations are rather unstable and therefore the interpretation of the result becomes uncertain (Brody and Bain, 1954; Brody, 1955; Aldridge and Parker, 1960).

It has also been shown in dogs that pentobarbital caused a reduction of about one-third in oxygen uptake and utilisation of glucose. Metrazol, a central nervous system stimulant restored these functions to normal.

It is however, difficult to correlate the action of depressant drugs with their inhibition of carbohydrate oxidation. It has been demonstrated that convulsant drugs (including certain barbiturates) often produce a similar inhibition in vitro.

An example is the convulsant barbiturate 5-(1,3-dimethylbutyl)-5-ethyl-barbituric acid (Swanson and Chen, 1939) which inhibits brain oxidative processes *in vitro*. In some cases, therefore, pharmacologically antagonistic substances produce almost identical biochemical effects.

The effects of barbiturates on various enzyme systems in brain and liver have been studied extensively, but these studies do not contribute much to understanding the mechanism of action of the drugs. For example, it has been shown that barbiturates inhibit the oxidation of glucose (Jowett and Quastel, 1937; Quastel, 1939) and of pyruvate, but that they have little effect on the oxidation of succinate or *p*-phenylenediamine. The greater sensitivity of respiration with glucose as a substrate compared to lactate and pyruvate may well be secondary to uncoupling effects, since glucose must first be phosphorylated by ATP (Quastel, 1953, 1955). It has therefore been suggested that interference with energy

production may be the mechanism by which barbiturates depress neuronal function. However, succinate oxidation remains unimpaired during barbiturate anaesthesia. Moreover, sodium succinate increases oxygen consumption in brain homogenates in vitro. It might therefore be supposed that the administration of succinate would diminish the central nervous depressant action of barbiturates. However, in in vivo studies sodium succinate administration failed to alter the degree of barbiturate anaesthesia (Soskin and Maubenhaus, 1943; Lardy, Hansen and Phillips, 1944; Pfinschmidt, Ramsey and Haag, 1949; Giarman, Rowe and Young, 1954).

Other biochemical effects have been described. For example, there is some evidence that barbiturates inhibit cytochrome reductase and that thiobarbiturates inhibit adenosine triphosphate synthesis (Bain, 1955). Nevertheless, it has not been established that any of these actions is responsible for the central nervous depression produced by barbiturates. It is also known that

some anaesthetic agents may interact with proteins (Featherstone et al, 1961). This may explain only how certain agents might enter the cells of the central nervous system and not how anaesthesia is produced.

Also more recently Pauling (1961, 1964) and Miller (1961) independently proposed that anaesthetic gases might form hydrate microcrystals or so-called clathrates within the central nervous system. This involves the interaction of the anaesthetic agents with water molecules in the brain. It would appear that such a formation of microcrystals occurs mainly in the synaptic regions.

1.41 DISTRIBUTION, METABOLISM AND EXCRETION

Barbiturates are transported through the blood, bound to plasma proteins to a varying degree (Goldbaum and Smith, 1954; Shideman, 1958; Block, 1961). Approximately twenty-five per cent of thiopental is present in the plasma in the free form during anaesthesia, while seventy-five per cent is bound to protein, mainly to plasma albumin. When the plasma concentrations become lower, i.e. several hours after the administration of thiopental, the proportion bound to protein may increase to eighty-eight per cent (Goldstein and Aranow, 1960; Brand et al., 1963). The complex is a loose easily reversible one, the bonds consisting of weak physico-chemical forces (Mark, 1963). This 'transport mechanism' for thiopental may be somewhat analogous to the oxygen transport by haemoglobin. Barbiturates are distributed throughout the body, with high concentrations in liver, kidneys and skeletal muscles. The distribution of intravenously injected thiopental has already been discussed.

It has been shown that several organs are capable of metabolising thiobarbiturates and this is especially true for thiopental (Shideman, Kelly and Adams, 1947; Kelly and Shideman, 1949; Dorfman and Goldbaum, 1947; Gould and Shideman, 1952; Cooper and Brodie, 1957).

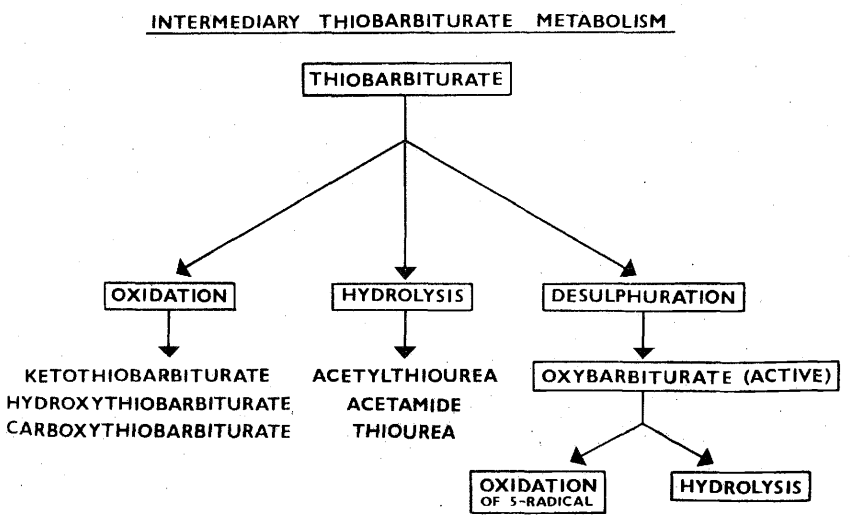
The following data were obtained by Cooper and Brodie.

TABLE 1

Tissue slices (350 mg)	µMol/3 hours. Barbiturate metabolised	
	Pentobarbital	Thiopental
Liver	0.70	0.73
Kidney	0.00	0.38
Brain	0.00	0.28
Heart	0.00	0.14
Skeletal Muscle	0.00	Trace

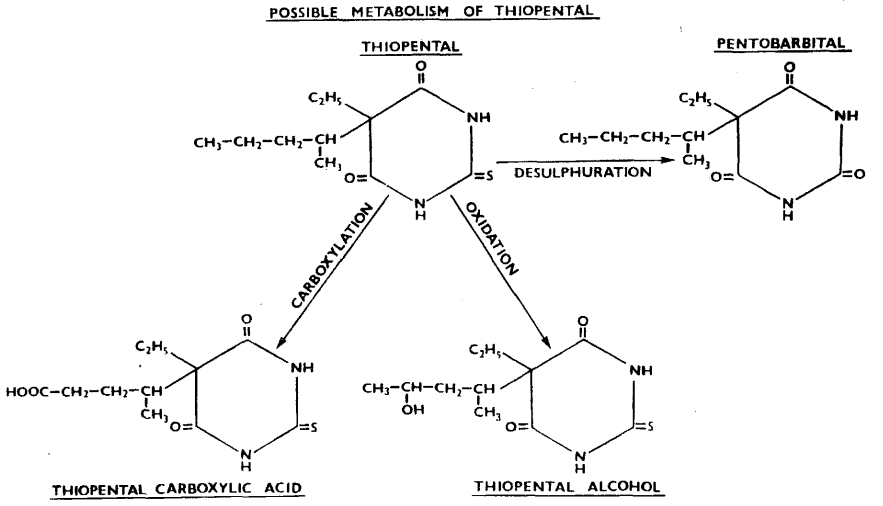
The above table demonstrates clearly the inability of kidney, brain and cardiac muscle to metabolise

FIGURE 4



Theoretically possible pathways of intermediary metabolism of thiobarbiturates.

FIGURE 5



Possible enzymatic biotransformations of thiopental.

pentobarbital *in vitro* while the same tissues are capable of metabolising thiobarbiturates.

Significant quantities of thiopental are metabolised *in vitro* especially in liver, kidney and brain tissue homogenates in the presence of nicotinamide, ATP, cytochrome C, and a Krebs's cycle intermediate (Gould and Shideman, 1952). This transformation is believed to take place in the microsomal fraction of cells. It has also been claimed that reduced nicotinamide adenine dinucleotide phosphate and oxygen are both necessary for the metabolism of barbiturates *in vitro* (Cooper and Brodie, 1955; Axelrod, 1955; Spector and Shideman, 1959). Several possible pathways of metabolism have been proposed (Fig.4). The most likely immediate metabolites appear to be thiopental carboxylic acid, thiopental alcohol and pentobarbital (Fig. 5). There is a considerable amount of evidence that the carboxylic acid derivative is formed (Brodie *et al*, 1950; Wood and Horning, 1953; Soehring and Dietz, 1956, Winters, 1957). Desulphuration to pentobarbital is disputed

by some workers, while oxidation to thiopental alcohol has been postulated, but not yet fully established (Winters et al, 1955; Block and Ebigt, 1960a; Mark, 1963).

Thiopental and its metabolites are excreted relatively slowly at a rate of 8-15% per hour. In man only approximately 0.3% of unaltered thiopental is excreted in the urine following a therapeutic dose while there are indications that some animal species excrete larger amounts of unaltered substances. (Personal unpublished data). Isotope studies with ^{35}S-labelled thiopental have demonstated that at least 9-12 sulphur-containing fractions are excreted in the urine of monkeys. (Taylor, Richards and Tabern, 1950a; 1950b; 1952). Nearly ninety per cent of the injected labelled ^{35}S is recovered in the urine of which about 65% was recoverable within the first 24 hours from rat urine. It has been found that 7% of the total radioactivity excreted during a period of 4 days could be extracted into chloroform, while in the monkeys 14%

of the total injected dose was excreted in a chloroform extractable form. Other sulphur fraction consisted of inorganic sulphate, ether sulphate and neutral sulphur. Only small concentrations were found in the faeces (about 5%) and approximately 2% of the radioactivity (^{35}S) could be deteced in the carcass. It is believed that some of the sulphur is made available for further synthesis in the organism via amino acids. Some artifacts may have been present (Block and Ebigt, 1960a).

1.50 EFFECT OF OTHER SUBSTANCES UPON DURATION OF BARBITURATE ANAESTHESIA

Various compounds are known to interact with barbiturate anaesthetic agents. Some of these compounds prolong the 'sleeping time' in animals while others decrease it. There are several theories to account for these interactions. The first relates to the possible role of 5-hydroxy-tryptamine (5-HT, serotonin). Many authors (Shore, Silver and Brodie, 1955; Fornaroli and Koller, 1955; Fastier, 1956) have demonstrated that 5-HT can prolong barbiturate anaesthesia, and that the action of other anaesthetics such as chloral hydrate can also be influenced by 5-HT (Fastier, Speden and Waal, 1957). It is now generally accepted that 5-HT can be released in a number of specialised tissues in the mammalian body, and it has been demonstrated that a great number of pharmacologically active substances (and some disease processes) may be responsible for the release of unphysiologically large amounts of 5-HT. Such increases of 5-HT levels have been implicated

in certain drug interactions.

It has been suggested that a number of the substances which prolong the 'sleeping time' may release 5-HT or nor-adrenaline from stores and inhibit monoamine oxidase, thus increasing the blood as well as brain-level of 5-HT and other physiologically active amines. This in turn could lead to a prolongation of barbiturate anaesthesia.

Well known brain 5-HT and nor-adrenaline liberators are several Rauwolfia alkaloids (Brodie, Shore and Pletscher, 1956; Shore et al., 1957), and large doses of amphetamine (Paasonen and Vogt, 1956), while monoamine oxidase inhibitors such as iproniazid and phenylisopropylhydrazine have been shown to rise the brain 5-HT and nor-adrenaline levels (Brodie, Spector and Shore, 1959; Horita, 1958). Bonnycastle, Giarman and Paasonen (1957) have studied a group of anticonvulsant drugs and have found that they release brain 5-HT. The same group of investigators (Bonnycastle, Anderson and Bonnycastle, 1959) also showed that a large number of chemically unrelated central nervous system (C.N.S.) depressants such as chloral hydrate,

ethyl alcohol, urethane, diethylether, chloralose and some barbiturates will cause a rise in the brain 5-HT levels (Andserson and Bonnycastle, 1960).

A second way in which drugs may alter the duration of anaesthesia is by modifying the breakdown of the drug by liver microsomal enzymes (Axelrod, Reichenthal and Brodie, 1954; Rümke, 1963; Rogers, Dixon and Fouts, 1963, and many others). This mechanism may play a part in hexobarbital or pentobarbital anaesthesia. Evidence in favour of this hypothesis that the liver microsomes are involved, is also provided by the paradoxical effect produced by administering the drugs some time earlier (Rümke, 1963). Some of the compounds which prolong anaesthesia may actually shorten the sleeping time if they are administered at at longer interval before the barbiturate. Rümke and Bout (1960) have investigated seventeen chemical compounds which significantly extend the anaesthetic time in mice when given one to one-and-a-half hours before hexobarbital. However, when thirteen of

these substances were administered from 24–48 hours before the barbiturate, the duration of anaesthesia was shortened. These drugs were hydroxyzine, chlorpromazine, promazine, orphenadrine, phenobarbital, glutethimide, iproniazide, SKF 525-A (Proadifen), carbutamide, urethane, trimethadione, methoin and primidone. Azacyclolol, tolbutamide, chlorpropamide and reserpine failed to exhibit this reduction in the duration of the sleeping time.

It is suggested that the shortening of anaesthesia produced by the above mentioned compounds may be due to their being metabolised by the same liver microsomal enzymes as the barbiturate. The activity of these enzymes is stimulated, leading to the more rapid metabolism of hexobarbital but other chemical compounds may also be affected.

Such an action is well established for phenobarbital and some steroids (Remmer, 1958a; 1958b; 1958c; 1959a; Conney et al., 1960; Mullen, Juchau and Fouts, 1966; and others). Phenobarbital may also enhance the metabolism of compounds

other than barbiturates, for instance antipyrine and strychnine (Remmer, 1959 and 1959b; Kato et al., 1962).

Some compounds may interfere with other enzyme systems - by acting as mild monoamineoxidase inhibitors for example. A typical example of this group is iproniazid, which has a well described biphasic action, primarily to prolong, and after a lag period of 24 hours to decrease the duration of barbiturate anaesthesia (Holtz et al., 1957 and others). The secondary effect is seen after approximately twenty-four hours, and depends largely on the dosage used.

Drugs may influence the effects of central nervous depressants in other ways. The effect of chlorpromazine on the sleeping time was investigated by Kato (1960) in a number of experiments in female rats. He found that the reduction in sensitivity to pharmacologically active substances was confined to pentobarbital and hexobarbital. No significant reduction in the effect of other C.N.S. depressants such as ethyl alcohol, glutethimide and hydroxy-

dione (Viadril) could be found. He also established that the decrease in sensitivity was not due to adrenal hormonal influences as it was clearly shown that the pentobarbital sleeping time remained significantly reduced after chlorpromazine pretreatment using adrenalectomised rats. That the same applies to the sex hormones was shown when immature rats were used. It is well known that barbiturate metabolism may be altered by corticosteroids as well as by sex-hormones (Brodie, 1956; Remmer, 1958a, 1958b and 1958c; Juchau and Fouts, 1966).

Chlorpromazine can decrease the body temperature significantly, especially if the room temperature is low. A lowered body temperature may by itself lead to anaesthetic prolongation, probably due to a reduced rate of metabolism of certain chemical compounds (Usinger, 1957).

It is possible to criticise much of the published work on the effect of drugs on the duration of barbiturate anaesthesia, since in few of the available publications has attention been paid to this factor. Both the room temperature where the

experiments have been carried out and the body temperature after the administration of the drugs should be monitored. Hexobarbital in higher doses is a relatively long-acting barbiturate in mice, and due to this long duration of anaesthesia there is inevitably a drop in the body temperature. In addition, Dandiya and Sellers (1961) showed that 5-HT administration interferes with temperature regulation. Indeed many other substances administered specifically for the study of the prolongation of barbiturate anaesthesia may decrease body temperature (Fastier, Speden and Wall, 1957; Dandiya and Sellers, 1961).

Only by the use of short-acting barbiturates or by carrying out such experiments in a thermostatically controlled observation cage, can the effects of room temperature changes be overcome and more reliable results obtained (Raventós, 1938). The author has personal experience of the effect of temperature on the duration of barbiturate anaesthesia. It was observed that there was a large variation in the response to identical amounts of

thiopental on a randomly bred strain of male mice with body weights between 22-25 gm. This variation occurred not only from day to day but from morning to evening. At first no consideration was given to the room temperature, but later it was found that the temperature fluctuated considerably.

Another interfering factor is dehydration. In the nocturnal animal there is little drinking during the day. It has been shown that even short periods of water deprivation may have a marked effect on the duration of anaesthesia (Bhide, 1960; Ramwell and Lester, 1961). In the present study this was avoided by injecting 0.3-0.4 ml. of saline per animal intraperitoneally 30 minutes before the barbiturate challenge. This modified the response and tended to standardise the duration of anaesthesia following a single dose of thiopental.

Because of the difficulties mentioned above, the author came to the conclusion that the results of earlier studies have to be very carefully scrutinised, as there is only limited information available about the conditions under which the experi-

ments were carried out.

There are other criticisms which can be made. Some of the substances cause hypotension, while others may induce a hypertensive state. In both circumstances this may result in decreasing the blood supply to such organs as the brain, the liver and the kidney, with the result that the anaesthetic agent remains in the tissue and body fluids for a longer period of time than would be the case under normal circulatory conditions.

Another factor in some previously reported investigations is that the intraperitoneal route was used for administration of all drugs tested. This method is subject to error, especially if the challenging dose of the barbiturate was also administered intraperitoneally shortly after the drug, i.e. within one-half to two hours (Rümke and de Jonge, 1962). It has been shown that even an inert fluid such as physiological saline solution given intraperitoneally can prolong hexobarbital anaesthesia if the drug is also given by this route. Presumably this is due to dilution and therefore a

delay in the absorption of the active compound.

The validity of some of these criticisms is illustrated by studies with reserpine. For many years it was believed that it prolongs barbiturate anaesthesia. More recently it has been demonstrated that reserpine has only little effect on the duration of barbiturate sleep when the experiments are carried out in an environment in which the temperature is maintained at 37°C or near body temperature. Thus the previous results must have been due partly to hypothermia. This decrease in temperature may have been the result of the massive release of 5-HT and other amines such as nor-adrenaline from tissue stores during the early phase of the reserpine action.

