A STUDY OF THE METABOLISM OF BARBITURATES IN THE HORSE

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A Thesis presented for the Degree of Master of Science in the University of Edinburgh.

July, 1967.

CONTENTS

SUMMARY

(a) The amounts of pentobarbitone and phenobarbitone able to be given to ponies, without risking respiratory arrest, was low in comparison with other species.

(b) Ultra-violet spectrophotometry has been shown to be of limited value for general use in quantitative and qualitative analysis of horse blood and saliva samples for pentobarbitone and phenobarbitone.

(c) Pentobarbitone had a mean blood clearance rate of 46%/hour (S.D. 8%/hour, n = 6) when given by intravenous injection and this was paralleled in the saliva.

(d) The blood clearance rate of phenobarbitone was found to be non-logarithmic over the time 0 to 6.5 hours after oral administration and this was paralleled in the saliva.

(e) The degree of plasma protein binding was shown to be 43% and I5% for pentobarbitone and phenobarbitone respectively. Pentobarbitone was shown to be not protein bound in saliva, but phenobarbitone was found to be bound to a small extent i.e. 7%.

(f) No effect was observed on the rate of normal saliva flow following barbiturate administration. The concentration of the two barbiturates in the saliva was found to be independant of the saliva flow rates, although phenobarbitone concentrations

were subject to considerable fluctuations.

(g) The concentration of barbiturate in the saliva was directly dependant on the concentration of non-protein bound barbiturate in the blood.

(h) Phenobarbitone, the presence of which was not easily demonstrated, was excreted in horse urine to the extent of about 10% in the 72 hours following oral dosage of the drug.

(i) p-hydroxyphenobarbitone was the only metabolite of phenobarbitone detected in horse urine and was conjugated with glucuronic acid.

(j) Pentobarbitone was metabolised in the horse to the 5-ethyl-5-(3-hydroxy-1-methylbutyl)barbituric acids and to 5-ethyl-5-(3-methyl-1-carboxypropyl)barbituric acid. The alcohol metabolites, of which no evidence of glucuronic acid conjugation was found, were completely excreted in urine over a period of 21 hours after oral administration and accounted for 33% of the administered drug.

GENERAL INTRODUCTION

Although the fate of barbiturates has been studied in many species there appears to be no published work specifically dealing with the horse. It was therefore of interest to determine the fate of barbiturates in the horse and to compare the results with those obtained by other workers for different species.

Of particular interest was the salivary secretion of barbiturates in the horse. This aspect of the fate of barbiturates in various species has only been subjected to limited investigations, a review of which is given, and no report could be found dealing with the saliva clearance of barbiturates in any species. The availability of two ponies with permanent parotid fistulae allowed the study of barbiturate salivary secretion to be approached with relative ease.

Since much data is available in the literature on barbiturate blood clearances in various species, but not apparently on the blood clearances in horses, their determination was considered to be of importance. Of secondary interest was the degree to which barbiturates were protein bound in horse plasma, a factor which has frequently been determined in conjunction with blood clearance experiments and which is

reviewed with particular emphasis on the conflicting information available in the literature.

The metabolic fate of many barbiturates in several species has been reported and slight species differences have been noted. In many cases the barbiturates are excreted in urine unchanged or metabolised either partially or completely. This posed two questions on the fate of barbiturates in the horse which it was desired to answer i.e. does the horse metabolise barbiturates in the same way as do other species and at what rate are the barbiturates or their metabolites excreted in urine?

In order to carry out the above described study it was necessary to investigate the suitability of previously described analytical methods which have been used by other investigators. Since the number of methods described in the literature for the analysis for barbiturate in biological specimens is large and varied a review of the literature was carried out and is reported. From this range of methods a selection of those suitable for the present study was made and their limitations established.

From the wide range of available barbiturates two of the most common were chosen for study - pentobarbitone and phenobarbitone. Before work was started on the fate of these

two barbiturates in the horse the known metabolism of each barbiturate in various other species was reviewed and is reported.

A REVIEW OF PHENOBARBITONE METABOLISM

Phenobarbitone metabolism did not attract the early attention that pentobarbitone was given, probably because phenobarbitone was excreted unchanged, at least to some extent, in urine whereas pentobarbitone was not.