1.60 SCOPE OF THE PRESENT INVESTIGATION

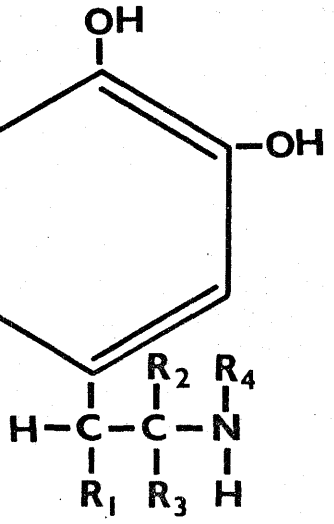
Since much of the published work relating to the effect of various drugs upon the duration of barbiturate anaesthesia can be critised along the lines indicated in the previous chapter, it was decided to undertake an investigation of a few of these drugs with due regard to avoiding the sources of error. The substances selected for study were alpha-methyldopa (a-MD), reserpine and proadifen (SKF 525-A). Alpha-methyldopa was specifically selected because it has been reported to reduce the levels of 5-HT in the brain. It was anticipated that it would afford an opportunity to test the theory that such substances modify the duration of barbiturate anaesthesia through this mechanism. Proadifen is believed to inhibit certain liver microsomal enzyme systems responsible for the detoxification of pharmacologically active compounds. It appears from current views that the main effect is on enzymes which oxidize the side-chains of barbiturates. The rate of inactivation of these

substances is therefore reduced, resulting in a prolongation of the anaesthetic time. This substance would therefore appear to act in a manner different from a-MD.

In addition to those two drugs, the effect of reserpine was also examined. Since this substance has been studied under adequately controlled conditions by other workers, it was thought that it would serve as a verification on the techniques employed in the present investigation.

The barbiturate selected for these studies was thiopental. Due to its very short duration of action (110-260 seconds in mice) there is no time for the body temperature to alter significantly during the experiment, provided the prolongation of anaesthesia does not exceed twenty minutes. The selection of this drug made it possible to dispense with a thermostatically controlled observation chamber, which was not available.

FIGURE 6



NAME	R_1	R_2	R_3	R_4
DOPA	H	H	COOH	H
DOPAMINE	H	H	H	H
α-METHYLDOPA	H	CH_3	COOH	H
ADRENALINE	OH	H	H	CH_3
NOR-ADRENALINE	OH	H	H	H

Structural relationships of various phenylethylamine derivatives.

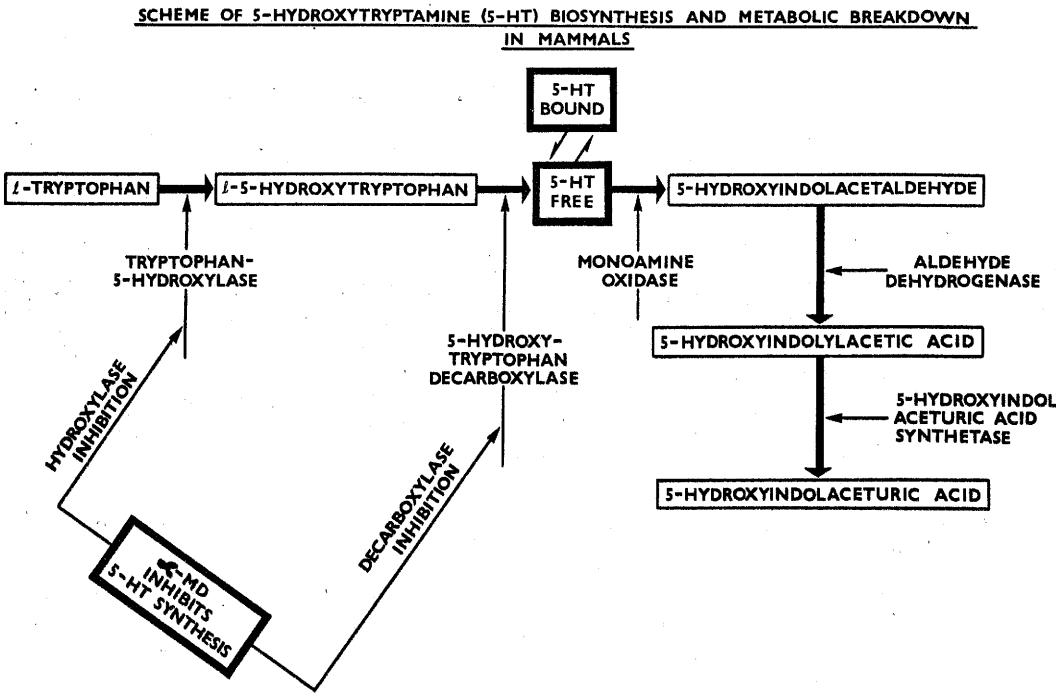
1.70 THE PHARMACOLOGY OF THE SUBSTANCES INVESTIGATED:

1.71 ALPHA-METHYLDOPA

In chemical structure a-MD closely resembles substances of the catecholamine group, being alpha-methyl-(3,4-dihydroxy-phenyl)-alanine (Fig. 6). Only the Laevo-isomer is pharmacologically active, and was used exclusively in these studies.

Initially it was demonstrated that a-MD is capable of inhibiting the decarboxylation of DOPA and 5-hydroxytryptamine (5-HTP), thus reducing the formation of the eventual end-products, nor-adrenaline and 5-HT respectively (Westermann, Balzer and Knell, 1958; Dengler and Reichel, 1958). There appears to be a better correlation between the 5-HT levels and the decarboxylase inhibition than with the nor-adrenaline depletion (Smith, 1960; Hess et al., 1961a and 1961b; Leroy, 1961; Brest and Moyer, 1962; Bayliss and Harvey-Smith, 1962). More recently it was shown that a-MD is a very potent hydroxylase inhibitor (Brukard, Gey and Pletscher, 1964) (Fig. 7). In both in vitro and in vivo experiments it was shown that hydroxylase

FIGURE 7


SCHEME OF 5-HYDROXYTRYPTAMINE (5-HT) BIOSYNTHESIS AND METABOLIC BREAKDOWN IN MAMMALS
5-HT BOUND
L-TRYPTOPHAN
L-5-HYDROXYTRYPTOPHAN
5-HT FREE
5-HYDROXYINDOLACETALDEHYDE
TRYPTOPHAN-5-HYDROXYLASE
MONOAMINE OXIDASE
ALDEHYDE DEHYDROGENASE
5-HYDROXY-TRYPTOPHAN DECARBOXYLASE
5-HYDROXYINDOLYLACETIC ACID
HYDROXYLASE INHIBITION
5-HYDROXYINDOL-ACETURIC ACID SYNTHETASE
DECARBOXYLASE INHIBITION
α-MD INHIBITS 5-HT SYNTHESIS
5-HYDROXYINDOLACETURIC ACID

was approximately 100 times more sensitive to a-MD than the decarboxylase.

It is questionable whether these mechanisms are responsible for its effect in the treatment of hypertension. In pursuing the literature it appears that most authors favour an interference with the storage mechanism of catecholamines. It has to be remembered that a-MD resembles DOPA closely enough to be metabolised itself, hence in the dopa-decarboxylase system it yields α-methyldopamine. Alpha-methyldopamine or a closely related compound (perhaps α-methyl-nor-adrenaline) might be stored in the sympathetic nerve-endings and released as a 'false transmitter' (Day and Rand, 1964; Pettinger et al., 1965).

The false transmitter would have a weaker adrenergic stimulant action than nor-adrenaline, and in this way the blood pressure would be lowered.

The effect of a-MD administration on the 5-HT

concentrations in various tissues, especially the brain and heart, has been well documented (Westermann, Balzer and Knell, 1958; Sjoerdsma, 1960; Porter, Totaro and Leiby, 1961; Carlsson and Lindquist, 1962; Pletscher, Gey and Burkard, 1963 and many others).

The peak action is reached 2-4 hours after administration, and the concentration of 5-HT remains lowered in the brain for approximately 3-5 hours. After this time it starts to rise and reaches normal levels after 16 hours. By doubling the dose of a-MD there is a further decrease in the concentration of 5-HT in the brain, and the levels remain depressed for approximately 8 hours before they start to rise again.

Due to its pharmacological action it also affects nor-adrenaline tissue levels, especially in the heart and to a lesser extent in the brain (Sjoerdsma, 1960; Sourkes et al 1961; Carlsson and Lindquist, 1962). The nor-adrenaline level remains low for approximately 2-4 days, while the 5-HT level in the brain recovers within about 16

hours. The loss of amines from the brain cannot result from the decarboxylase inhibition alone, but is probably also due to their release from the storage sites, as was pointed out by Murphy and Sourkes (1959).

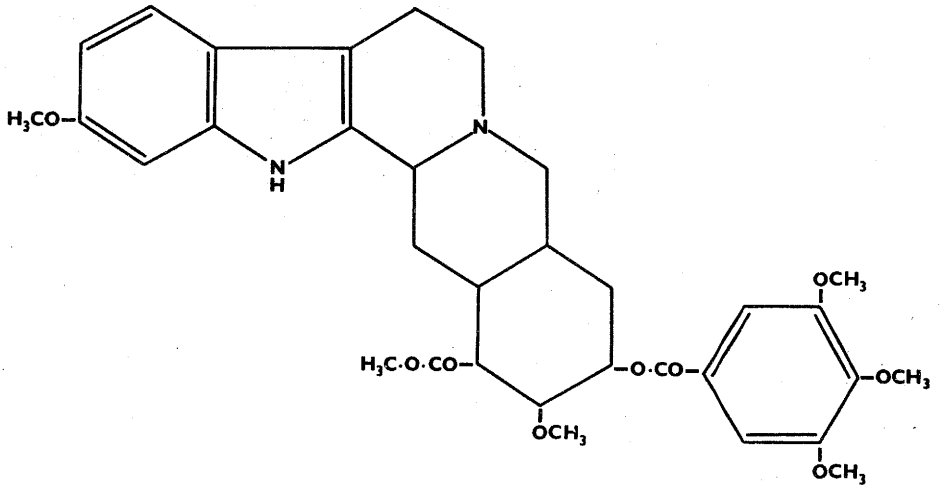
In small doses a-MD does not lead to a marked central nervous depression, although there is some decrease in normal motor activity in the tested animals.

However, if a-MD is administered to mice in large doses, it produces a marked sedation, and a drop in the body temperature is apparent (Smith, 1960). The same author has also demonstrated that hexobarbital anaesthesia is prolonged after the administration of a-MD, and Herold and his coworkers (1962) have shown an increased sleeping time under pentobarbital anaesthesia.

In this study pure l-alpha-methyldopa powder was made available from the manufacturer (Merck, Sharp and Dohme, South Africa). This substance was dissolved in 1/N HCl and diluted with distilled

water to the required concentration. The resulting solution was strongly acid, this created problems when administered parenterally. Attempts to raise the pH resulted in a colour change and precipitation.

FIGURE 8



3,4,5-Trimethoxybenzoyl Methyl Reserpate (Reserpine; Serpasil)

1.72 RESERPINE

Reserpine is an alkaloid derived from Rauwolfia serpentina (Benth)(Fig.8). A plant extract has been in use in India for several hundred years as a sedative.

This compound also leads to release and consequent depletion of catecholamines and 5-HT stores throughout the body, including the brain. Brain concentrations of dopamine (Carlsson, 1959), nor-adrenaline (Holzbauer and Vogt, 1956) and 5-HT (Brodie, Shore and Pletscher, 1956) are decreased. It has been suggested that reserpine might compete with 5-HT as well as other amines for binding sites, or prevent their storage. The synthesis of 5-HT and nor-adrenaline is not affected.

Release of 5-HT is progressive over many hours. It was formerly believed that the release of 5-HT continued long after the reserpine had disappeared from the organism (Hess, Shore and Brodie, 1956). However, studies by Sheppard et al., (1957)

in which labelled reserpine was used, have shown that the drug remains in the body for a longer time than previously suspected. In view of this it is no longer necessary to postulate that reserpine acts by a secondary mechanism.

Spector and his co-workers (1960) as well as others have demonstrated that there is a certain interaction between monoamine oxidase inhibitors and reserpine.

A decrease in amines only occurs after the effect of an administered MOA-inhibitor has passed off.

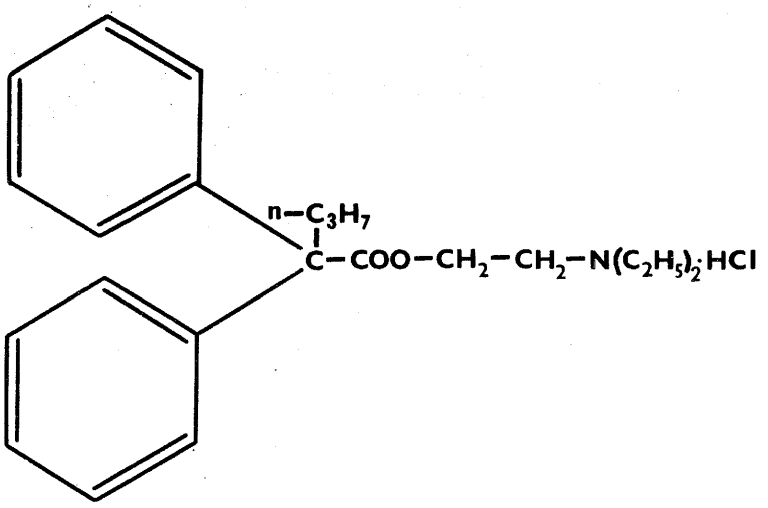
Most of the effects of reserpine in animals and man are still not well understood. Some of its actions are explained as being due to peripheral depletion of catecholamines. After giving reserpine, especially at high dosage levels, animals are sedated and hypothermic. It has been demonstrated that the sedation is closely related to those Rauwolfia alkaloids which affect the level of 5-HT in the brain (Brodie, Shore and

Pletscher, 1956; Shore et al., 1957). The observed diarrhoea may be due to the large amounts of 5-HT liberated from stores. The miosis and salivation are possibly due to increased central parasympathetic activity. The onset of action of reserpine is very slow. Even after an intravenous dose some of its effects may only be seen in 2-6 hours. Sedation persists over long peroids and is still marked after 24-48 hours.

It is possible that these centrally mediated effects are related to the depletion of brain amines.

The form of reserpine used in this study was the commercially available 'Serpasil-Ampoules' (Ciba, Basel).

FIGURE 9



β-Diethylaminoethyl Diphenyl-n-propylacetate
Proadifen HCl (SKF 525-A)

1.73 PROADIFEN (SKF 525-A)

With the exception of a hypocholesterolaemic effect demonstrated in several animal species, Proadifen (Fig. 9) is claimed to possess minimal direct pharmacological action (Dick et al., 1960; Brodie, Gillette and La Du, 1958).

This compound is of great interest because of its intriguing ability to act synergistically with some chemical substances of diverse structure and pharmacological activity. Drugs whose characteristic activities have been shown to be enhanced by proadifen include both barbiturates and non-barbiturates (Cook, Macko and Fellows, 1954); analgesics (Cook, Navis and Fellows, 1954); anti-epileptics (Swinyard, Madsen and Goodman, 1954); spinal cord depressants and central nervous stimulants (Macko et al., 1953); skeletal muscle relaxants (Navis et al., 1953) and antihypertensives (Goldstein and Rossi, 1959).

It has been shown that proadifen may enhance certain pharmacological actions of some compounds without influencing their LD_{50} values. For

example, the analgesic action of morphine and meperidine and some other analgesics are significantly increased, while the toxicity is not altered. It has also been demonstrated that morphine exhibits an action in morphine tolerant animals following proadifen pretreatment. However, respiratory depression and tolerance develop in a comparable manner whether proadifen plus morphine, or morphine alone were administered (Cook, Navis and Fellows, 1954; Cook et al., 1953). This clearly demonstrated that different facets of narcotic action can be influenced selectively.

Although proadifen has proved to be an extremely useful tool in the experimental investigation of pathways of drug metabolism, it is not used clinically, due to its high toxicity in man, especially to the liver (Fouts, 1963).

It is of interest that the chronic administration of proadifen is associated with hepatotoxicity in rats (Tognoli, 1969) and in dogs (Dick et al., 1960). Holmes and Bentz (1960) have suggested that inter-

ference with an important electron transfer co-enzyme (Coenzyme Q) may account for the proadi-fen-induced accumulation of lipid material in the liver of animals.

From the evidence so far collected it may be assumed that proadifen exerts its potentiating action mainly on drugs which are detoxified in the body.

Proadifen Hydrochloride was supplied from the manufacturers (Smith, Kline and French) as a powder and was freshly prepared as an aqueous solution when required.

TABLE 2

ALPHA-METHYLDOPA TOXICITY IN MICE

Dose mg/Kg.	Route of Administration	No. of Animals	Form *	Survived	Died	Remarks and Symptoms
1000	Oral	25	Solution	25	0	Mild to moderate sedation.
1500	Oral	30	Solution	30	0	Moderate sedation.
2500	Oral	25	Solution	25	0	Moderate sedation.
5000	Oral	30	Solution	30	0	Sever sedation, hypnotic-like state
6000	Oral	10	Suspension	8	2	Death occurred probably due to dehydration after 48 hours.
6000	Oral	20	Suspension	20	0	Saline injected to prevent dehydration.
7500	Oral	10	Suspension	7	3	Death occurred probably due to dehydration. 1 animal died after 24 hours and 2 after 48 hours.
7500	Oral	20	Suspension	20	0	Saline injected to prevent dehydration.
1000	Subcutaneous	25	Solution	25	0	Moderate sedation.
2000	Subcutaneous	25	Solution	25	0	Moderate to severe sedation.
3000	Subcutaneous	30	Solution	30	0	Severe sedation, solution very acid - caused irritation, animals were killed after 48 hours.
1000	Intraperitoneal	25	Solution	25	0	Moderate sedation.
2500	Intraperitoneal	25	Solution	25	0	Moderate to severe sedation.
5000	Intraperitoneal	25	Solution	25	0	Animals were killed after 24 hours because of irritation due to the high acidity of the solution.
250	Intravenous	20	Solution	20	0	Sedated.
500	Intravenous	25	Solution	25	0	Severe sedation.
1000	Intravenous	20	Solution	20	0	Severe sedation, later hypnotic like state.

* Solutions were made up in 1/N HCl, and 1/N NaOH was added to adjust pH to ±3-3.5.
Suspensions were made up in distilled water with gum arabic using the base of a-MD.

2.00 TOXICOLOGICAL STUDIES

2.10 METHOD

Only healthy male mice bred non-selectively and weighing 22-30 gm. were used. They were kept on a standard diet in the form of compressed cubes and water ad libitum. Before experiments were performed they were kept for at least two weeks in the room where the investigation was carried out.

To establish if the strain of mice which were to be used was unduly sensitive to any of the drugs, acute toxicity studies were carried out.

The observation period was limited to 72 hours following drug administration. Where possible the LD_{50} was established.

2.20 ALPHA-METHYLDOPA

It was found that in the doses used α-methyldopa was virtually non-toxic, and no LD_{50} could be determined (Table 2).

The drug was administered either in a suspension

made up in gum arabic or by forming the hydrochloride from the free base. The hydrochloride solution was very acid (pH about 3). It was not possible to neutralise it, as there was a colour change which might have represented a change in the potency of a-MD, and if the pH was raised to near neutrality, precipitation took place. Since this solution caused severe irritation when administered parenterally, the oral route was used, except for acute experiments. It was well tolerated, even when oral administration was continued for a long time. Mild sedation was noted at doses above 400 mg/Kg. orally or above 100 mg/Kg. intraperitoneally and marked central nervous system depression was observed with oral dosages above 1500 mg/Kg. When 6,000 and 7,500 mg/Kg. was administered, 2 and 3 mice respectively of the 10 died after 24-48 hours. It was thought that this might have been due to dehydration resulting from the severe sedation. Evidence in support of this was obtained when the experiment was repeated, but oral saline was given to counter the dehydration. On this occasion none of the animals died,

and all eventually recovered from the hypnosis-like state. It was also noted that a white sediment was excreted in the urine with the higher dosage levels. No analysis of this excretory product was undertaken, but it might have been unaltered a-MD crystallised in the urine.