One of the first reports on phenobarbitone metabolism was that of Butler,[1] who first isolated p-hydroxyphenobarbitone as a metabolite of phenobarbitone from the urine of dogs receiving daily doses of the parent drug. He found that the metabolite was excreted largely in a conjugated form, the exact nature of which was not established, and this had to be subjected to acid hydrolysis before extraction of the free metabolite with ether could be carried out. Complete identification of the metabolite was achieved by comparing it with a synthetic sample of p-hydroxyphenobarbitone i.e. 5-ethyl-5-(4-hydroxyphenyl)-barbituric acid.

Curry,[2,3] in toxicological post-mortem investigations, also isolated the same metabolite as did Butler. The metabolite was found to be unconjugated in the urine of humans which was contrary to Butler's findings in the dog. No indication of the presence of the metabolite in blood, brain, liver or cerebro-spinal fluid was given in paper chromatographic analysis of the appropriate extracts. From the ultra-violet absorption curves given by Curry [3] it is obvious that the metabolite cannot be determined by differential ultra-violet spectrophotometry of

solutions at pH I3 and pH IO, since the absorption curves at these pH values are almost superimposable. Solutions at pH I3 or IO and pH 2 or lower may however be used with this technique.

Algeri and McBay [4] isolated p-hydroxyphenobarbitone from the urine of two persons who had died as a result of phenobarbitone intoxication. The investigators were able, in each case, to determine the relative amounts of conjugated and unconjugated metabolite present. In one case 46% was conjugated and in the other 20% was conjugated. No metabolite was found in liver, blood or fluid stomach contents.

It is of interest to note that Curry [2,3] and Algeri and McBay,[4] although using the same conditions for paper chromatography, report differing R_f values for the metabolite (see Table I).

Svendson and Brockmann-Hanssen [5] detected p-hydroxyphenobarbitone in human urine using gas-liquid chromatography. With a polyester column only the original drug was eluted, but when Apiezon L was used as the stationary phase both metabolite and phenobarbitone were eluted. No quantitative determination of the metabolite was made.

Butler [6] later reported a fuller investigation on the metabolic hydroxylation of phenobarbitone in man and dog. He was able to show that p-hydroxyphenobarbitone was conjugated with glucuronic acid when excreted in dog urine, although a smaller amount was also excreted unconjugated. Following intravenous injection of the metabolite into a dog, the plasma level fell

very rapidly, the greater part of the substance having disappeared from the plasma in one hour. When the subsequent 24-hour urine was examined almost complete conjugation of the metabolite was found. It was therefore concluded that conjugation was responsible for the rapid removal of p-hydroxy-phenobarbitone from the plasma. Since the rate of disappearance of phenobarbitone from plasma is slow the consequent production of the metabolite must be slow, and it follows therefore that the plasma concentration of the metabolite, formed from the parent compound, must always be low.

In man the position was shown to differ from that in the dog. Almost half the metabolite was excreted unconjugated. The remainder was shown not to be conjugated with glucuronic acid, and it was assumed that in all probability the sulphate was formed.

Benakis and Glasson,[7] in a more recent report, used phenobarbitone-2-C^{14} and phenobarbitone-5-C^{14} for investigating metabolic transformations in the parent molecule with particular reference to opening of the pyrimidine ring of which they found no evidence. Using rats, they were able to demonstrate the presence in the urine of p-hydroxphenobarbitone and its glucuronate. Four other metabolites were detected using paper chromatography. Two were considered to be, without direct proof, the o-hydroxy metabolite and its glucuronate. It was suggested that the remaining two might be sulpho- or mercapto- conjugates. Previously [8] they had reported the relative amounts of each

Table I. Physical properties of p-hydroxyphenobarbitone as reported in the literature.