TABLE 3

RESERPINE TOXICITY IN MICE

Dose mg/Kg.	Route of Administration	No. of Animals	Survived	Died	Remarks and Symptoms
2.5	Subcutaneous	12	12	0	Sedation.
5.0	Subcutaneous	12	10	2	Moderate-severe sedation (at room temperature.
10.0	Subcutaneous	20	12	8	Severe sedation at room temperature, 2 died after 49 hours, 3 died after 72 hours and 3 after 96 hours.
10.0*	Subcutaneous	15	15	0	Kept at 36°C. (± 1°C).
5.0*	Intraperitoneal	15	15	0	Kept at 36°C. (± 1°C). Severe sedation.
10.0*	Intraperitoneal	20	20	0	Kept at 36°C. (± 1°C). Severe sedation.

* The last three groups received fluid (i.e. NaCl phys. or Glucose 2.5%) orally to prevent excessive dehydration.

2.30 RESERPINE

Due to the limited amount of this compound available, a complete toxicity study was not carried out. Reserpine was therefore only studied in the dosage ranges which would make it possible to compare the sensitivity of the strain of mice used in the present study, with data from the literature. Doses of up to 10 mg/Kg. intraperitoneally and subcutaneously were used. No differences between the two routes of administration were observed (Table 3). The peak action was seen after approximately 4-8 hours. The animals were never in a state of hypnosis, but were deeply sedated. However, they could be aroused at all times by disturbances. There was no food intake during this period and probably very little fluid. This resulted in a large loss of body weight, which was regained after the effect of reserpine had worn off. Of the subcutaneously administered reserpine group, two of the 12 mice given 5 mg/Kg. dose and 8 of the 20 given 10 mg/Kg. dose died. It seemd likely once again that these deaths might have been the result of general circulatory collapse, due to severe dehydration.

Another factor might have been hypothermia, as the animals were kept at room temperature. The experiments were therefore repeated with the mice in a temperature controlled heat box, and saline or 2.5% glucose solution was administered by oesophageal cannula. On this occassion none of the animals died.

TABLE 4

PROADIFEN - SKF 525A - TOXICITY IN MICE

Dose mg/Kg.	Route of Administration	No. of Animals	Survived	Died	Remarks
250	Oral	25	24	1	
500	Oral	35	32	3	Hyperexcitable, twitching.
750	Oral	25	18	7	Hyperexcitable, twitching.
1000	Oral	50	18	32	Hyperexcitable, twitching, convulsion-like state, respiratory failure.
1500	Oral	40	2	38	Respiratory depression, clonic-tonic convulsions - leading to death due to apnoea - forced respiration.
25	Intraperitoneal	25	25	0	Restless, no visible effect.
50	Intraperitoneal	25	23	2	Hyperexcitable, restless, twitching.
100	Intraperitoneal	50	36	14	Hyperexcitable, respiratory depression, twitching (6 animals showed convulsions) death was probably due to anoxia.
150	Intraperitoneal	100	8	92	Hyperexcitable, respiratory depression, twitching, clonic-tonic convulsions, apnoea and died probably due to anoxia, laboured respiration in surviving animals.
175	Intraperitoneal	50	1	49	
200	Intraperitoneal	25	0	25	

2.40 PROADIFEN (SKF 525-A)

It was found that this substance was very toxic when compared with alpha-methyldopa. The graphically estimated LD_{50} by oral administration is 945 mg/Kg. (Table 4). However, death occurred in some animals with doses as low as 250 mg/Kg. orally when observed for longer than 72 hours. The reason for such wide individual variation in the toxicity of the drug have not been fully investigated, again for the lack of sufficient pure material.

After intraperitoneal administration, it was found that proadifen exhibits an LD_{50} of 114.5 mg/Kg. When the observation period was extended beyond 72 hours, it was noted that several animals, treated with less than 50 mg/Kg., died.

While it took up to 30 minutes to produce observable symptoms after oral administration of a toxic dose, these symptoms appeared within 2-3 minutes in all animals treated intraperitoneally. These results confirm earlier reports (Cook, Toner and Fellows, 1954). Death occurred generally within the first

sixty minutes. Animals which did not die within this time usually recovered, apparently fully; however, there was some delayed toxic effect with additional mice dying during an observation period of seven days. The possibility of reversible microscopic tissue damage has not been investigated.

The toxic symptoms observed in the animals following a lethal dose of proadifen included severe hyperexcitability, depressed respiration, twitching of the skin and convulsions, eventually followed by death probably due to complete paralysis of the respiratory system. The convulsions could have been due to a direct action of the drug, but in view of the respiratory depression it appeared possible that they might be the result of cerebral anoxia. This possibility was subjected to further investigation, since this type of acute toxic effect of proadifen dose not appear to have been fully documented previously (Cook, Toner and Fellows, 1954).

Based on the toxicity study, it was decided to use a dosage schedule of maximal doses of 100 mg/Kg.

intraperitoneally and up to 200 mg/Kg. orally for further investigations.

2.50 INVESTIGATION INTO THE CONVULSIVE PROPERTIES OF PROADIFEN

The available literature on proadifen has hitherto made little mention of a convulsive action in the larger or lethal doses (Cook, Macko and Fellows, 1954; Cook, Toner and Fellows, 1954). On the contrary, it has been demonstrated that small doses enhance the anti-convulsant action of various compounds (Swinyard, Madsen and Goodman, 1954). Together with the observation that proadifen produced a prolongation of barbiturate anaesthesia, this raised the possibility that proadifen possesses a central nervous depressant action. The convulsions which are observed might then be the result of cerebral anoxia secondary to respiratory depression. Whether they were due to depression or stimulation of the central nervous system, they were presumably the result of a direct effect of proadifen on the brain. The elucidation of their causation therefore appeared relevant to the understanding of the action of proadifen in prolonging barbiturate anaesthesia.

In order to achieve this, it was decided to study the effect of the anticonvulsant phenobarbital on the convulsions caused by proadifen. Phenobarbital diminishes or abolishes convulsions produced by central nervous stimulants such as pentylenetetrazol (Cardiazol, Leptazol). If proadifen caused convulsions by a stimulant action, it would be anticipated that phenobarbital would exhibit a similar protective effect. On the other hand, if the proadifen convulsions were due to cerebral anoxia from the respiratory depression, phenobarbital would have no beneficial effect and might even aggravate them. A direct comparison was therefore made between the effect of phenobarbital on pentylenetetrazol convulsions and proadifen convulsions in mice.

METHODS

In preliminary studies it was found that the LD_{99} for this strain of mice for intraperitoneal administration of pentylenetetrazol was 100 mg/Kg. It was also observed that 50 mg/Kg. phenobarbital administered orally, protected between 50-70% (mean 60%) of the mice against the pentylenetetrazol challenge.

TABLE 5

PROADIFEN CHALLENGE FOLLOWING PRETREATMENT WITH ORAL PHENOBARBITAL

Test Substance (Convulsants) in mg/Kg. intraperitoneally	Dose of Phenobarbital mg/Kg.	No. of Animals	Time interval before challenge (in minutes)	Cumulative % Died in 60 mins.	Cumulative % Died in 24 hours	Died in 60 hours	Survival
Pentylenetetrazol							
100	Saline	25	-	100	-	-	0
100	50	25	60	40	0	0	60
100	100	25	60	0	0	0	100
100	100	25	120	0	0	0	100
Proadifen							
100	Saline	12	-	25	58	83	17
100	50	24	60	0	21	75	25
100	100	24	60	0	25	83	17
125	Saline	36	-	54	64	89	11
125	50	24	60	50	71	96	4
125	50	24	120	58.5	58.5	92	8
125	100	24	60	62.5	71	100	0
125	100	12	120	7	35	92	8
150	Saline	12	-	67	83	100	0
150	100	12	120	0	8	100	0
150	100	12	180	25	75	100	0

When 100 mg/Kg. of phenobarbital was given orally at least one hour before the pentylenetetrazol, 100% protection of the mice was achieved; there was no tendency to clonic-tonic convulsions and extensor spasms did not occur. Pentylenetetrazol administration to unprotected animals induced death within 20-40 minutes.

As a result of these observations it was decided to pretreat groups of 12-24 mice with 50 and 100 mg/Kg. phenobarbital orally before the intraperitoneal administration of 100, 125 and 150 mg/Kg. proadifen.

RESULTS

Phenobarbital in the lower dose of 50 mg/Kg. had no protective effect against the subsequent proadifen challenge of 125 mg/Kg. intraperitoneally, carried out 1-3 hours later. The clonic-tonic twitching observed in the control groups was also present in the treated groups (Table 5). However, when phenobarbital was administered in a dose of 100 mg/Kg. at least 2 hours before proadifen, there appeared

to be some protection against the early toxic phase. In spite of the lesser mortality within the first 24 hours, however, by 60 hours nearly all the animals in both the treated and non-treated groups were dead. When a lower dose of proadifen (100 mg/Kg.) was administered, only 50 mg/Kg. phenobarbital was required to exhibit this protective action. The 60 hour mortality again failed to show a distinct difference. Nevertheless, when enough phenobarbital was administered a sufficiently long time before the proadifen, it unequivocally controlled the convulsions and reduced the early mortality, and it therefore seems reasonable to conclude that the spasms were the result of central nervous stimulation by proadifen. In spite of this, however, there was some evidence that proadifen also produces depression of the central nervous system. Within the first two hours after the oral administration of the phenobarbital, several mice exhibited marked hyperactivity and hyperexcitability combined with severe ataxia. Soon after the proadifen injection they became sedated, adopted a flat belly posture, and changed position only when

disturbed by painful stimuli. Proadifen in these doses therefore apparently has both a sedative and a convulsant action.

Although the early toxicity of proadifen was reduced by phenobarbital, the eventual survival of the animals was not improved. The late toxic effects may be due to damage to vital organs (such as the liver), and the resultant failure of normal physiological functions eventually leads to death. The investigation of this aspect of the actions of proadifen appeared outside the scope of this dissertation and was not attempted.

2.60 THIOPENTAL

The sensitivity of the strain of mice to thiopental, was found to be much higher than had previously been reported (Shideman, Kelly and Adams, 1947; Giarman et al., 1954; Archor and Geiling, 1954; Cook, Macko and Fellows, 1954; Kushinsky et al., 1957; Block and Ebigt 1960a). For example, doses of up to 100 mg/Kg. are reported in the literature to produce no unusual responses, whereas in our laboratory many of the animals died when dosages less than half this were used.

No anaesthesia was observed with doses below 10 mg/Kg. Thiopental in doses of 15 mg/Kg. produced a loss of the righting reflex in a few animals for a few seconds only, while doses of 20 mg/Kg. produced anaesthesia in more than 80% of the mice, lasting for an average of about 95 seconds. By increasing the dose of thiopental to 25 mg/Kg. a constant loss of righting reflex was produced for an average of about 162 seconds. Doses of 35 mg/Kg. and higher led to respiratory arrest in a large number of animals and in some, death due to prolonged

TABLE 6

INTRAVENOUS THIOPENTAL TOXICITY IN MICE

Dose mg/Kg.	No. of Animals	Survived	Died	Remarks
10.0	30	30	0	
12.5	40	40	0	In 8 animals a very brief loss of righting reflexes was observed.
15.0	40	40	0	In 14 animals a very brief loss of righting reflexes was observed.
20.0	40	40	0	Produced anaesthesia in more than 80% of animals. The loss of righting reflexes lasted approximately 95 seconds.
25.0	100	100	0	Anaesthesia was achieved in all animals and lasted approximately 162 seconds.
35.0	30	19	11	All animals exhibited anaesthesia; some died due to anoxia (respiratory depression).
37.5	30	17	13	All animals exhibited anaesthesia; some mice died due to anoxia (respiratory depression).
40.0	40	21	19	All animals exhibited anaesthesia, some mice died due to anoxia (respiratory depression).

All animals received an arbitary amount of saline 0.3-0.4 ml/per mouse, intraperitoneally 30 minutes before the thiopental administration.

anoxia (about 40% died with 35 mg/Kg.) (Table 6). As a result of this investigation, a dose of 25 mg/Kg. of thiopental was used throughout the investigation unless otherwise stated. It was shown that this dosage produced consistent effects and rarely caused respiratory depression and then only for a few seconds. When it did occur, this depression was probably due to a too rapid injection of thiopental.

The reduction in variation obtained by using a preceding injection of an arbitary amount of saline intraperitoneally is also worth mentioning. This phenomenon can probably be explained by the variation in biological rhythm in the course of the day as described by some workers (Carlsson and Serin, 1950; Aschoff, 1963). The saline possibly serves the purpose of partially counteracting the dehydration taking place in these animals during the day, as mice are typical nocturnal animals and there is a very little food and fluid intake during the day.

No attention was paid in this investigation to post-anaesthetic sedation and ataxia nor to the duration of these, following anaesthesia. However, these 'hangover' symptoms appear regularly and it would be of some interest to pursue them further, as their duration seems to be largely extended after pretreatment with some of the compounds investigated.

TABLE 7

RE-INJECTION OF A SECOND THIOPENTAL DOSE (10 mice per group)

Time interval between 1st and 2nd challenge	Duration of Anaesthesia in seconds		% Prolongation of 2nd over 1st challenge	Duration of Anaesthesia in control group in seconds.	Time of day
	1st challenge Mean (Range)	2nd challenge Mean (Range)			
30 minutes	176 (153–205)	476 (258–617)	170.5	–	9.30–10.00am
60 minutes	189 (142–241)	331 (240–465)	75	178	9.50–10.50am
120 minutes	109 (105–230)	274 (188–390)	62	173	4.30–7.00 pm
180 minutes	181 (142–217)	218 (173–278)	20	178	11.15am – 2.40pm
360 minutes	145 (95–180)	158 (118–298)	9	167	1.30–7.30pm

2.70 RE-INJECTION OF THIOPENTAL

It was thought initially that it would be advantageous if a group of animals could serve as their own controls. For this reason it was decided to investigate the action of thiopental when a second challenge was given within pre-selected time intervals of 30, 60, 120, 180 and 360 minutes. It soon became apparent (Table 7) that this technique could only be applied if the time interval between the first and the second challenge was 360 minutes or more. It was demonstrated that in a period of up to three hours there was an additive effect noted with the second challenge as compared to a control group receiving equivalent volumes of saline without the barbiturate. This additive effect, however, disappeared when the interval was extended beyond six hours between the first and second challenge.

There are several possible explanations for this mechanism of action. In the first two to three hours there might still be enough unaltered free thiopental in the circulation to cause a direct

additive effect on re-injection of the second dose.

Another possibility is that the fat stores have already been saturated so that none of the second dose of thiopental can be inactivated by re-distribution. Hence the duration of anaesthesia is extended. Still another theoretical possibility is that the active metabolite pentobarbital, formed by the desulphuration of the thiopental was present in sufficient quantity to produce an additive effect.

As the peak action of most of the compounds studied appears to be within the first three hours the attempt to use each animal as its own control had to be abandoned. It was felt that the disadvantage resulting from carrying out the two experiments at different times of the day outweighed the possible benefits. It was therefore decided to use random groups of animals as control and test subjects for each experiment.

TABLE 8

INTRAVENOUS PENTOBARBITAL TOXICITY IN MICE

Dose mg/Kg.	No. of Animals	Survived	Died	Remarks
10.0	30	30	0	Minimal drug effect noted, mild ataxia for short period only.
15.0	50	50	0	Minimal drug effect noted, mild ataxia in 28 out of 50 mice.
25.0	50	50	0	Complete loss of righting reflex in 7 out of 50, partial loss of righting reflex in about 50% of all animals.
37.5	50	49	1	Complete loss of righting reflex in 26 out of 50; all animals exhibited partial loss of righting reflex
50.0	50	48	2	All animals exhibited a deep anaesthesia; no stimuli could arouse them. Recovery of righting reflex after ± 18 minutes.

2.80 PENTOBARBITAL (Nembutal)

The sensitivity of the laboratory strain to pentobarbital was also established because it was considered that this substance might be of importance later in the investigation, since it is a possible metabolite of thiopental. It was decided to establish a dose which would induce loss of righting reflex in approximately 50% of the animals; thus, doses between 10 and 50 mg/Kg. intravenously were tested (Table 8). The procedure was the same as for thiopental.

RESULTS

At a dosage level of 10 mg/Kg. intravenously, no animal exhibited a loss of righting reflexes, while a dose of 25 mg/Kg. produced it in roughly 50% of the animals. When 50 mg/Kg. was injected a deep anaesthesia developed within about 40-60 seconds after the administration and lasted for approximately 18 minutes. The delay of onset as compared to thiopental is probably due to the fact that pentobarbital has a much lower lipid solubility, hence the penetration of this substance into the

brain will not be as fast as in the case with thio-barbiturates. The anaesthesia produced was very deep once it had acted fully, with slowing of the rate of respiration and some decrease in tidal volume. It is, however, interesting to note that it was difficult to induce a respiratory arrest as so often occurs after thiopental administration. This again suggests that there might be a slower penetration through the blood-brain barrier; there is as a result a more generalised distribution throughout the body and high initial concentrations in the brain are not easily attained.

3.00 THE PROLONGATION OF THIOPENTAL ANAESTHESIA

3.10 GENERAL METHOD

Thiopental was injected intravenously into a tail vein. The substance under study was given at various intervals prior to the thiopental. The room temperature was between 26°C and 29°C. The concentrations of the majority of the substances used were so adjusted that 0.1 ml. of solution contained the dose for an animal of 10 gm body weight. Only male animals were used throughout this study, as females may exhibit lower enzyme activities and could thus influence the investigation unduly (Holck et al., 1943). The standard dose of thiopental was 25 mg/Kg. intravenously as mentioned earlier, but some experiments were carried out with lower or higher doses of thiopental for comparison of its actions at different doses. The control groups were kept under identical conditions, and were treated with the relevant diluting vehicle, i.e. normal saline or the suspending agent, gum arabic etc., orally, subcutaneously or intraperitoneally. In the case

of a-MD the exact amounts of HCl and NaOH used in the preparation of the drug were also added to the diluting fluid alone, and this was then used as the control solution. In every other respect the control animals were handled identically to the test animals. Saline(0.3 to 0.4 ml.)was administered intraperitoneally 25-30 minutes before the thiopental to all animals for the reasons already discussed.

The sleeping time was determined as already defined. The change from sleeping to waking appeared to be rapid, but there was nevertheless some residual ataxia and sedation. A control group, of 5-6 mice, was studied at the same time as the experimental groups which normally consisted of 10-20 animals.

TABLE 9

THIOPENTAL CHALLENGE FOLLOWING PRETREATMENT WITH SUBCUTANEOUS RESERPINE

Dose mg/kg.	Time before challenge	No. of Animals	Duration of Anaesthesia in seconds Mean (Range)	% Prolongation Control = 100%	Remarks
2.5	1 hour	10	276 (195 - 346)	137	
5.0	1 hour	10	215 (145 - 380)	106	
2.5	5 hours	10	503 (324 - 880)	249	1 out of 10 died*.
5.0	5 hours	10	481 (175 - 925)	233	1 out of 10 died*.
5.0	5 hours	10	306 (195 - 415)	151	In incubator $\pm$ 36°C.
Control Saline	-	10	202 (189 - 247)	100	

* Death followed anaesthesia.

3.20 THE EFFECT OF PRETREATMENT WITH RESERPINE

Reserpine pretreatment was observed to prolong thiopental anaesthesia. Reserpine was administered subcutaneously since it had previously been established that the effect by this route was similar to when it was given intraperitoneally. Five mg/Kg. reserpine subcutaneously had an effect comparable with 50 mg/Kg. a-MD intraperitoneally but there was a longer interval before the prolongation could be observed (5 hours instead of 2 hours). Doubling the dose of reserpine did not increase the duration of thiopental anaesthesia. It became clear during these experiments that some hypothermia developed in the animals pretreated with reserpine. However, the fall in body temperature was small within the first few hours (especially when the room temperature was kept above 27^{o}C), whereas the moderate sedation observable then increased continously. The animals became very apathetic and immobile, but could readily be disturbed. When the hypothermia was prevented by keeping the mice in an incubator at 37^{o}C, there was considerably less prolongation of the thiopental anaesthesia (Table 9).

TABLE 10

THIOPENTAL CHALLENGE FOLLOWING PRETREATMENT WITH INTRAPERITONEAL PROADIFEN

Dose mg/Kg.	Time after Proadifen	No. of Animals	Duration of Anaesthesia in seconds. Mean (Range)	% Prolongation Control =100%	Sleeping time in control group in seconds. Mean (Range)
50	1 hour	10	179 (95 - 198)	95	189 (115 - 224)
50	3 hours	10	248 (178 - 367)	107	231 (145 - 263)
100	1 hour	10	790 (620 - 1090)	520	152 (105 - 187)
100	2 hours	10	391 (264 - 696)	177	221 (185 - 275)
100	3 hours	10	366 (237 - 583)	158	231 (145 - 263)
10*	30 mins.	10	156 (70 - 450)	65	241 (155 - 495)

* Administered intravenously.

3.30 THE EFFECT OF PRETREATMENT WITH PROADIFEN

Since only limited amounts of proadifen were made available for study, the nubmer of experiments had to be curtailed. Nevertheless, it was demonstrated that proadifen prolongs the thiopental sleeping time in mice when adequate doses are administered. It was established that the compounds had to be injected intraperitoneally in large doses and at least 60 minutes before the thiopental challenge in order to produce this effect. Doses of 100 mg/Kg. intraperitoneally at this time increased the sleeping time to approximately five times the control value (Table 10). However, it has to be remembered that this high dose of proadifen produces severe toxic effects in some sensitive animals, and may kill some mice within one hour (as was established in the toxicity study). With lower doses of proadifen in the range of 25-50 mg/Kg. intraperitoneally, no distinct prolongation could be established.

It appears from these results that the action of proadifen in prolonging thiopental anaesthesia

depends largely on the dose selected as well as the time interval between the administration and the barbiturate challenge. This view is also supported by the results obtained when proadifen 10 mg/Kg. was administered intravenously 30 minutes before the thiopental challenge. Instead of a prolongation of the thiopental anaesthesia, a reduction in the sleeping time was noted, when compared to the control group. This might explain some of the contradictory results obtained by other investigators, whereby some claimed a prolongation of thiopental anaesthesia due to proadifen, while others failed to demonstrate such an action. It appears that the prolongation of thiopental anaesthesia is in relationship, at least in part, to the acute toxicity of proadifen, in mice.

TABLE 11

THIOPENTAL CHALLENGE FOLLOWING PRETREATMENT WITH INTRAPERITONEAL ALPHA-METHYLDOPA

Dose mg/Kg.	Time after a-MD in mins.	No. of Animals	Duration of Anaesthesia in seconds. Mean (Range)	% Prolongation Control = 100%	Remarks
25	30	10	178 (104-307)	110	
25	60	10	308 (125-526)	190	Severe post-anaesthetic sedation.
25	120	20	356 (135-690)	220	
25	180	10	181 (90-245)	112	
25	360	20	187 (90-368)	115	
50	120	10	373 (217-487)	230	
50	180	10	386 (257-480)	238	Severe post-anaesthetic sedation.
50	240	10	360 (244-620)	222	
50	360	10	292 (183-366)	180	
200	120	10	627 (390-870)	387	Sedation after anaes-
400	120	10	863 (422-1478)	533	thesia for more than 30 minutes.
Control Saline		100	162 (95-275)	100	Received saline intra-peritoneally only.

3.40 THE EFFECT OF PRETREATMENT WITH ALPHA-METHYLDOPA

It was established that relatively small doses of a-MD prolong thiopental anaesthesia distinctly. However, an effect was only apparent 60 minutes after the drug was injected intraperitoneally. A dose of 25 mg/Kg. extended the anaesthetic time to 220% of the control time.

When 50 mg/Kg. intraperitoneally was administered only a further insignificant increase of 18% in the duration of the thiopental anaesthesia was noted. Only by increasing the dosage to 200-400 mg/Kg. (8-16 times the original dose) could the sleeping time be approximately doubled to 387% and 533% respectively (Table 11). Even though the duration of anaesthesia was not further prolonged at the 50 mg/Kg. dose level, however, the effect of the a-MD could be observed for a longer period of time. Three hours after 25 mg/Kg. a-MD the effect had disappeared, while it was still possible to demonstrate a considerable prolongation of anaesthesia 4-6 hours after the administration of a 50 mg/Kg. dose.

TABLE 12

THIOPENTAL CHALLENGE FOLLOWING PRETREATMENT WITH INTRAVENOUS ALPHA-METHYLDOPA VERSUS INTRAPERITONEAL ALPHA-METHYLDOPA

Dose mg/Kg.	Route of Administration	Time after a-MD in mins.	No. of Animals	Duration of Anaesthesia in seconds Mean (Range)	% Prolongation Control = 100%
25	Intraperitoneal	60	10	352 (180 - 615)	208
25	Intraperitoneal	120	10	432 (269 - 721)	267
10	Intravenous	10	10	147 (85 - 185)	91
10	Intravenous	30	10	141 (115 - 158)	87
10	Intravenous	60	10	404 (267 - 621)	249
10	Intravenous	120	10	463 (286 - 789)	286
Control (Saline)			10	169 (105 - 223)	100

If a-MD was administered by the intravenous route it also took a full hour before any prolongation of thiopental anaesthesia could be demonstrated. It is therefore obvious that the rate of absorption of a-MD from the intraperitoneal site was not responsible for the delay in appearance of an effect. Several possible reasons for this delay will be considered at a later stage.

However, it also appeared that intravenously administered a-MD was distinctly more potent than intraperitoneally administered a-MD (Table 12). The prolongation of thiopental anaesthesia after 2 hours with 25 mg/Kg. intraperitoneally was 267% while with 10 mg/Kg. intravenously it was 286% over the control values. It seems possible that the slower or incomplete absorption of a-MD from the peritoneal cavity might be responsible for this difference. When administered intraperitoneally the maximal plasma and tissue concentrations are presumably much lower.

3.50 ALPHA-METHYLDOPA PRETREATMENT AND PENTOBARBITAL CHALLENGE

Since the possibility existed that thiopental is desulphurised in the organism to form pentobarbital, it was interesting to examine the effect of a-MD pretreatment on pentobarbital-induced sleep. The effect of 3 different doses of a-MD was studied, namely 25 mg/Kg., 50 mg/Kg. and 100 mg/Kg. intraperitoneally. In each case the duration of pentobarbital-induced sleep was determined 1 hour and 3 hours after administering the a-MD to separate groups of mice. Only a few animals completely lost the righting reflex even with the largest dose of a-MD and so the presence of partial lose of reflex and the assumption of the 'belly position' was also recorded. In addition, the duration of sedation, ataxia and hyperexcitability was determined.

Approximately 50% of the control mice exhibited a partial loss of righting reflex, while all animals showed this symptom after a-MD pretreatment with a dose of 50 mg/Kg. intraperitoneally. The residual sedation and ataxia in pretreated animals was also

TABLE 13

PENTOBARBITAL CHALLENGE FOLLOWING PRETREATMENT WITH INTRAPERITONEAL ALPHA-METHYLDOPA

Dose mg/Kg.	Time after a-MD	Righting Reflex % of animals exhibiting Complete loss	Righting Reflex % of animals exhibiting Partial loss	Recovery in seconds.	Number of animals exhibiting ataxia, sedation and/or hyperexcitability etc.
25	1 hour	33	67	346	6 out of 12
25	3 hours	33	83	468	8 out of 12
50	1 hour	50	75	537	12 out of 12
50	3 hours	40	100	635	10 out of 10
100	1 hour	50	100	1183	12 out of 12
100	3 hours	60	100	±1800	10 out of 10
Saline 0.9%	3 hours	17	42	117	20 out of 30

All animals exhibited severe ataxia and hypermotility, therefore an accurate assessment cannot be given.

All animals were injected intravenously with a pentobarbital challenge dose of 25 mg/Kg.

notably longer. If the dose of a-MD was increased to 100 mg/Kg. intraperitoneally, full recovery of the righting reflex was considered delayed, compared with both the control groups and the lower dose a-MD groups (Table 13). These results indicate that a-MD has an effect on pentobarbital anaesthesia as well as on thiopental anaesthesia.

A very interesting observation was that a sub-anaesthetic dose of pentobarbital given after pretreatment with a-MD would lead to the loss or partial loss of the righting reflex; furthermore, the central nervous depression appeared 20-40 seconds after the injection of the pentobarbital.

Hence, when a-MD preceded a sub-anaesthetic dose of pentobarbital the potentiating effect of the pretreatment can clearly be demonstrated. The response is similar to an anaesthetic dose of pentobarbital, where the lag period following the injection is about 40-60 seconds. Thus the penetration of pentobarbital through the blood-

brain barrier appears not to be facilitated by the pretreatment with a-MD. The only explanation for the observed potentiation could be postulated on the basis that a-MD pre-sensitizes certain brain areas responsible for the anaesthetic effect exhibited by the barbiturates. Another explanation may be that sedative properties of a-MD are sufficiently additive to pentobarbital as well as to thiopental to produce the prolongation of anaesthesia.

3.60 PROLONGATION OF ANAESTHESIA BY ALPHA-METHYLDOPA IN RABBITS

There were two reasons why it was thought to be necessary to examine the effects of a-MD upon the duration of thiopental anaesthesia in rabbits as well as in mice. Firstly, it appeared likely that the effect of a-MD upon the body temperature of the experimental animals might play a part in prolonging the anaesthetic time, and the accurate electrical thermometer available was suitable only for a larger animal. Secondly it was thought to be of interest to study the urinary excretion of barbiturates after injecting thiopental, and the possible effect of a-MD on the appearance of these substances in the urine. While it would have been possible to do this in mice, large numbers would have been necessary and it was much easier from a technical point of view to use rabbits. Furthermore, a pilot experiment revealed that large quantities of thiopental and other thio- and oxybarbiturates appeared in the urine after injecting thiopental. This species therefore appeared suit-

TABLE 14

THIOPENTAL CHALLENGE FOLLOWING PRETREATMENT WITH SUBCUTANEOUS ALPHA-METHYLDOPA

(Mean values of 5 rabbits)

Dose mg/Kg.	Time in Seconds: Duration of Respiratory Arrest Mean (Range)	Time in Seconds: Start Moving Mean (Range)	Time in Seconds: Recovery of Righting Reflex Mean (Range)	% Prolongation Control= 100%	Remarks
Solvent Vehicle only	28 (14- 42)	510 (448-585)	918 (763-1048)	100	Post anaesthetic sedation ± 1 hour.
200	72 (43-110)	929 (773-1130)	1740(1457-1964)	189.5	Post anaesthetic sedation more than 2 hours.
400	112 (74-135)	3015(2263-3705)	5321(4197-6194)	580	Respiratory depression ± 10 minutes (assisted respiration). Post anaesthetic sedation and ataxia more than 2 hours.

able for investigating any possible effects on the drugs on the metabolism and excretion of thiopental.

It was decided to administer the a-MD subcutaneously to the rabbits, because the intraperitoneal or intravenous injection of the acid solution was obviously painful for these animals. All animals were sacrificed for further investigations after or during awaking.

The same dose of thiopental as used on the mice was administered to the rabbits i.e. 25 mg/Kg. intravenously. The mean recovery time of the control group was fifteen minutes, while that of the group given a-MD 200 mg/Kg. subcutaneously was 29 minutes. Using 400 mg/Kg. subcutaneously the recovery of the righting reflex was prolonged by more than five-fold (Table 14). Respiratory depression was marked in these animals, and it seems likely that cerebral anoxia may have played some part in the prolongation of anaesthesia.

TABLE 15

THIOPENTAL CHALLENGE FOLLOWING PRETREATMENT WITH INTRAVENOUS 5-HYDROXYTRYPTAMINE

Dose mg/Kg.	Time after 5-HT in mins.	No. of Animals	Anaesthesia in seconds Mean (Range)	% Prolongation Control = 100%	Remarks
5	20	10	259 (146 - 424)	180	
10	20	10	472 (175 - 2,140)	328	
20	20	8	570 (170 - 1,005)	396	2 out of the 10 animals died.
Control (Saline)	20	10	144 (100 - 175)	100	

4.00 ROLE OF 5-HYROXYTRYPTAMINE IN THE PROLONGATION OF THIOPENTAL ANAESTHESIA BY ALPHA-METHYLDOPA

As a-MD is a potent depletor of endogenous 5-HT, it was necessary to test the reaction of the strain of mice used in these present studies to pre-injected doses of 5-HT followed by a standard dose of thiopental. Since the blood-brain barrier is relatively impermeable to 5-HT large doses were administered intravenously to ensure reasonably increased levels in the brain.

Moderate to severe diarrhoea was observable in all animals approximately 5-7 minutes after the administration of 5-HT. Some animals exhibited clonic-tonic convulsions shortly after the intravenous injection followed by weakness of the extremities.

When the mice were challenged with thiopental twenty minutes after the 5-HT injection, a marked prolongation of the anaesthetic time was demonstrated (Table 15). It was therefore established that the mice used reacted to 5-HT pretreatment in the manner described by various workers using pentobarbital,

TABLE 16

COMBINED EFFECT OF ALPHA-METHYLDOPA AND 5-HT ON PROLONGATION OF THIOPENTAL ANAESTHESIA

Substance	Dose mg/Kg.	No. of Animals	Route of Administration	Time before challenge (in minutes)	Total Anaesthesia in seconds Mean	(Range)	% Control = 100%
a-MD	25	12	Intraperitoneal	180	649	(410-1080)	345
5-HT	10	10	Intravenous	20			
a-MD	50	10	Intraperitoneal	180	681	(515-1280)	362
5-HT	10	10	Intravenous	20			
Control Saline	10 ml.	10	Intraperitoneal	180	188	(135-212)	100
	10 ml.		Intravenous	20			

Remarks: All treated animals exhibited a phase of respiratory arrest for a period of 11-54 seconds immediately after the thiopental injection.

hexobarbital or thiopental in rat experiments.

The mice showed slight sedation which was detectable without the use of the actograph but it was less than that observed with reserpine.

This experiment was then repeated in animals pretreated with a-MD. If the prolongation of thiopental anaesthesia produced by a-MD was due to depletion of 5-HT, replacing the 5-HT just before the thiopental challenge should restore the duration of anaesthesia to normal. However, 5-HT 10 mg/Kg. intravenously failed to counteract the effect of a-MD 25 and 50 mg/Kg. intraperitoneally on thiopental anaesthesia.

Indeed some evidence was obtained that the combination of a-MD with 5-HT actually prolonged anaesthesia more than a-MD alone (Table 16). It was also interesting to note that the doses of a-MD required to antagonise the laxative effect of the intravenously administered 5-HT were 500 mg/Kg. and more. These results suggested that the prolongation of thiopental anaesthesia by a-MD was not due to depletion of 5-HT in the brain.