Property	Value	Ref.
Melting point	225-226°	6
	220-223°	I
	222°	2
Partition Coefficient; ether/water	5	I, 6
benzene/water	0	I, 6
pK_I	7.3	6
pK_2	9.38	6
pK_3	I2.28	6
R_f (paper chromatography using n-butanol saturated with 5N ammonia)	0.I7	2
	0.29	4
$\lambda_{max.}$ at pH I3	249, 290 mμ	3
$\lambda_{min.}$ at pH I3	23I mμ	3
$\lambda_{max.}$ at pH I0	244.5, 290 mμ	3
$\lambda_{max.}$ at pH 2	ca. 273 mμ	3
Solubility in water	I.24 g/I	6

metabolite excreted in the 24-hour urine of rats receiving 90 mg/kg of the labeled drug (see Table II). 45% of the total administered radioactivity was excreted in this time. No labeled carbon dioxide was exhaled during this period of investigation.

Glasson et al.,[9] have investigated the distribution of phenobarbitone in the rat at various time intervals after treatment with the drug. They found that there was a marked concentration of C^{14} in the blood and liver during the first 6 hours. The concentration in kidney, spleen, lungs, heart and stomach remained low and the concentration in the brain reached its maximum after 2 hours. Elimination of the drug and its metabolites in the urine was at its maximum rate between the sixth and eighth hours. After 48 hours the concentration in the body of the rat was found to be insignificant.

Pharmacological properties of p-hydroxyphenobarbitone

Butler [I] showed that p-hydroxyphenobarbitone did not have anaesthetic properties in mice when given in doses up to I g/kg. Later [6] this statement was modified as it was found that, although giving no response to doses of 500 mg/kg, mice did show sluggishness, tremor of the limbs and difficulty in righting following an intravenous dose of I g/kg. These symptoms disappeared within a few minutes. A dog, receiving I00 mg/kg by rapid intravenous injection, showed no noticeable effects.

Table II. Composition of the 24-hour urinary excretion of phenobarbitone-C^{14} in rat urine amounting to 45% of the total administered activity. Dose, 90 mg/kg.

Metabolite	Relative Amounts
I. Phenobarbitone	27.5%
2. p-Hydroxyphenobarbitone	I9%
3. o-Hydroxyphenobarbitone	2.5%
4. Glucuronide of 2	27%
5. Unidentified	20%
6. Conjugate of 5	4%
7. Unidentified	<I%

Site of phenobarbitone metabolism

Investigations to discover the site of phenobarbitone metabolism do not appear to have attracted the same degree of interest as did the corresponding work with pentobarbitone. However, the general procedure has been the same and in recent years considerable work has been done on the effect of phenobarbitone on the liver.

Masson and Beland [10] and Butler et al., [11] carried out experiments on hepatectomised animals and showed that the clearance rate of phenobarbitone was not markedly affected. Using carbon tetrachloride to produce liver damage in rats Cameron and de Saram [12] showed that acute liver damage did not prolong the action of the barbiturate. Glasson and Benakis [13] demonstrated that carbon tetrachloride intoxication in rats resulted in a decrease in the urinary elimination rate of C^{14}, given as phenobarbitone-C^{14}, and also in the rate of production of p-hydroxyphenobarbitone and its glucuronide.

Dorfman and Goldbaum [14] have shown that rabbit muscle or brain brei did not destroy phenobarbitone.

Nephrosis and nephrectomy have been shown by several authors to greatly reduce phenobarbitone clearance rates. Murphy and Koppanyi [15,16] produced nephrosis in mammals using nephropathogenic agents and found that fatality was normally incurred when phenobarbitone was subsequently administered. Similar results were achieved by Hirschfelder and Haury [17] who found that phenobarbitone administration in nephrectomised animals

usually resulted in death. Brodwall and Stoa [I8] have emphasised the danger of giving phenobarbitone to humans with diseased kidneys since clearance rates are reduced.

The conclusion drawn from the above observations is that, unlike pentobarbitone, phenobarbitone is detoxified in the kidneys. However, this may not be strictly true since phenobarbitone is excreted to the extent of about I0 to 20% in the urine. If the kidneys are, for some reason, unable to excrete this unchanged drug then the concentration in the body will not decrease as rapidly as in normal subjects. This would prolong the risk of respiratory arrest and other adverse effects, since the subjects would remain under the action of the drug for a much longer time as complete metabolism of the barbiturate in the liver will be the only means by which it can be inactivated. This is supported by the results of Glasson and Benakis [I3] who showed that liver damage decreased excretion rates of p-hydroxyphenobarbitone.