TABLE 17

EFFECT OF THIOPENTAL, ALPHA-METHYLDOPA, RESERPINE AND PROADIFEN ON THE BODY TEMPERATURE OF RABBITS

(Rectal temperatures in ^{o} Centigrade)

Substance	Dose mg/Kg.	Route of Administration	No. of Animals	Initial Temperature	TEMPERATURE AFTER ADMINISTRATION – MEAN (RANGE) 30 mins.	60 mins.	90 mins.	120 mins.	180 mins.	240 mins.	300 mins.
Thiopental a-MD-Vehicle only Control	25 2 ml	Intravenous	5	39.08 (38.8-39.4)	38.94 (38.6-39.2)	38.94 (38.7-39.2)	38.88 (38.6-39.2)	38.88 (38.6-39.1)	38.94 (38.6-39.1)	38.92 (38.5-39.2)	38.94 (38.6-39.2)
Vehicle Only Control	2 ml	Subcutaneous	10	39.04 (38.8-39.4)	39.93 (38.5-39.2)	38.92 (38.4-39.2)	38.93 (38.5-39.2)	38.87 (38.4-39.1)	38.88 (38.4-39.0)	38.90 (38.4-39.1)	38.86 (38.4-39.1)
Alpha-methyldopa	100	Subcutaneous	5	38.90 (38.6-39.2)	38.86 (38.5-39.1)	38.84 (38.5-39.0)	38.82 (38.6-39.0)	38.78 (38.5-39.0)	38.88 (38.6-39.1)	38.90 (38.6-39.1)	38.96 (38.7-39.2)
Alpha-methyldopa	200	Subcutaneous	5	38.94 (38.7-39.2)	38.78 (38.5-39.0)	38.78 (38.6-39.0)	38.74 (38.6-38.9)	38.68 (38.5-38.9)	38.72 (38.1-38.6)	38.72 (38.1-38.6)	38.68 (38.1-38.7)
Alpha-methyldopa	400 (2ml/Kg)	Subcutaneous	10	38.94 (38.6-39.1)	38.62 (38.4-39.0)	38.52 (38.2-38.8)	38.36 (38.0-38.7)	38.30 (38.0-38.6)	38.88 (38.4-39.0)	38.90 (38.4-39.1)	38.86 (38.4-39.1)
					60 mins.	120 mins.	180 mins.	240 mins.	300 mins.	360 mins.	420 mins.
Reserpine	1	Subcutaneous	5	38.80 (38.6-38.9)	38.80 (38.7-38.9)	38.74 (38.6-38.8)	38.70 (38.6-38.8)	38.42 (38.2-38.5)	38.34 (38.2-38.4)	38.24 (38.1-38.4)	38.16 (37.9-38.3)
Reserpine	1	Intravenous	5	38.66 (38.2-38.9)	38.66 (38.7-38.9)	38.76 (38.4-38.9)	38.70 (38.4-38.9)	38.48 (38.1-38.7)	38.36 (38.0-38.6)	38.16 (37.8-38.4)	38.08 (37.6-38.3)
Proadifen	100	Subcutaneous	5	38.86 (38.6-39.1)	38.84 (38.6-39.0)	38.84 (38.5-39.1)	38.88 (38.5-39.2)	38.88 (38.5-39.1)	38.88 (38.6-39.1)	38.92 (38.6-39.1)	38.86 (38.6-39.0)
				Before a-MD	After	10 mins.	15 mins.	20 mins.	30 mins.	60 mins.	90 mins.	120 mins.
Thiopental (25mg/Kg.) (after a-MD pretreatment	(200 mg/Kg a-MD)	Subcutaneous	5	39.02 (38.9-39.4)	38.76 (38.6-39.0)	38.66 (38.6-38.8)	38.58 (38.3-38.8)	38.52 (38.3-38.7)	38.50 (38.2-38.8)	38.54 (38.2-38.8)	38.70 (38.5-38.9)	38.80 (38.6-39.1)
Thiopental	(400 mg/Kg a-MD)	Subcutaneous	5	39.02 (38.8-39.1)	38.76 (38.6-39.0)	38.56 (38.4-38.7)	38.46 (38.3-38.7)	38.46 (38.3-38.7)	38.42 (38.3-38.6)	38.28 (38.2-38.4)	38.18 (38.0-38.3)	38.18 (38.1-38.3)

5.00 EFFECTS OF ALPHA-METHYLDOPA, RESERPINE AND PROADIFEN UPON THE BODY TEMPERATURE

METHOD

Initially mice were used for this study, the rectal temperature being measured by means of a small glass thermometer. However, the results were unsatisfactory due to the insensitivity and the size of the thermometer and the struggling of the animals, and it was decided to carry out this study in rabbits using an Electric Universal Thermometer (Type TE 3 by Ellab, Copenhagen). The weights of the rabbits varied from 2,100-2,630 gm. Experiments were carried out with a-MD, reserpine and proadifen as well as with thiopental alone, and the dosages, time intervals and routes of administration are set out in Table 17; the figures in brackets are the lowest and highest recorded temperatures in each group. The room temperature was between 24-30^{o}C. Control groups received normal saline or the vehicle in which the a-MD was made up.

5.10 THIOPENTAL

The rectal temperature did not change significantly when thiopental was administered together with the a-MD vehicle (Table 17).

5.20 ALPHA-METHYLDOPA

It was only with the higher dosage of a-MD that there was a drop in rectal temperature (Table 17). The main decrease was observed between 60-240 minutes after a dose of 400 mg/Kg. subcutaneously. After 180 minutes the rectal temperature started to return to normal, and after eight hours it reached the initial baseline value. Alpha-methyldopa in doses of 100-200 mg/Kg. subcutaneously had no significant effect on body temperature.

5.30 RESERPINE

Reserpine in a dosage of 1 mg/Kg. intravenously or subcutaneously lowered the body temperature slightly. The lowest values appeared only about 6-8 hours after the administration of reserpine, regardless of the route of administration (Table 17). Twenty-four hours later the rectal temperatures of these animals were still slightly below the control values. The lower

room temperature during the night might have contributed to this finding.

Another group of rabbits were injected with 2.5 mg/Kg. subcutaneously. No change in body temperature was noted when compared to the fluctuation of the values recorded in the control group. The room temperature was much higher than it was during other investigations, namely the experiments started when the room temperature had reached 29°C. In all animals, sedation, miosis, ptosis, and other side-effects of reserpine e.g. diarrhoea, were noted to occur within 2-3 hours after the administration of the drug. It was attempted to replace at random, the loss of fluid during the experiment. There was no action on the body temperature when the animals received 20-30 ml. of saline in divided doses during a period of 3 hours, subcutaneously administered.

5.40 PROADIFEN

It was established that proadifen at a dose of 100 mg/Kg. subcutaneously did not influence body temperature in rabbits during an observation period of 5 hours (Table 17).

6.00 ROLE OF ADDITIVE CENTRAL NERVOUS SYSTEM DEPRESSION IN THE PROLONGATION OF THIOPENTAL ANAESTHESIA BY ALPHA-METHYLDOPA AND PROADIFEN

Reserpine has an established sedative action, and this is probably responsible, at least in part, for its action in prolonging thiopental anaesthesia. In order to assess whether a-MD or proadifen produced sedation, the locomotor activity was investigated by means of an actograph (Jaquet, Switzerland). This apparatus records on an electrical totalizator the movement of animals enclosed in a dark cage resting on an airmail letter balance. The movements are transmitted by a lever to a mercury bridge, closing an electrical circuit. The mice were males weighing 22-24 gm.

They were placed singly into the dark cage. After a stabilising period of five minutes, a ten or fifteen minute period of movement was recorded. The figure obtained served as the baseline. The animal was then removed and injected with the drug being tested. After an hour it was again put into the dark cage, and after a similar stabilisation period

TABLE 18

LOCOMOTOR ACTIVITY OF MICE

Measured by means of the 'Actograph'

Substance	Dose mg/Kg. Intraperitoneal	Value Pretest = 100%	60 minutes	% Decrease	120 minutes	% Decrease	180 minutes	% Decrease
a-MD	50	1401	611	56	540	61	529	62
	100	1612	510	68	483	70	600	63
	200	1594	220	86	189	88	265	83
Proadifen	50	1605	364	77	340	79	416	74
	75	1643	290	82	265	84	318	81
	100	1374	111	92	99	93	119	91
Control (Saline)	10.0 ml.	1406	1346	4	1298	8	1291	8

Average motility (movement) of groups of 6 mice as counted by a totalisator.

the activity was again recorded. Five or six animals were studied at each dosage level, and a control group was tested with each of the treated groups to eliminate possible daily fluctuation.

The experiments were carried out over several days between 8.30 a.m.-12.30 p.m. because it was found that afternoon values varied too much. The probable reason for this is that the animals normally sleep at this time.

When a more accurate instrument became available at a later date, it was used to check some of the observations. The instrument was the Actophotometer (Metro Scientific Inc. New York) and a group of six mice was studied at a time.

RESULTS

It was observed that both a-MD and proadifen reduced the locomotor activity in mice in the doses studied (Table 18).

There was a very small reduction in spontaneous motility in the smaller dosage range with a-MD.

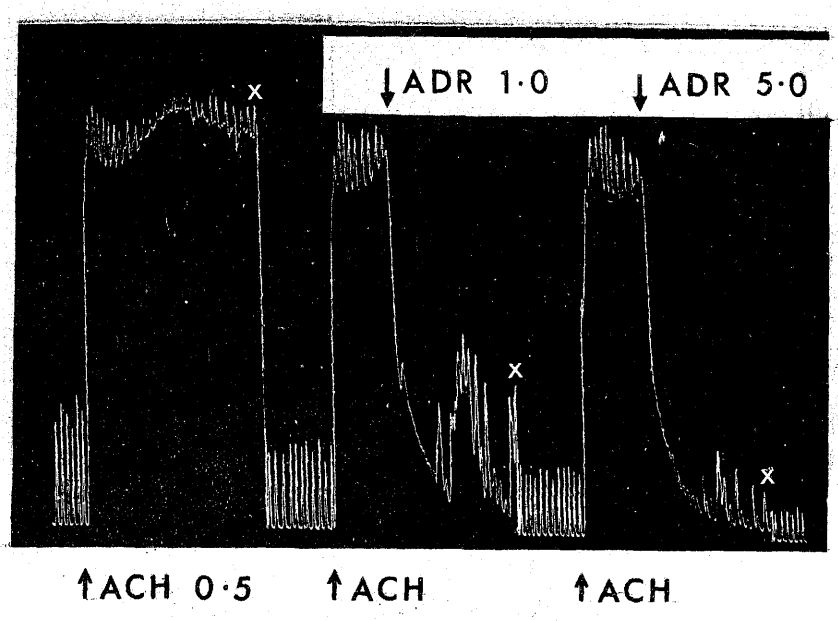
However, if the dose was increased to 100 or 200 mg/Kg. intraperitoneally a large decrease was noted, which persisted for a prolonged period of several hours.

These decreases in locomotor acitvity were only observed when tested by means of the actograph and were not readily noticeable in the observation cage.

An interesting finding was noted with proadifen. All animals exhibited a strong inhibition of their activity, as compared with a control group. This confirmed the direct observations made during the toxicity study, when sedation was noted in spite of the fact that convulsions also occurred. Convulsions suggest stimulation and not depression of the central nervous system. Moreover, the fact that the convulsions were inhibited by phenobarbital (see 2.50) provided support for the view that proadifen does stimulate the central nervous system. It therefore appears that this drug has both stimulatory and depressant actions on the central nervous system.

The proadifen and the a-MD results were checked by repeating the study using the more sensitive Actophotometer. A similar effect was noted. Shortly after the intraperitoneal administration of the compound, an inhibition of the motor activity was produced. Hence, a central nervous system depressive effect appears to be present, and may be additive to the barbiturates anaesthetic action.

FIGURE 10



The physiological antagonism between acetylcholine (the agonist and adrenaline is demonstrated on isolated rabbit ileum.

7.00 ROLE OF POSSIBLE ADRENERGIC ACTION OF ALPHA-METHYLDOPA IN THE PROLONGATION OF THIOPENTAL ANAESTHESIA

Adrenergic stimulants in high dosage may prolong barbiturate anaesthesia (Dandiya & Sellers, 1961), the high dosage being necessary in order to obtain an adequate concentration across the blood-brain barrier. Alpha-methyldopa is a close chemical relative of adrenaline and nor-adrenaline (Fig. 6). Adrenaline and nor-adrenaline are physiological antagonists (Fig. 10) of acetylcholine, and it was therefore thought to be of interest to determine whether a-MD was also antagonistic to acetylcholine. There was a further reason for studying the possible relationship between the actions of a-MD and acetylcholine. Some substances, such as anticholinesterase, which potentiate the action of acetylcholine may also prolong barbiturate anaesthesia (Greene, 1964). This prolongation can be antagonised by atropine. It was therefore considered necessary to investigate the possibility that a-MD might act in this way. Studies with

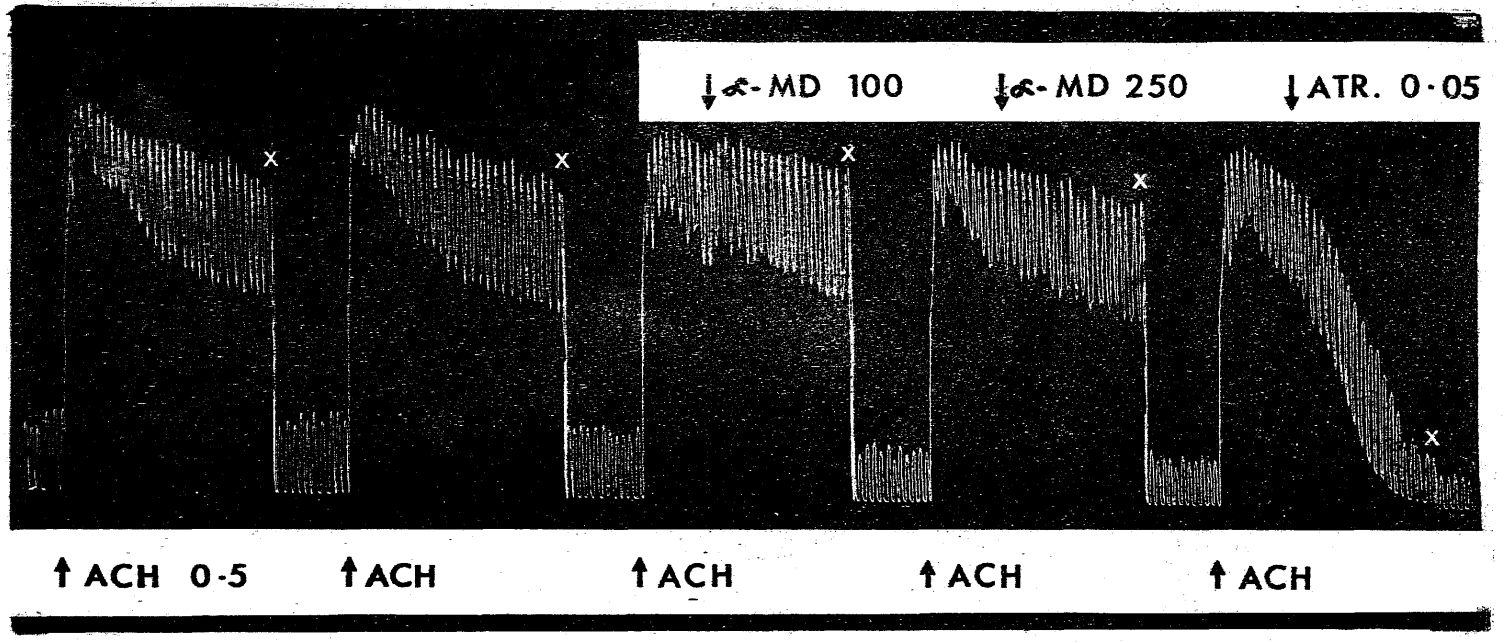
isolated rabbit ileum were undertaken, the muscarinic action of acetycholine being selected because atropine antognises the acetylcholine prolongation of barbiturate anasethesia.

METHOD

Pieces of ileum, approximately 2 cm. in length, were excised after killing the animals. After removal, the pieces were cleaned with Tyrode's solution (Dittler; Kochmann).

After storing the ileum in the Tyrode's solution at room temperature for approximately three hours, a single piece was suppended in a 50 ml. glass vessel containing Tyrode's solution at $37^{o}C$. aerated with 95% oxygen and 5% CO_2. A stabilisation period of 15 minutes was usually sufficient before the acetylcholine spasm was induced. The response to two individual standard doses of 5×10^{-7} gm/ml. acetylcholine was tested before a-MD was added to the bath. Adrenaline and atropine served as control compounds. The dose of acetylcholine was so selected that only a sub-

FIGURE 11



Alpha-methyldopa at high doses does not exhibit an antagonistic effect on acetylcholine-induced spasms of isolated rabbit ileum. Atropine is used as a standard and specific competitive antagonist. Doses are given in microgram per millilitre bath fluid.

maximal spasm was induced to enable a possible further agonistic effect due to a-MD to be detected.

The contraction of the smooth muscle was recorded with a frontal writer which was counter-balanced with 2 gm. The lever ration was 1:4, i.e. 1 cm. contraction of the muscle exhibited 4 cm. recording on the smoked kymograph paper.

RESULTS

As can be noted from Fig. 11, doses of up to 2.5 $\times 10^{-4}$ gm/ml. of a-MD failed to influence the spasm induced by acetylcholine. Atropine in a dose of 5×10^{-8} gm/ml. and adrenaline 10^{-6} gm/ml. produced a 100% antagonism within 1-1½ minutes. It was also noted that there was no further increase in the acetylcholine-induced sub-maximal spasm. A synergistic action between acetylcholine and a-MD can therefore be eliminated as well. It is thus unlikely that a-MD would have a direct action on acetylcholine transmission in the central nervous system comparable with that of physostigmine or large doses of catecholamines.

8.00 THE SEPARATION OF OXY- AND THIO-BARBITURATES BY THIN-LAYER CHROMATOGRAPHY

INTRODUCTION

A theoretically possible explanation for the prolongation of thiopental anaesthesia by a-MD and the other drugs was that they delayed the metabolic degradation of the barbiturate and so extended its duration of action. In order to investigate this possibility, it was necessary to be able to detect the degradation products in the urine of rabbits.

Paper chromatography methods have been described for the separation of barbiturates (Wickström and Salvesen, 1952; Dietz and Soehring, 1957; Frey, 1959; and others). The techniques, however, have certain shortcomings; they are time-consuming (approximately 18 hours per separation) and the R_f value of some of the oxy- and thio-barbiturates lie very close to each other. For example, it was found to be impossible to separate methohexital (an oxybarbiturate) from thiopental (a thiobarbiturate) by any existing method. Since there is

evidence that pentobarbital (an oxybarbiturate) may be one of the metabolites of thiopental, it was clearly desirable to be able to separate this and any other possible breakdown products in order to determine whether there was any effect of a-MD upon thiopental degradation.

A method was therefore developed by which a mixture of several oxy- and thio-barbiturates can be separated in less than an hour. Furthermore, the separation is clear. It is not necessary to rely upon R_f values, which are only of value when standards are run simultaneously. The separation was achieved by the use of two reagents for detecting the barbiturates. Thiobarbiturates are rendered visible with copper sulphate-dietylamine, which does not react with the oxybarbiturates. The second reagent, mercurous nitrate, is applied after drying the plate. It is not necessary to wash the plate after applying the copper-dietylamine reagent. With the application of the mercurous nitrate the oxybarbiturates became visible.

Copper sulphate-diethylamine was first described as a reagent for the quantitative determination of thiobarbiturates by Raventós (1946). The reagent was somewhat modified by Dietz and Soehring (1957), and has been listed in Stahl's book in Thin-layer Chromatography (1965).

Up to the present time, the most common 'spotting' reagent used for barbiturates is an aqueous 1% $Hg_2(NO_3)_2$ solution. Other reagents which have been used are bromophenol/silver nitrate (lemon-yellow spots when viewed under UV), and potassium permanganate which gives yellow colour reactions with some barbiturates as well as with some non-barbiturate hypnotics.

The sensitivity of the new method is high: less than 10 ug. can readily be detected, whereas up tp 20 ug. was formerly considered by some investigators to be the minimum quantity. An added advantage of the method is that oxy- and thio-barbiturates appear as separate 'classes' on the developed thin-layer chromatogram.

8.10 MATERIALS AND METHODS

MATERIALS

Modified Copper Reagent:

Seven hundred and eighty milligrams of $CuSO_4.5H_2O$ (Merck G.R.) were dissolved at room temperature in 97 ml of A.R. methanol. Anhydrous $CuSO_4$ - as recommended by Dietz and Soehring (1957) and Frey (1959) was in no way superior to the pentahydrate.

To the light blue solution, 3 ml. of diethylamine (B.D.H. Laboratory Reagent) were added. A dark blue precipitate formed. On shaking the precipitate, a suspension in the supernatant solution resulted. The reagent was stored in the deep freeze. Contrary to earlier authors (Waldi, 1965 and others) this reagent keeps perfectly satisfactorily for several months.

Mercurous Nitrate Solution

A saturated aqueous solution of $Hg_2(NO_3)_2$ (B.D.H. - A.R. Grade) in distilled water was prepared (Deininger, 1955; Randerath, 1963).

Solvent System

A chloroform A.R. - acetone A.R. mixture (85:15 v/v) gave good and reproducible results.

Absorbents

30-32 gm. of Kieselgel (Silica Gel G) 'Camag' and in later experiments 'Merck' was suspended in 60 ml. distilled water. No advantage was gained by rendering the slurry alkaline with either 0.1/N - NaOH or dilute sodium acetate solution.

Barbiturates Tested

All reference compounds for this study were made up usually in concentrations of 0.05-0.25% in distilled water from the relevant sodium salts. To test the specificity of the method the following barbiturates were investigated.

(a) Amobarbital (Amytal)

(b) Barbital (Veronal)

(c) Buthalitone (Ubreval)

(d) Hexobarbital (Evipan)

(e) Methohexital (Brietal)

(f) Pentobarbital (Nembutal)

(g) Phenobarbital (Luminal)

(h) Thialbarbital (Kemithal)

(i) Thiopental (Pentothal)

METHODS

Solutions of the above barbiturates were applied to the TLC plates in suitable concentrations in the usual manner, but preferably under a stream of nitrogen to prevent oxidation of the compounds, which might create artifacts. The maximal volume applied to the plates was never more than 0.02 ml. by means of a micropipette.

Activation of Plates

Silica Gel G was applied to the plates to a thickness of 0.25-0.30 mm. using a 'Shandon Unoplan' leveller and spreader. The plates were left to dry at room temperature for 45-60 minutes and were then placed in an oven at 95-105^{o}C. for 1-1$\frac{1}{2}$ hours. The best results were obtained when the plates were used within 1-2 days after activation. The plates were stored in an air-tight metal box, sealed with pressure tape. The solvent front was generally

kept within 100–150 mm. from the origin, depending on the separation desired.

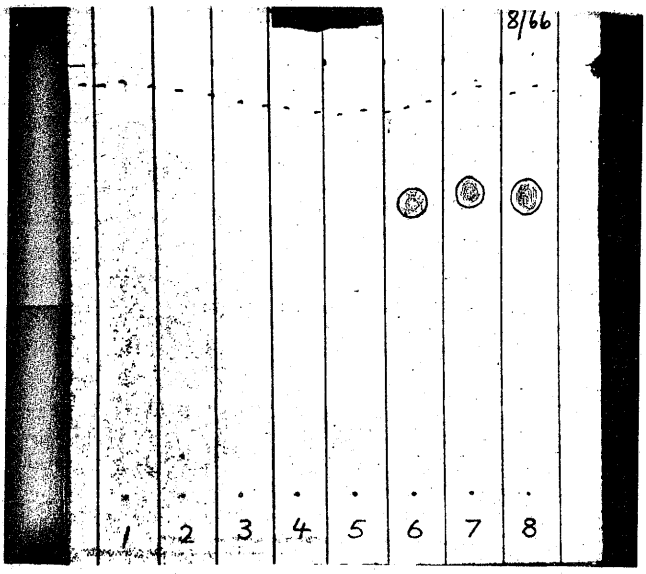
Vapour Saturation of the Chamber

It was not necessary to equilibrate the chamber before developing the TLC plates, but it was advisable to place filter paper (Whatman No.1) soaked in the developing fluid around half the inner walls of the chamber.

Development

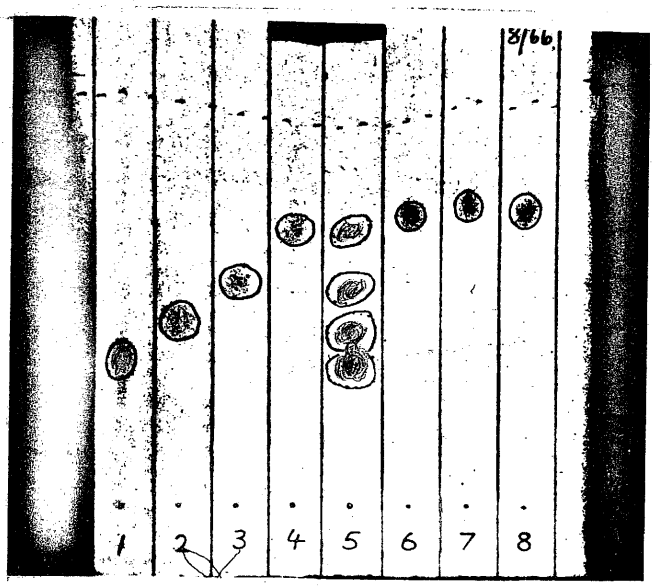
The plates were introduced into the chamber containing chloroform–acetone mixture, and the time recorded. The chamber was covered with a glass plate to ensure full vapour saturation and to prevent evaporation of the solvents. When the solvent reached a predetermined height, e.g. 150 mm. the plates were removed from the solvent chamber and were left for a few minutes on the bench-top to allow complete evaporation of the solvent. They were then first sprayed with the copper–diethylamine reagent, and all spots which appeared after this treatment were dully marked for the correct location.

FIGURE 12



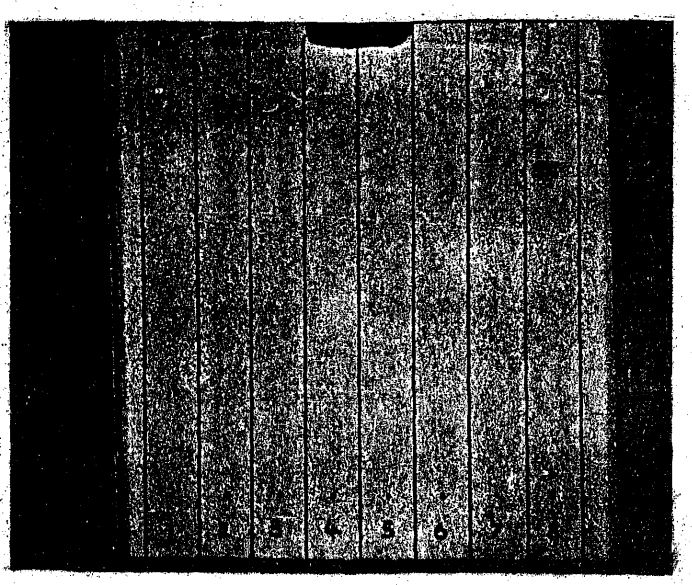
TLC plate after spraying with copper/diethylamine reagent. The thiobarbiturates appear immediately as yellowish green spots with relatively high R_f values.
6 = thiopental; 7 = kemithal; 8 = buthalitone.

FIGURE 12a



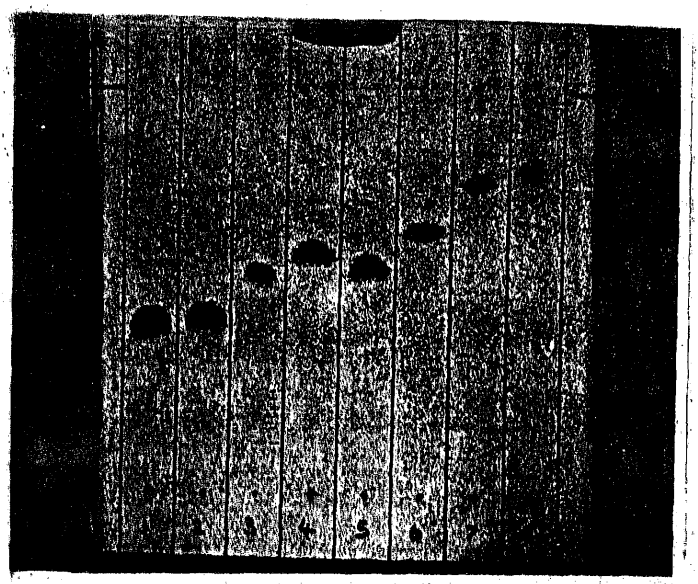
Same plate after application of mercurous nitrate reagent. The oxybarbiturates appear within 60–90 seconds as white to dark grey areas, while the thiobarbiturate spots are intensified. 1 = barbital; 2 = phenobarbital; 3 = pentobarbital; 4 = methohexital; 5 = mixture of 1–4. 6–8 as for figure 12.

FIGURE 13



TLC plate after spraying with copper/diethylamine reagent
Only thiopental (8) is visible.

FIGURE 13a



Same plate after application of mercurous nitrate reagent
The oxybarbiturates appear as a group.

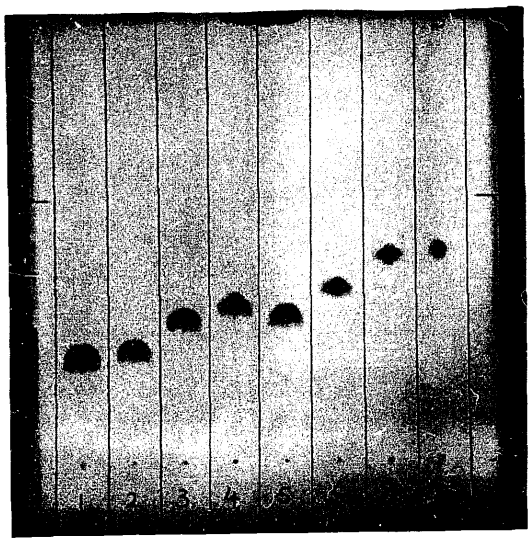
25 µg of wach has been applied.
1 = barbital; 2 = phenobarbital; 3 = pentobarbital;
4 = secobarbital; 5 = amobarbital; 6 = hexobarbital;
7 = methohexital.

After the plates were dry again they were treated with the second spray, namely with the saturated $Hg_2(NO_3)_2$ solution. After spraying with the copper-diethylamine reagent, thio-barbiturates appear instantaneously as distinct yellow to yellow-green spots with relatively high R_f values (Figs. 12 and 12a), while some oxybarbiturates may manifest as transient, very faint blue spots (especially if the plates have been over-loaded with barbiturates). These spots rapidly disappear as the plates dry.

After the plates have been treated with the mercurous nitrate reagent, oxybarbiturates can be seen as white to dark grey spots, some of which are transient, depending on the amount or type of barbiturate applied (Figs. 12 and 13). For this reason it is advisable to mark the exact location, as they do not reappear if the mercury reagent is applied a second time. Usually the thiobarbiturate colour intensifies to brown or brown-green (Fig. 12 and 12a). These latter spots are stable for several weeks, fading only slightly.

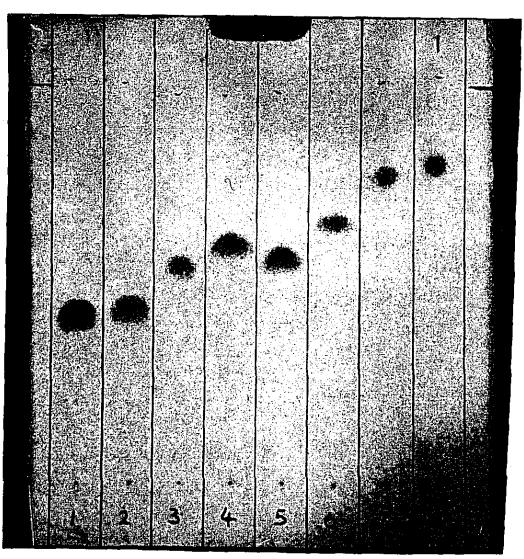
COMPARISON OF DEVELOPMENT DISTANCE

FIGURE 14



Development distance 100 mm.

FIGURE 14a

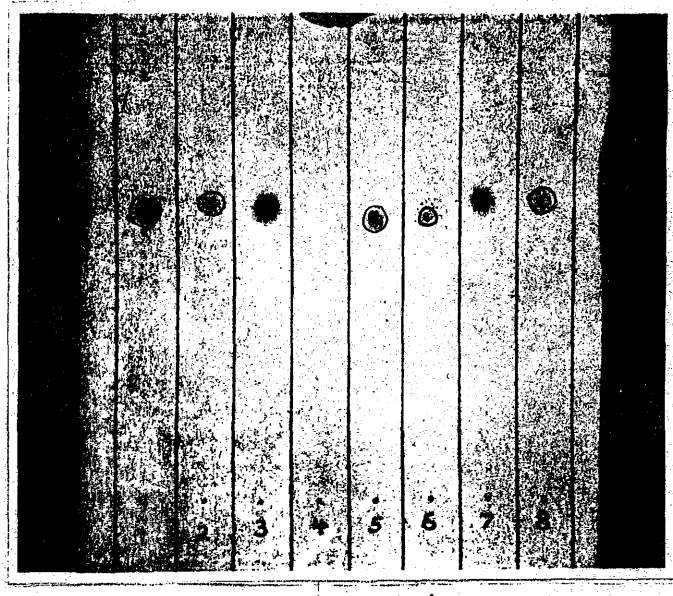


Development distance 150 mm.

For both figures:-
25 ug of each has been applied to the TLC plate.
1 = barbital; 2 = phenobarbital; 3 = pentobarbital;
4 = secobarbital; 5 = amobarbital; 6 = hexobarbital;
7 = methohexital; 8 = thiopental.

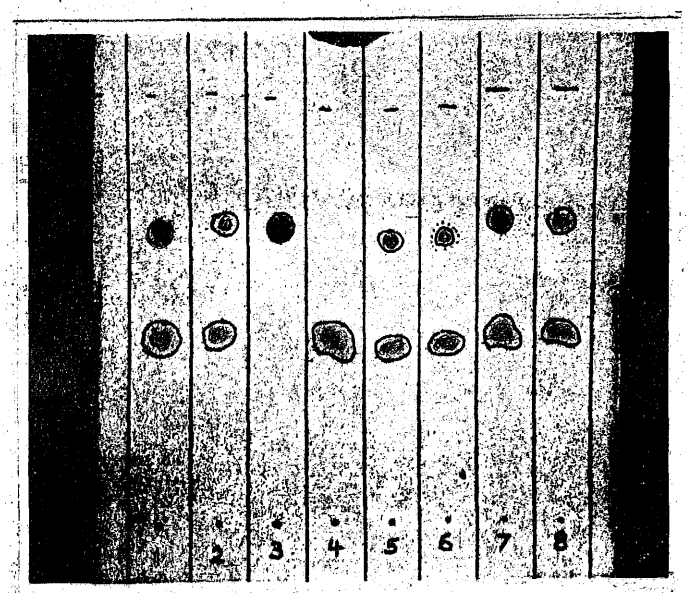
THIOPENTAL AND PENTOBARBITAL SEPARATION

FIGURE 15



After spraying with copper/diethylamine reagent only.
Only thiopental is visible.

FIGURE 15a



The same TLC plate after spraying with mercurous nitrate solution. Pentobarbital now becomes visible.

For both figures:-
1-6 sodium salts; 7,8 acid forms.
1=50 µg both substances; 2=12.5 µg both; 3=50 µg thiopental only; 4=50µg pentobarbital only; 5=25µg both; 6=6.25µg both; 7=25µg both (acid); 8=12.5µg both (acid).

RESULTS

As shown in Table 19 and Figures 14 and 14a, a developing distance of 150 mm. was found to produce a clear separation of a group of barbiturates, especially in the case of pentobarbital and thiopental, where there was a R_f (x100) difference of 26 for a total solvent front distance of 150 mm. from the point of origin (Fig. 15 and 15a). The duration for such a distance is usually between 40-45 minutes. (A shorter time and distance may be selected for routine investigation of barbiturate poisoning cases).

The R_f values are given in Table 19, but special care has to be taken when interpreting results, since these may vary with factors such as the thickness and evenness of the layer, the degree of activation of the layer, the amount of substance applied, the impurities present in the substance, the chamber temperature and the quality of the solvents used for the development of the plates, etc. (Brenner et al, 1962). It is therefore recommended that standard preparations should be used wherever possible (Paulus, 1963).

TABLE 19

A COMPARISON OF DEVELOPING DISTANCE AND R_f VALUES AT $21^{0}C$.

Development Time	19–22 minutes	42–45 minutes
	R_f values x 100	
Substance (12.5 µg) (in aqueous solution as the Na-salt)	100 mm. Distance	150 mm. Distance
Barbital	41	43
Phenobarbital	44	45
Pentobarbital	55	55
Amobarbital	56	57
Secobarbital	60	61
Hexobarbital	65	67
Methohexital	77	79
Thiopental	80	81

Each value represents the mean of three individual experiments. The pair of plates were from the same batch.

Inspection of the R_f values obtained for the various barbiturates studied in the present investigation revealed an interesting phenomenon.

It appeared that there is an inverse relationship between these values and the duration of action of the drugs. Thus, of the oxybarbiturates, barbital had the lowest R_f value, while methohexital had approximately the same R_f value as the thiobarbiturate thiopental. The duration of methohexital and thiopental anaesthesia is similar.

The R_f values of barbital, phenobarbital, pentobarbital, hexobarbital, methohexital and thiopental increase in this order, while their lag period of onset and the duration of action in a number of species are in decreasing order.

There is good evidence that the duration of action of the barbiturates depends to a considerable degree upon their lipid solubility (Brodie and Hogben, 1957; Brodie, 1952; Mark et al., 1958; Mayer, Maickel and Brodie, 1959; Brodie, Kurz and Schanker, 1960 and others). Those drugs with the highest R_f value in this system are obviously most soluble in the chloroform/acetone mixture. It therefore seems reasonable to postulate that the inverse correlation which has been observed is a consequence of the solubility properties of these compounds.

8.20 QUALITATIVE ANALYSIS OF BARBITURATES IN THE URINE OF RABBITS AFTER INJECTING THIOPENTAL SODIUM

COLLECTION AND EXTRACTION OF URINE

Urine was collected by direct bladder puncture. Specimens from several animals were pooled so as to obtain sufficient material. The urine was acidified with HCl to a pH of less than 2 and then extracted three times with sufficient volume of freshly prepared chloroform/ether 1:1 v/v. The mixture of solvent was found to minimise emulsification. Some emulsification nevertheless frequently occurred, especially when small amounts of the solvent mixture were used. However, separation of the emulsion could easily be achieved by centrifugation at approximately $\pm$ 900-1,200 x g. It was desirable to use as small a volume of the solvent mixture as possible both because of the expense and the inconvenience of evaporating a large volume. The so-called organic solvent fractions were filtered through Whatman No. 1 filter paper to remove large fat globules which were sometimes present even after centri-

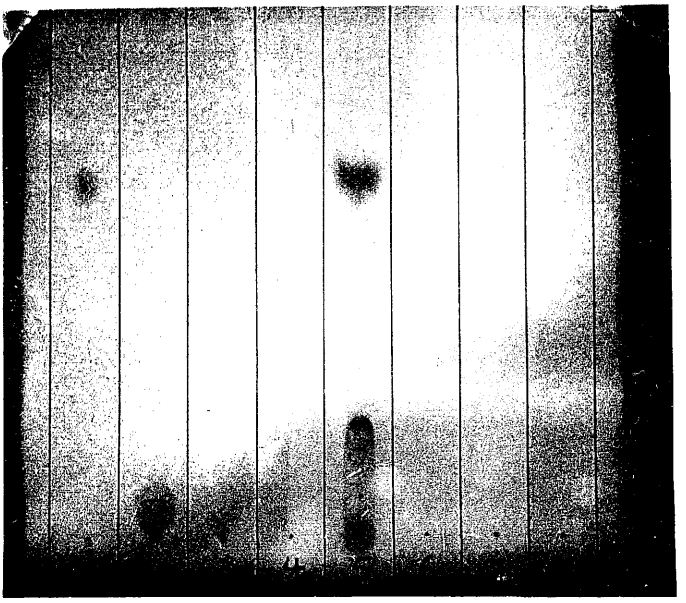
fugation. Filtering through charcoal was abandoned after it was found that approximately 20 to 30% of the extracted barbiturates was lost by this method. It was observed that the extracted urinary pigments did not interfere with the TLC separation of barbiturates.