Recent reports by Orrenius and other workers have indicated that the liver is in fact the site of phenobarbitone hydroxylation. Herdson et al., [I9] were first to report the production of fine structural changes in the livers of rats after the incorporation of sodium phenobarbitone into their diet. Later Burger and Herdson [20] showed that the induced liver enlargement and hepatic fine structural changes occurred regardless of the route of administration of the drug.

Orrenius et al.,[2I] investigated the properties of liver microsomes isolated from phenobarbitone treated rats. The microsomes showed increased oxidative demethylation activity and TPNH-cytochrome c reductase activity. Increased amounts of CO-binding pigment, microsomal protein, RNA and phosopholipid were found and the concentration of the participating enzymes rose. Although an increase in the various TPNH-linked reductase reactions, parallel to the increased drug hydroxylation activity, was noted the TPNH-oxidase activity did not change appreciably.[22] Treatment of rats with phenobarbitone (I00 mg/kg) for five days was found to enhance the activity of the demethylation of aminopyrine in rat liver microsomes five times.[23] Further treatment did not increase the effect nor did larger doses of the drug. Lower doses gave a less rapid increase in activity. The fine structural changes, which were incurred, were studied.

Phenobarbitone blood clearance and urinary excretion rates

Blood clearance: As with pentobarbitone, experiments have been carried out to determine the blood clearance rates of phenobarbitone in certain species. The various results quoted in the literature are summarised in Table III.

Butler et al.,[24] have pointed out that plasma clearance rates vary markedly both from animal to animal in the same species and in the same animal on different occasions. Thus, with one dog, receiving intramuscular or intravenous doses of

of phenobarbitone for 25 days, the plasma-barbiturate concentration fell, during the period one hour to 24 hours, at at rates varying between I6 and 50%. The variations were irregular and their cause uncertain. It was suggested that the rate of urine formation might have had some influence.

Urinary excretion: The ultraviolet determination of phenobarbitone in urine is subject to interference from free p-hydroxyphenobarbitone. Thus, Waddell and Butler [25] have shown that, in the method used by Lous, [26] a concentration of I00 mg/l of the metabolite in the urine would be interpreted as 8 mg/l of phenobarbitone. They further suggested that the method of Wright and Johns [27] would be subject to greater interference than that of Lous.

The effect of urinary pH on phenobarbitone urinary excretion has been observed, [25,28] and it has been shown that excretion is higher when the urine is alkaline than when it is acid. Wright [29] noted no significant increase in the output of phenobarbitone in casual samples of urine from two patients suffering from barbiturate intoxication.

Results extracted from the literature for urinary excretion rates are quoted in Table IV.

Tolerance to phenobarbitone

Tolerance to phenobarbitone has been studied by Butler et al., [24] who found that man and dog developed a tolerance to

successive doses of phenobarbitone. This tolerance was not solely due to an increased clearance rate because the plasma-barbiturate concentration of the drug increased with successive doses which were required to produce the same effect as the initial dose. Similar results were quoted by Wright.[29]

Table III. Phenobarbitone blood clearance rates.

Species	Number used	Dose	Clearance Rate (%/day)	Reference
Man	I	0.75g	27	26
	I	0.75g	I4	26
	I	0.75g	I5	26
	5*	varied	26 ± 8"	30
	II+	?	5 to I9	28
	6*	?	I3.8	24
	5*	?	I8.6	24
	6	0.8 to Ig each	ca. 30	3I
	3+	?	I2,55 and 3I"	I8
	2+	2.73; other unknown	I5"	29
Dog	I	5I mg/kg	25	24, 32
	I	?	I8	24, II
Mouse	2	I50 mg/kg	33	I4
Rat	I0	47 mg/kg	77"	II
Rabbit	I	400 mg/kg	I8"	33

*cases of continuous administration

"estimated from data

+cases of acute intoxication

Table IV. Urinary excretion of phenobarbitone.