After filtering the chloroform/ether extract it was evaporated at a temperature of 50-55^{o}C under vacuum by means of a Rotary Evaporator. The residue was dissolved in a sufficient quantity (usually 2-5 ml.) of chloroform/acetone and applied to the plate in various volumes.

EXPERIMENT

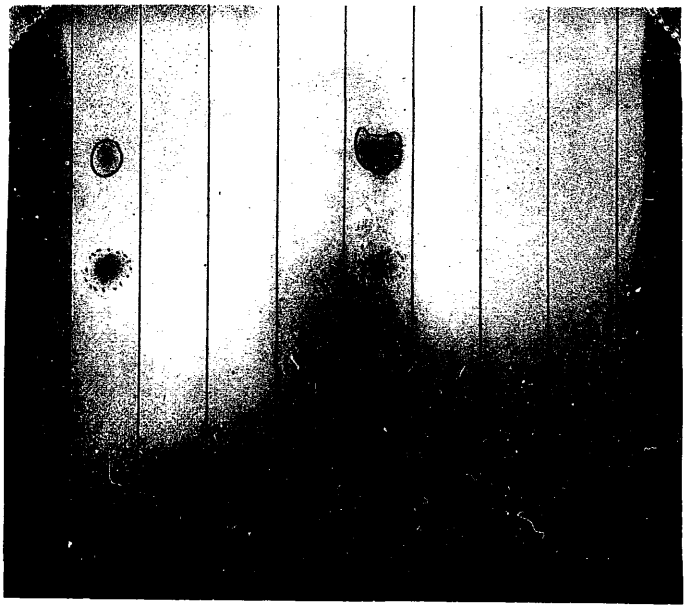
Five rabbits were used. Thiopental sodium was injected slowly intravenously until the animals were anaesthetised as judged by complete loss of corneal reflexes and loss of righting reflex. The dosage required was 20-25 mg/Kg. Urine was collected 45 minutes later and pooled. Urine was also collected from unanaesthetised animals, pooled and a mixture of thiopental sodium and pentobarbital sodium in concentrations of 50 mg/100 ml. was added to serve

FIGURE 16



TLC plate after spraying with copper/diethylamine reagent. 1 = thiopental; 2 - 4 = thiourea in decreasing amounts, i.e. 50, 25 and 10 µg; 5 = urine extract (rabbit anaesthetized with thiopental); 6 - 8 = urea in decreasing amounts, i.e. 50, 25 and 10 µg.

FIGURE 16a



Same plate after application of mercurous nitrate reagents. The oxybarbiturates appear and the thiourea and thiopental spots are intensified.

as a standard. The various specimens were then extracted, and the extracts applied to a TLC plate. In addition, aqueous solutions of thiourea and of thiopental sodium and pentobarbital were applied.

RESULTS

After development and spraying with copper-diethylamine reagent, the thiopental and thiourea standards became visible (Fig. 16). Thiourea was barely visible and had not moved from the point of application, for all practical purposes, and the colour was dark brown compared with the yellow or yellow-green of thiobarbiturates. A thiobarbiturate spot was visible in the extract from the rabbits injected with thiopental and the R_f value was identical with the thiopental standard 78-82 ($R_f \times 100$). In addition, a second thiobarbiturate with a R_f value of 13-15 was present in this urine. The mercurous nitrate reagent was then applied, and the oxybarbiturates became visible (Fig. 16a). There were two definite spots in the urine of the animals injected with thiopental. One had the same R_f value as pentobarbital (54-56 - $R_f \times 100$) and the other was very near the origin (R_f = 6 to 7).

Similar spots were not present in the urine to which thiopental or pentobarbital were added before the extraction procedure.

DISCUSSION

From the evidence presented, it seems clear that thiopental and the oxybarbiturate pentobarbital were present in the urine of rabbits injected with thiopental sodium. It is of interest that rabbits excrete large quantities of both thiopental and desulphurised thiopental (pentobarbital) in the urine. This is in contrast to humans, in whom only 0.3% of the administered thiopental can be recovered in the urine in an unaltered form over 72 hours.

In addition to thiopental and pentobarbital, two other unidentified barbiturates were observed in the rabbit urine. One was a thiobarbiturate and the other an oxybarbiturate. It is possible that these were the carboxylic acid derivatives of thiopental and pentobarbital, as suggested by Brodie et al., (1950). They were not artifacts

arising during the extraction procedure, because they were not seen in the urine from control animals to which thiopental, pentobarbital or a mixture of the two were added prior to extraction.

8.30 EFFECT OF PROADIFEN, RESERPINE AND ALPH-METHYLDOPA ON THE QUALITATIVE URINARY BARBITURATE EXCRETION AFTER INJECTING THIOPENTAL SODIUM

In order to investigate the possibility that the prolongation of barbiturate anaesthesia by proadifen, reserpine and a-MD was due to altered metabolism or excretion of thiopental, specimens of urine from rabbits treated with these drugs were compared with those from control animals.

DOSAGE OF PROADIFEN, RESERPINE AND ALPHA-METHYLDOPA

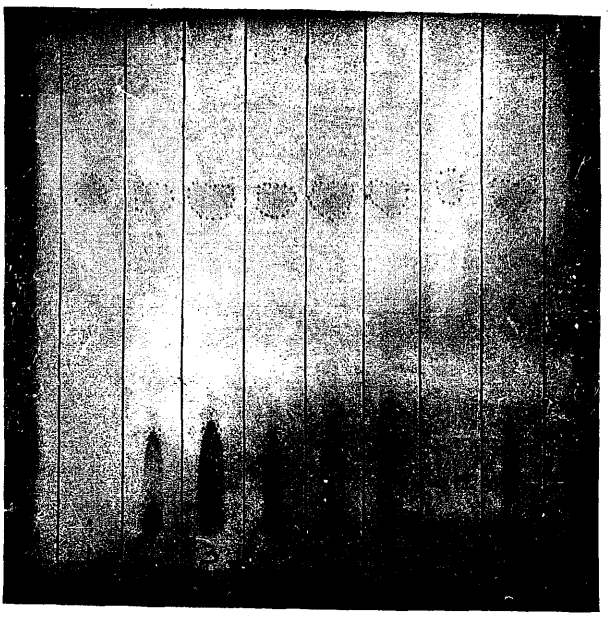
Proadifen 100 mg/Kg. was injected subcutaneously 2 hours before administering the thiopental. The dose of reserpine was 2.5 mg/Kg. given subcutaneously 5 hours before the thiopental, and that of a-MD 200 mg/Kg. subcutaneously 2 hours before. Three rabbits were used in each case.

EXPERIMENT

Thiopental sodium was injected intravenously so as to produce anaesthesia in the same way as before. Three control animals were also used, and urine

QUALITATIVE URINARY BARBITURATE EXCRETION FOLLOWING THIOPENTAL INJECTION (See text)

FIGURE 17



TLC plate after spraying with copper/diethylamine reagent 2 and 3 = a-MD pretreatment, different volumes of urine extract; 4 = reserpine pretreatment; 5 and 6 = proadifen pretreatment, different volumes of urine extract; 8 = extract of urine to which thiopental has been added before extraction.

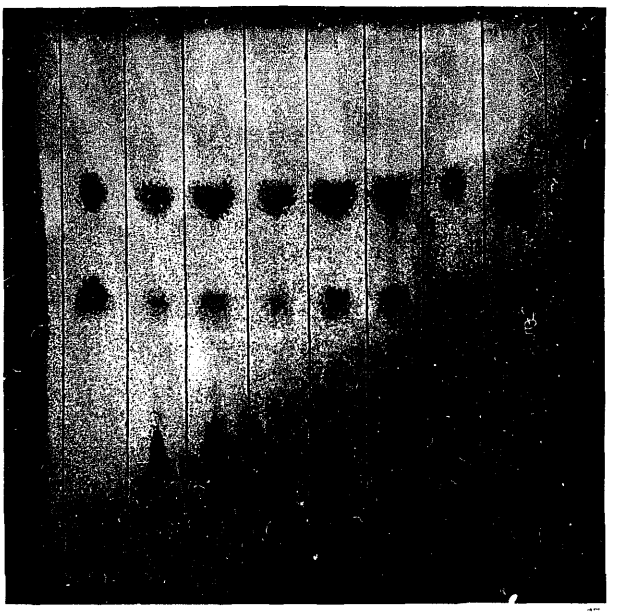


FIGURE 17a

The same plate after application of mercurous nitrate reagent. The oxybarbiturates appear.

For both figures:-

1 = thiopental and pentobarbital standards 50 µg each;
7 = 25 µg each.

from each group of animals was pooled as usual. The same extraction procedure was followed, and the same standard employed.

RESULTS

There were no detectable difference between the urinary extracts from the two groups of rabbits pre-treated with a-MD and proadifen respectively and the control group. The same oxy- and thiobarbiturates could be identified, and there did not appear to be any gross differences in the quantities excreted (Fig. 17), when the total volumes of urine extracted from each group were compared.

Some difficulty was encountered in interpreting the results obtained in the reserpine group. Reserpine produces a relative parasympathetic hyperactivity and this results in voiding of urine and faeces. Very little urine could be recovered from the bladders of the animals, even When fluid was administered during the course of the experiment. Consequently the results should be treated with reserve. Thiopental and pento-barbital were undoubtedly present, and the same

unidentified thiobarbiturate noted in the control urines probably also occurred (Fig. 17 and 17a). The oxybarbiturate derivative observed in the other specimens could not be identified with certainty. When large volumes of the urinary extract were applied, the overloading of the plate resulted in 'tailing' of the spots in question due to interference by other compounds such as urinary pigments.

DISCUSSION

Within the technical limitations of this method, no definite evidence was obtained that proadifen, reserpine or a-MD alter the metabolic degradation or urinary excretion of intravenously injected thiopental sodium in rabbits.

9.00 GENERAL DISCUSSION

Many attempts have been made to understand the mechanism by which anaesthetic agents reversibly depress the function of excitable cells within the central nervous system. However, in spite of better understanding of cellular function in respect of enzyme systems, the transmission of impulses, the transport of drugs through membranes and the metabolism within the cells, there is still no satisfactory explanation available for the mode of action of general anaesthetic agents (Pittinger and Keasling, 1959; Butler, 1950).

Even though the actual mechanism of action is not yet understood, however, a great deal of experimental evidence has accumulated which gives support to the old idea that simple physico-chemical factors determine the absorption, distribution and elimination of active compounds. A certain amount of solubility in water is necessary in order that the compound be absorbed and ultimately reach the site of action to produce the desired pharmacogical effect or effects. In addition, many potent anaes-

thetic drugs possess a high lipid solubility, a fact which was recognised as long as sixty years ago, by Meyer and Overton as well as others. It appears from the Meyer-Overton generalisation that there exists a direct correlation between the potency and the lipid-aqueous partition coefficient of anaesthetic agents. It is postulated that the lipid in cell membranes, especially in the brain, plays a significant role, the more lipid-soluble drugs crossing the membranes more readily.

Other investigstors have postulated that the potency of an anaesthetic agent is a function of the ability to lower surface tension in aqueous solution (Traube, 1904). It is claimed that the anaesthetic substance accumulates on the cell surfaces, resulting in a decreased electrical contact potential and impedence of nerve impulses. However, chloroform and some hydrocarbons have little effect on the surface tension, and it would appear that this theory is untenable. Höber (1907) as well as Lillie (1909) and others proposed that the permeability of the brain cell is altered by

anaesthetic agents. The membrane permeability is essential for ionic exchange during excitation and depolarization. An interference with the cell membrane permeability may thus render the cells less irritable or may prevent depolarization. Although such observations may add to the understanding of the mechanism of action of anaesthetics, this effect is not limited to such agents alone. In spite of much study for many years, there is still no satisfactory explanation for the mode of action of general anaesthetic drugs.

Whatever the mechanism of action of anaesthetic agents, drugs which prolong their effects may clearly do so in a number of different ways. If a chemical compound alters the permeability of the membranes it may have a direct influence on the action and duration of the anaesthetic drug, either by increasing the penetration into the brain cells or by blocking its passage out of the cell. An alteration in membrane permeability may also affect the duration of anaesthesia more indirectly. An important factor in the termination of the anaes-

thetic activity of barbiturates is the physical redistribution of the drug. This is especially so with thiopental administered by intravenous injection. The diffusion of thiopental out of the plasma and into the tissues other than brain results in a lowering of the plasma concentration. Thiopental therefore diffuses out of the brain cells into the plasma, and the patient wakes up. Any interference with this redistribution process would prolong the action of the anaesthetic agent. Redistribution of the drug is of much more importance than the rate of metabolic degradation in determining the duration of thiopental anaesthesia.

Nevertheless, another way in which the action of an anaesthetic agent might be prolonged is by delaying its breakdown in the body. It has been recognised that the metabolic alteration of a number of anaesthetic agents to less active or inactive substances is a factor in terminating their action. This process generally plays a much greater part than the usually slow renal excretion (Brodie, Maickel and Jondorf, 1958). By far the most active

site in the body for the metabolism of drugs is the liver. It has been demonstrated by various investigators that the hepatic microsomal enzymes are responsible for the metabolism of barbiturates, since compounds which inhibit or stimulate the action of the microsomal enzymes alter the duration of barbiturate anaesthesia. It is of interest that the more fat-soluble barbiturates (e.g. thiopental) are more easily metabolised than compounds which exhibit a lower order of lipid solubility (e.g. phenobarbital). This is consistent with the postulation of Gaudette and Brodie (1959) that the 'microsomal oxidative systems are protected by a lipoid barrier which only fat-soluble substances can penetrate'.

A further way in which a drug may prolong the duration of action of an anaesthetic agent is by pharmacological synergism. Such a drug would depress the central nervous system, so that its action would be additive with that of the anaesthetic agent. A fourth possibility is that the drug might alter the physiology of the body in any

of a number of different ways so as to produce hypoxia, hypotension, hypothermia, etc. The altered physiological state then directly or indirectly prolongs the action of the anaesthetic agent. Combinations of all these mechanisms are obviously possible and indeed frequently occur.

The effects of various substances on the duration of barbiturate anaesthesia has been the subject of numerous investigations. A great variety of substances and methods has been employed. For example, the effect of the prior administration of a drug on the duration of anaesthesia after a standard dose of the anaesthetic agent has been studied. Alternatively, its effects on the dose of barbiturate needed to produce anaesthesia has been examined. A third method is to determine whether the injection of the drug during the awakening phase restores sleep. In the present investigations only the first two methods were applied because the third technique is subject to various uncontrollable errors.

9.10 NON-SPECIFIC TOXICITY

The first step was to exclude the possibility that the drugs studied did not prolong anaesthesia simply by a direct toxic effect, altering normal organ function in a non-specific way. It was found that the mice tolerated ten to twenty times the amount of a-MD used in the subsequent experiments without any serious untoward effects, except for some sedation in the higher dosage range. The prolongation of thiopental anaesthesia brought about by a-MD was therefore not due to direct toxic damage to some vital tissue such as the liver, especially as the study was carried out on acute basis and the compound was administered only once.

The prolongation produced by proadifen may, however, be due to some direct damage to tissues, as the dosages selected exhibited severe and in some instances fatal effects. It is felt that most fatalities observed were due to respiratory depression resulting in anoxia. Such an action could lead to severe cellular damage even on an acute basis. During the toxicity study it was

initially observed that proadifen stimulated the central nervous system, producing convulsions. A stimulant effect was confirmed by the fact that the convulsions were inhibited by phenobarbital. However, after extensive testing of the compound by means of an actograph, a sedative component could also be established. This sedative action of proadifen has not hitherto been recognised. The suggestion that proadifen may have some C.N.S. depressant action received further support from the observation that lethal doses produce a phase of laboured respiration which later led to full apnoea resulting in death. Furthermore, others have noted that proadifen possesses a slight anti-convulsant activity (Swinyard, Madsen and Goodman, 1954).

These findings suggest that the prolongation of the barbiturate anaesthesia by proadifen may be caused at least in part by a generalised toxic effect. It cannot, however, be denied that proadifen exhibits a well-documented inhibitory action on the breakdown of certain chemical com-

pounds both in vitro and in vivo, thus leading to potentiation or prolongation of the action of such substances. Part of its effect may therefore result from this action, even though no positive evidence to support this hypothesis was obtained in the present study.

Cook and his co-workers (1954a) found that proadifen prolongs hexobarbital (but not thiopental) anaesthesia, while Archor and Geiling (1954) as well as Mirsky and Giarman (1955) demonstrated a definite prolonging effect on the anaesthesia induced by thiopental. The latter authors explained the disagreement with Cook on the basis of the difference in the time interval between the administration of proadifen and the thiopental challenge. To this explanation the present author would like to add another factor which appeared to be of some importance, namely the relationship of the dose administered to the LD_{50} of the particular species being studied. The present study has revealed that prolongation of thiopental anaesthesia can be produced, but that the dose required produces acute

toxic phenomena in some animals. As already discussed, the toxic effect may at least in part be responsible for the observed prolongation of thiopental anaesthesia. Care had therefore to be taken in assessing the prolongation of the barbiturate anaesthesia induced by proadifen, as there is a strong possibility that a non-specific toxic action to the brain may be responsible.

No real evidence of a direct toxic effect due to reserpine was uncovered. When hypothermia was prevented by controlling the environmental temperature and dehydration by administering fluids, all the animals survived. Reserpine (Serpasil) has been shown to have a low toxicity in most species, and as much as 7,000 mg/Kg. intraperitoneally has been tolerated by rats at room temperature (Keplinger, Lanier and Deichmann, 1959).

9.20 ADDITIVE PHARMACOLOGICAL ACTIVITY

Reserpine produces obvious sedation after an initial phase of increased activity. Investigation with an actograph established that both a-MD and proadifen also induce a marked degree of an apparent sedation. The effect of all three drugs in prolonging thiopental anaesthesia might therefore simply be due to an additive central nervous depressant action, whether the site of action of each drug on the central nervous system were similar to that of thiopental or not. It may be postulated that the concentration of thiopental in the brain necessary to maintain unconsciousness would be lower if another depressant drug were acting simultaneously. The same rate of redistribution, metabolism and excretion of the thiopental might be present, and yet anaesthesia be prolonged.