Species	Dose	Number Used	Period of Observation (days)	Amount Excreted	Analysis*	Ref.
Man	0.75g	I	6	I4%	U.V.	
	0.75g	I	9	I3%	and	26
	0.75g	I	I0	37%	grav.	
	0.2g	I	2	23.4%	GLC	5
	?	?	?	II to 24.5%	grav.	34
Dog	I00 mg/kg	2	II4 hours	I3 to I6%	color.	35
Rat	90 mg/kg	?	I	27.5%	C^{I4}	8

* The abbreviations used are; U.V. - ultra-violet method

grav. - gravimetric

GLC - gas-liquid chromatography

color. - colorimetry

C^{I4} - radioactive labeling used

A REVIEW OF PENTOBARBITONE METABOLISM

Schonle et al.,[36] first showed that pentobarbitone was excreted unchanged to only a very small degree, if at all, in the urine of humans and dogs receiving oral doses of the drug. They reasoned that in all probability the barbiturate was completely metabolised to carbon dioxide, water and ammonia and excreted as such.

Roth et al.,[37] were first to use C^{14} labeled pentobarbitone to study the barbiturate's metabolism in mice. Since no C^{14} was found in the exhaled air, it was proved conclusively that pentobarbitone was not metabolised to carbon dioxide and disposed of via the lungs. These investigators were also able to show that 90% of the administered C^{14} was excreted in the urine in the first I2 hours following drug administration. A radioautograph of a paper chromatogram of this urine revealed at least 5 radioactive metabolites and demonstrated that less than 0.5% of the original drug was present. It was also shown that less than 0.5% of the barbiturate was excreted as urea. Only 2% of the recovered C^{14} was found in the faeces.

Van Dyke et al.,[38] using dogs, were able to show that Schonle's postulation of complete metabolic conversion of pentobarbitone to carbon dioxide, water and ammonia was untenable. After a dose of N^{15} labeled pentobarbitone less than

8% of the N^{15} isotope was found in the urinary ammonia and urea, although the 24-hour urine which gave this result contained 60% of the total administered N^{15}.

By considering the various hydrolysis products of pentobarbitone which could possibly be formed in the body and then excreted in the urine, and then carrying out a search for them, Maynert and van Dyke [39] were able to show that only negligible amounts of ethyl-(I-methylbutyl)acetyl urea and the corresponding acetamide and malonamide were present as metabolic products of the drug. Ethyl-(I-methylbutyl)malonuric acid was shown to be absent.

Maynert and van Dyke [40] were the first to report the isolation of a metabolite of pentobarbitone from the urine of dogs, and were able to attribute to it the formula (+)-5-ethyl-5-(3-hydroxy-I-methylbutyl)barbituric acid. Brodie et al.,[46] were also able to isolate from human urine a metabolite of pentobarbitone which, on comparison with an authentic sample, appeared to be the same as that isolated be Maynert and van Dyke.[40]

Maynert and Dawson,[41] using N^{15} labeled pentobarbitone succeeded in isolating both diastereoisomeric alcohols, (+)- and (-)-5-ethyl-5-(3-hydroxy-I-methylbutyl)barbituric acid, from the urine of dogs receiving the drug. They determined several of the physical constants of these metabolites and prepared the acetate derivatives of both and the di-p-nitrobenzyl derivative of the

(+)-isomer. In dogs, 33 to 36% of the drug was found to be excreted as the (-)-alcohol and I5% as the (+)-alcohol.

Algeri and McBay [42] made use of the discovery of Maynert and Dawson [4I] to establish the first recorded medico-legal death established through recognition of barbiturate metabolic products. They used the characteristic paper chromatographic pattern of pentobarbitone and its metabolites after their extraction from the urine of the deceased. This pattern compared exactly with the pattern obtained from the urine of dogs and humans receiving the drug.

Algeri and McBay [42] observed a third unclassified metabolite of pentobarbitone on the paper chromatograms of urine extracts. This substance possessed the barbituric acid ring and gave indications of being a carboxylic acid. All attempts at purification were resisted. These authors were able to show that the metabolites were not present in stomach contents or in blood.

Algeri and McBay [43] later described in fuller detail their previous investigations on the paper chromatography of pentobarbitone metabolites. When silver acetate was used to detect the metabolites on chromatograms it was found that the previously reported acidic metabolite could be detected in amounts in the order of 30 μg, whereas more than I00 μg of the alcohols was required for positive identification. Concentrations were determined by ultra-violet spectrophotometry and were

quoted as pentobarbitone.