9.30 EFFECTS ON BODY TEMPERATURE

In experimental animals a decrease in body temperature significantly prolongs anaesthesia. This phenomenon has been neglected in many published studies of the effect of drugs on the duration of barbiturate anaesthesia, and is obviously especially important when studying anaesthetic agents which have a long duration of action. The activity of most enzymes is temperature-dependent, so that a drop in body temperature could lead to a prolongation in sleeping time by decreasing the rate of breakdown of the barbiturate. It is well established that a number of drugs lower the body temperature and/or enhance the hypothermic action of some anaesthetic substances (Fastier, Speden and Wall, 1957; Dandiya and Sellers, 1961). These drugs include atropine (Burn and Dutta, 1948), ergot alkaloids (Githens, 1917; Flacke, Mülcke and Schulz, 1953), histamine (Fabiniyl- Szebehely and Szebehely, 1952, Kind, 1954), 5-hydroxytryptamine and other physiologically active 'amines' (Dandiya and Sellers, 1961) and tolazoline (Horwitz et al.,

1949). All these compounds are vaso-active drugs.

The effect of body temperature on the action of drugs was reviewed by Fuhrman as far back as 1946. However, it is obvious from later investigations that no general agreement exists on the subject. It is extremely difficult to assess or to compare any of the earlier findings, because some authors measured only environmental temperatures while other workers observed and recorded the body temperature. Shaw and Shankley (1948) reported that the duration of action of pentobarbital in high dosage is greater at high body temperatures. It is unfortunate that no mention was made in their paper of whether or not the animals which were not anaesthetised by low doses were included in the calculation of the average sleeping time. In another investigation, Raventós (1938) demonstrated that there was no change in the LD_{50} of hexobarbital in mice kept at environmental temperatures ranging from 20° to $30^{\circ}C$; however, phenobarbital was more toxic at $20^{\circ}C$ than at $30^{\circ}C$. In contrast, Richards (1941) found a steady re-

duction in the toxicity of thiopental in mice when the temperature was progressively decreased from 38°C. to 22°C.

Smith (1960) reported that there was a moderate decrease in body temperature following the subcutaneous administration of 100 mg of a-MD to mice, but no effect was observed in guinea-pigs. In the present study such a hypothermic state was produced in mice only when the room temperature was low e.g. 19°C to 22°C., but did not occur in rabbits at either high or low temperatures. However, the evidence obtained in the present investigation suggests that the prolongation of thiopental anaesthesia by a-MD is not brought about by a decrease in body temperature, since it occurred even when the temperature did not fall. There may be some tendency for the rectal temperature to decrease if the animal remains under anaesthesia for a long period of time, or if the room temperature is below 27°C. This drop in temperature, however, occurs even if no a-MD was administered but anaesthesia is prolonged by additional small amounts of thiopental.

In contrast, part at least of the effect of

reserpine appears to be due simply to hypothermia. As has been found by other workers using various barbiturates, reserpine did not alter the thiopental sleeping time considerably when the thiopental challenge was carried out after one hour; at this time the body temperature was normal, but sedation was obvious. When the thiopental was administered at a later stage there was a much greater effect. Doubling the dose of reserpine did not further increase the duration of anaesthesia. It seems reasonable to deduce that the fall in body temperature which had occurred by this time was largely responsible for the prolongation of anaesthesia, since the effect was much less if the animals were placed in a warm environment. However, there was almost certainly some effect even if the temperatures were not allowed to fall. It therefore seems likely that hypothermia due to reserpine may only be partly responsible for the increased sleeping time when challenged with thiopental.

This is supported by the results obtained in

rabbits in which the body temperature did not alter during a five hour observation period. Anaesthesia induced by thiopental was however, greatly prolonged when compared with a group of control rabbits. These observations provided an example of species variation in drug response, an extremely common phenomenon in pharmacology (Quinn, Axelrod and Brodie, 1954; Brodie, 1956; Quinn, Axelrod and Brodie, 1958; Mark, 1958). In mice the reduction in body temperature produced by reserpine appears to be the most important reason for the prolongation of thiopental anaesthesia, although a second mechanism also plays a part. In rabbits hypothermia is not a factor.

Mice appear to have a very sensitive body-temperature regulating mechanism as many different chemical compounds may lower the rectal temperature by several degrees.

Similar results were obtained with proadifen. While in rabbits no change in the body temperature could be observed over a period of several hours,

mice exhibited a sharp decrease in temperature. This drop of the body temperature in the latter animals lasted for several hours and had not returned to the initial values after 24 hours. Again species differences have to be accounted for which may be responsible in part at least for the prolongation of anaesthesia in mice as also noted by other investigators.

9.40 ROLE OF 5-HYDROXYTRYPTAMINE AND OTHER AMINES

Because a-MD produced a decrease in the concentration of 5-HT in the brain, the possibility was considered that this might play some part in its action in prolonging thiopental anaesthesia. During the period of maximum depletion of 5-HT by a-MD, the prolongation of thiopental anaesthesia is also maximal. However, the present study has demonstrated that the administration of 5-HT twenty minutes before the challenge did not counteract the prolongation of thiopental anaesthesia in mice pretreated with a-MD. Indeed the duration of anaesthesia was probably lengthened. Furthermore, in another experiment the administration of 5-HT in quantities believed to be sufficient to raise the concentration in the brain produced a considerable degree of sedation, confirming observation made by others (Shore *et al*, 1957; Chessin, Kramer and Scott, 1957; Cronheim and Gourzis, 1960 and others). A marked degree of inhibition of motor activity was noted. Thiopental anaesthesia was also prolonged in these experiments. Similar observations have

been reported by other workers (Brodie, Pletscher and Shore, 1955; Sturtevant, 1956; Salmoiraghi, Sollero and Page, 1956 and others). Some investigators query whether injected 5-HT reaches the brain in sufficiently high concentration to exert any distinct action (Udenfriend, Weissbach and Bogdanski, 1957; Southern, 1960; Erspamer, 1961). Others conclude that in some species 5-HT does cross the blood-brain barrier and reaches certain parts of the brain (Costa and Aprison, 1957; Kärki and Paasonen, 1959; McIsaac and Page, 1959). Erspamer (1961) however, criticises the last report as not generally accepted. The sedation which was noted in this and previous studies appears to support the opinion that 5-HT does enter the brain.

These considerations make it extremely unlikely that the depletion of brain 5-HT by a-MD is responsible for its effect in prolonging thiopental anaesthesia. Indeed, anaesthesia itself has been shown to be accompanied by an increase in the concentration of 5-HT in the brain, the level rising

as the depth of anaesthesia increases (Anderson and Bonnycastle, 1960). Since a-MD alters the concentration of 5-HT in the opposite direction, it is interesting that it prolongs rather than shortens the duration of anaesthesia. There appear to be two possible explanations for this apparent anomaly. Either the mechanism by which a-MD does prolong anaesthesia is sufficiently powerful to overcome its action in depleting the brain of 5-HT, or else the change in concentration of 5-HT in the brain plays no part in determining the level of consciousness. The second possibility seems unlikely in view of the fact that the administration of 5-HT was observed to produce a marked degree of sedation and inhibition of motor activity.

Reserpine also produces a decrease in the concentration of both 5-HT and nor-adrenaline in the brain. The possibility that these actions might be responsible for the central nervous depressant effect of reserpine has been considered by a number of authors (Brodie et al, 1960; Carlsson, 1961; Holtz, 1961). The depression of the central

nervous system has been ascribed to both too low a concentration of the amines at the recptor sites and too high a concentration. Those who favour the 'too little' theory say that depletion of amines from their normal storage sites means that there is less chemotransmitter available to transmit impulses. The opposite school of thought maintains that it is storage of the amines in the nerve endings which is inhibited, but that synthesis continues. The amines are therefore continually being released and there is actually a higher concentration than normal at the recptor sites.

Other considerations appear to favour the 'too little' theory. For example, the administration of amines such as adrenaline in dosage sufficiently high to penetrate the blood-brain barrier produces a stimulation of the central nervous system, possibly including convulsions. Futhermore, monoamine oxidase inhibitors act as central nervous stimulants, not central nervous depressants. While it is naïve to ascribe the effects of these drugs entirely to delayed degradation of endogenous amines, they

presumably do not diminish the concentration of these substances.

The effect of administering reserpine appears to be biphasic. The initial phase is short, and is due to the release of amines from storage sites. In mice a piloerection and slightly increased mobility can usually be noted, especially with the higher dosage range. This phenomenon is ascribed to the increased circulating adrenaline and noradrenaline (Muscholl and Vogt, 1957) resulting in a direct adrenergic receptor stimulation. During this early phase of the reserpine action no prolongation of thiopental anaesthesia could be noted; on the contrary, there was an indication that the barbiturate-induced central nervous depression was of shorter duration (Matthies and Schmidt, 1961, 1962). These workers injected mice intracerebrally with reserpine and observed a significant shortening of the duration of barbiturate anaesthesia during the half-hour thereafter. These observations provide support for the concept that increased concentrations of the endogenous amines stimulate the

central nervous system, while decreased concentrations are associated with central nervous depression.

The second phase of the action of reserpine is seen after the amine stores have been depleted, and continues for many hours. This is the so-called 'vagotonic' phase. During this phase the animals are markedly sedated, and the duration of thiopental anaesthesia is prolonged. This has been observed by previous workers as well as in the present study. It has, however, to be remembered that several factors may be responsible for the prolongation of barbiturate anaesthesia by reserpine. These include hypotension, which results in a decreased blood flow to various tissues, and a domination of the parasympathetic nervous system due to an only partially functioning adrenergic system. It has been shown that acetycholine and related compounds readily produce a prolongation of barbiturate anaesthesia which can be antagonised by atropine (Lenke, 1958). In addition, the reduction in body temperature plays an impor-

tant part, as already discussed, and so does dehydration resulting from the prolonged sedation. There does not appear to be any evidence to suggest that proadifen may affect the endogenous amines.

9.50 CHOLINERGIC AND ADRENERGIC ACTIVITY

As already discussed, it has been suggested that part or all of the prolongation of barbiturate anaesthesia during the vagotonic phase of reserpine action might be due to excess acetylcholine in the central nervous system, since cholinergic substances have been shown to have this effect. It was therefore thought to be worth while to exclude the possibility that a-MD might have direct cholinergic activity. A more likely possibility was that it might have direct adrenergic activity, since its molecule closely resembles those of adrenaline and nor-adrenaline. These substances in large dosage may also prolong thiopental anaesthesia. The available evidence suggests that the relevant cholinergic receptors in the central nervous system are of the muscarinic variety, since the prolongation of anaesthesia induced by acetylcholine can be blocked with atropine. For this reason the effect of a-MD was studied on isolated rabbit ileum. However, it had no agonistic action and was also not antagonistic to

acetylcholine. There is therefore no evidence that a-MD prolongs thiopental anaesthesia by a direct cholinergic or adrenergic action. However, it remains possible that it might act in an indirect way. Since it reduces the concentrations of both nor-adrenaline and 5-HT within the central nervous system, there will be a relative preponderance of acetylcholine. As may be the case with reserpine, this might be responsible at least in part for its action in prolonging thiopental anaesthesia.

9.60 METABOLIC DEGRADATION AND RENAL EXCRETION OF THIOPENTAL

The possibility that the prolongation of thiopental anaesthesia by the three drugs might be due to delayed metabolic degradation and/or the renal excretion of the injected thiopental was examined. The method used was the qualitative identification of barbiturates in the urine of rabbits after injecting thiopental. A thin-layer chromatographic technique was developed for this purpose, and thiopental, its oxy-derivatives pentobarbital and two other barbiturates, one a thiobarbiturate and the other an oxybarbiturate, were observed. The two unidentified barbiturates were probably the carboxylic acid derivatives of thiopental and pentobarbital respectively.

Pretreatment of rabbits with either a-MD, reserpine or proadifen did not alter the appearance of these barbiturates in the urine. While the possibility that the drugs do influence the metabolism of thiopental cannot be excluded, no evidence in favour of

this was obtained. On theoretical grounds it seems unlikely that the duration of thiopental anaesthesia would be increased even if the rate of metabolic degradation were retarded. The most important factor in the termination of thiopental anaesthesia is the redistribution of the drug, not the metabolic degradation or the renal excretion. After injecting thiopental intravenously it moves into the central nervous system and unconsciousness rapidly supervenes. The concentration of thiopental in the plasma falls progressively as the drug is taken up by other tissues, such as skeletal muscle and body fat depots. As the plasma concentration decreases, the gradient between the brain and the plasma concentrations changes, and thiopental begins to move out of the brain into the plasma. Within minutes consciousness returns. During this brief period only minimal metabolism and excretion can have taken place, so that an inhibition of either process would not be expected to produce a marked effect.

9.70 DISTRIBUTION OF THIOPENTAL

The action of all drugs is markedly influenced by the freedom with which they cross membranes. As already discussed, this is of particular importance in the case of general anaesthetic agents, and the critical factor in determining the duration of unconsciousness after injecting thiopental intravenously. Clearly, therefore, any agent which altered membrane permeability or which changes the thiopental molecule in such a way as to affect its lipid solubility would have a marked effect on the duration of thiopental anaesthesia. The investigation of these aspects was beyond the scope of the present study, but they remain possibilities.

10.00 SUMMARY

When two or more drugs are administered simultaneously, they frequently exert an effect on each other's action. This happens with drugs of many different types, including those used to produce general anaesthesia. The present investigation was concerned with the mechanisms by which certain drugs prolong the duration of barbiturate anaesthesia.

There are many different ways in which a drug might lengthen the period of unconsciousness produced by a barbiturate. For example, it might simply possess a central nervous system depressant action of its own, and the additive pharmacological action would then enhance the effect of the barbiturate. Secondly, the drug might interfere in a toxic way with the normal functioning of vital organs such as the liver or heart, and the resultant physiological upset in the form of hypoglycaemia, hypothermia or hypoxia might render the organism as a whole less resistant to the action of the barbiturate. Thirdly,

the drug might delay the metabolic degradation or excretion of the barbiturate. Fourthly, it might affect its movement across membranes in several parts of the body, including the blood-brain barrier. Finally, the drug might have some pharmacological effect which would not in itself produce central nervous depression, but which might affect the response of the central nervous system to depressant drugs. Examples of this type of action might include the depletion of biogenic amines from the brain.

In the present study the barbiturate selected for investigation was thiopental sodium, and mice and rabbits were used. The effects of reserpine, alpha-methyldopa and proadifen on the duration of unconsciousness were examined. These particular drugs were selected from the many which have been reported in prolonging barbiturate anaesthesia for certain reasons. Proadifen is claimed to be a substance which inhibits hepatic microsomal enzyme activity without itself exerting a significant effect on the central nervous system. Alpha-methyldopa

produces a depletion of 5-hydroxytryptamine and other amines from the brain, and the 5-hydroxy-tryptamine concentration in the central nervous system is believed to exert a considerable influence on its function. Reserpine was chosen because there is a large volume of published work about the effect of this substance on barbiturate anaesthesia, and so it could serve as a standard for comparison.

Initial toxicity studies were performed and two points of interest emerged. Firstly, the strain of mice which were to be used in the experiments was found to be more sensitive to thiopental than anticipated from published reports. The dosage was, therefore, adjusted accordingly. Secondly, proadifen was found to exhibit a direct pharmacological effect on the central nervous system in the form of convulsions followed by sedation, in spite of the fact that it had previously been claimed not to have any such action. Furthermore, many of the mice died up to 72 hours after receiving the drug, probably from irreversible

hepatic damage. In view of these observations, its action in prolonging thiopental anaesthesia can certainly not be ascribed simply to inhibition of the hepatic microsomal enzymes.

The effects of the three drugs on the duration of thiopental anaesthesia was then examined. Previous reports that the period of unconsciousness was prolonged were confirmed in each case. During these experiments the important influence of the environmental temperature was noted. Reserpine was observed to produce a marked fall in the body temperature in mice if the ambient temperature were low. If this hypothermia were prevented by keeping the mice warm, anaesthesia was not prolonged to the same extent, although some effect was still apparent. In rabbits hypothermia was much less marked, and did not appear to play any part in the prolongation of anaesthesia. Both alpha-methyldopa and proadifen in large dosage also lowered the body temperature in mice but not in rabbits. As commonly occurs in pharmacology, the effect was different in different species. It appears that

much of the confusion in the published reports concerning the effect of reserpine on anaesthesia derives from the fact that inadequate attention has been paid to the possibility of hypothermia.

Another component of the effect of reserpine in prolonging the duration of thiopental anaesthesia was undoubtedly its central nervous depressant action, which must contribute an additive pharamcological effect. The mechanism of this action may be *via* depletion of biological amines such as *nor*-adrenaline and 5-hydroxytryptamine from the brain.

By means of an actograph it was shown that *alpha*-methyldopa also exerts a definite sedative action. It is known that *alpha*-methyldopa reduces the concentration of 5-hydroxytryptamine in the brain, and the possibility that this might be responsible for its action in prolonging thiopental anaesthesia was investigated. However, the injection of 5-hydroxytryptamine alone was found to prolong barbiturate anaesthesia. Furthermore, the injection of this substance together with *alpha*-methyldopa produced a

greater prolongation than alpha-methyldopa alone. It therefore seemed unlikely that the effect of alpha-methyldopa was brought about by its depleting the brain of 5-hydroxytryptamine.

The possibility that alpha-methyldopa might have a direct adrenergic stimulant activity or a cholinergic effect was also investigated because large doses of adrenaline and of acetylcholine both prolong barbiturate anaesthesia. Alpha-methyldopa is a close chemical realtive of adrenaline. However, no evidence of either adrenergic or cholinergic activity was detected with the isolated rabbit ileum preparation.

The possibility that any of the three drugs might alter the metabolic degradation or renal excretion of thiopental was investigated by studying the appearance of barbiturates in the urine of rabbits after injecting thiopental. In order to do this, a new thin-layer chromatography technique was developed.

With this technique, the separation and identifi-

cation of a large number of thio- and oxybarbiturates can be achieved. Thiopental, pentobarbital and two further barbiturates, one a thiobarbiturate and the other an oxybarbiturate were observed in the urine of rabbits injected with thiopental sodium. The unidentified barbiturates were probably the carboxylic acid derivative of thiopental and pentobarbital respectively. The desulphuration of thiopental to pentobarbital has not previously been described in rabbits. Rabbits are particularly suitable for this type of study because they excrete large amounts of unaltered thiopental as well as its metabolites in the urine.

The three drugs did not produce any definite change in the pattern of barbiturate excretion after injecting thiopental sodium. The possibility that there was some alteration in metabolism or excretion can not, however, be excluded, since a qualitative rather than a quantitative method was used. Nevertheless, on theoretical grounds it seems unlikely that a delay or alteration in the metabolism of thiopental would sig-

nificantly prolong the duration of unconsciousness, since it is the redistribution of the drug from the brain to other tissues which allows consciousness to return rather than the metabolic degradation of the drug. The possibility that any of the three compounds might delay the redistribution of the thiopental by some action on membranes was beyond the scope of this dissertation.

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