They also found in the urine a non-barbiturate material which possessed the barbiturate spectrum at pH 9.5 but retained the same spectrum on acidification. This substance was not further classified.

The two alcoholic metabolites were isolated from dog urine and so added confirmation to the results of Maynert and Dawson.[41]

Titus and Weiss,[44] using biologically prepared C^{14} pentobarbitone metabolites as radioactive indicators, were able to examine more efficiently than previous investigators the nature of urinary pentobarbitone excretion. In the urine of dogs receiving the C^{14} labeled drug they were able to demonstrate the existence of ten C^{14} labeled compounds. Five of these were shown to be, unchanged pentobarbitone, the (+)- and (-)-alcohols of Maynert and Dawson,[41] urea and the glucuronide of the (+)-alcohol. The acid previously detected by Algeri and McBay [42] was isolated and characterised as 5-ethyl-5-(1-methyl-3-carboxypropyl)barbituric acid. Three other compounds were in too low a concentration to allow identification. A tenth compound was found to be insufficiently stable to be isolated, though present to the order of 10% of the total C^{14}.

The structure of the acid metabolite was confirmed by comparison with a synthetic sample prepared by Wood and Horning.[45] The infra-red absorption spectra and X-ray powder

Table V. Physical properties of the three characterised metabolites of pentobarbitone.

Property	(+)-alcohol	(-)-alcohol	Acid	Ref.
Melting point	209-2I0	-	-	40
	209-2I0	I52-I53	-	4I
	209-2I0	I52-I53	-	43
	208.5-2II	I52.5-I53.5	I94-I96	44
	-	-	I92-I94	45
pK_a	7.4	8.0	-	4I
	-	-	4.5*	44
$[\alpha]_D^{28}$	+26.6	-5.56	-	4I
ε(0.5N NaOH, 255 mu)	6840	6790	-	4I
	7000	-	-	43
R_f (n-butanol sat. with I% aqueous ammonia)	0.63	0.54	0.07	44
R_f (n-butanol sat. with 5N aqueous ammonia)	0.46	0.46	0.08	43
% of metabolite excreted in dog urine	2I**	4I	4.5	44
M.P. of acetate derivative	I48-I49	I47-I48	-	4I
	I47-I48	I47-I48	-	43

* Stronger acid function.

** A further I3.6% is excreted as the glucuronide.

diagrams of the synthetic and biological metabolites were identical.

Some of the physical properties of the three characterised metabolites of pentobarbitone, elucidated by the foregoing investigators are collected in Table V.

All three characterised metabolites of pentobarbitone are water soluble. The alcohols are insoluble in hexane, benzene, chloroform and carbon disulphide, slightly soluble in ether, more soluble in water and ethyl acetate, and very soluble in ethanol, acetone, acetic acid and dioxan.[4I] The acid is not appreciably extracted from aqueous media at pH 7 by ether, but almost complete removal is achieved at pH 2.[43] The alcohols do not appear to be readily extracted from acidified urine by ether unless continuous liquid-liquid extraction is used,[4I] or the urine is reduced by evaporation *in vacuo* to small bulk and extracted several times with ether[43] or *n*-butanol and *iso*-butanol.[44]

Pharmacological properties of pentobarbitone metabolites

The pharmacological action of the diastereoisomeric alcohol metabolites of pentobarbitone has been studied. Maynert and Dawson,[4I] using material which they isolated from dog urine, found no anaesthetic or hypnotic effect, even in high doses, in mice. The levels used were intraperitoneal injections of I80 mg/kg of the dextrorotatory alcohol and 393 mg/kg of the

laevorotatory alcohol (both as their sodium salts). A dose of 36.3 mg/kg of sodium pentobarbitone was quoted as capable of anaesthetising 9 out of 20 mice.

Dickert et al.,[47] used a synthetically prepared mixture of the alcohols in investigations on mice, the material being administered by stomach-tube or by intraperitoneal injection as a I% Methocel suspension. Using a maximal electroshock test [48] they were able to show no indication of anaesthesia or ataxia at doses of I g/kg. A very weak anticonvulsant activity was noted following administration at a dose of 500 mg/kg.

No results appear to have been published regarding the pharmacological activity of the carboxylic acid metabolite of pentobarbitone.

Site of pentobarbitone metabolism

Much work has been carried out to determine in which organ or organs metabolism of pentobarbitone occurs. This work has been concentrated principally on *in vitro* and *in vivo* studies of liver and kidney, but has also included investigations of other organs and tissues.

Hirschfelder and Haury[I7] found that the duration of action of pentobarbitone on rabbits, nephrectomised under ether anaesthesia, was the same as with normal rabbits. The same result was observed by Koppanyi et al.,[35] using bilaterally nephrectomised dogs. Murphy and Koppanyi [I5,I6] used nephro-

pathogenic agents to produce nephrosis in mammals, and then examined what effect this had on barbiturate action. The results were similar to those obtained from bilaterally nephrectomised animals. A 40% increase in the sleeping time of rabbits, after nephrectomy, with pentobarbitone was observed by Taylor et al.,[49] but Masson and Beland [10] observed little change in the sleeping time of animals following complete nephrectomy. Scheifley and Higgins [50] found that unilateral nephrectomy in rats did not affect the duration of action of pentobarbitone.

The conclusion reached by these investigators was that the kidneys play little or no part in the metabolism of pentobarbitone, since partial or complete inactivation of the kidneys has no significant effect on the sleeping time of animals following pentobarbitone administration.

Pratt et al.,[51] and Pratt [52] found that liver damage, caused by chloroform or the destruction of hepatic circulation, resulted in prologation of pentobarbitone induced sleep in dogs by 8 to 12 times. Liver poisoning by phosphorus or carbon tetrachloride resulted in similar effects. Since the anaesthetic effect of barbitone, which is normally excreted largely unmetabolised, suffered little change under the same conditions, these investigators concluded that the liver was the site of pentobarbitone metabolism.

Koppanyi et al.,[53,54] demonstrated that chloroform anaesthesia of 2 hours duration, 24 hours before pentobarbitone

administration, usually resulted in the death of the animal involved. Analysis of the blood and organs showed that the chloroform treatment had reduced the rate of destruction of the pentobarbitone. While acknowledging liver damage as a principal cause, they also considered injury of cells in the central nervous system resulting in their increased susceptibility to barbiturates. However, the investigators claimed the use of liver poisons to be superior to hepatectomy which involves the incident factors of post-operational shock and prolonged use of anaesthesia during operation.

Cameron and de Saram [12] used carbon tetrachloride, which they claimed was a specific liver poison, to damage the liver of rats. They found that acute liver damage prolonged the action of pentobarbitone, but as the rats gradually returned to normal (in about seven days) so also did the period of action of the barbiturate. Tetrachloroethylene, a toxic substance which does not produce liver damage, had no effect on pentobarbitone action. Rae [55] found that carbon tetrachloride prolonged the sleeping time of sheep by almost three times and delayed plasma clearance.

From these observations, using liver poisons, it was concluded that the liver was the principal site of pento-barbitone metabolism. The same conclusion was also reached by investigators who later used hepatectomy in place of liver poisoning.

Scheifley and Higgins [50] considered that partial hepatectomy was a means of eliminating a constant amount of functioning hepatic tissue without introducing other immeasurable factors, as is the case with liver poisoning. They found that rats, with 70% of their liver removed, slept four times longer than normal under the action of pentobarbitone. As the liver gradually built up to its preoperative condition, the length of anaesthesia returned to its previous value.

Masson and Beland [10] using both partially hepatectomised and completely nephrectomised animals reached the same conclusions as did Scheifley and Higgins,[50] but were also able to go further. Barbiturates were classed into four groups according to their site of detoxification which was usually either the liver or kidney. No relation was found between the duration of action of a barbiturate and its main site or sites of detoxification.

The in vitro examination of organs and tissues has superceded previous methods as a means of examining pentobarbitone metabolism. This procedure does not involve the uncertainties of previously mentioned techniques.

Delmonico [56] was the first to carry out in vitro studies with pentobarbitone. He found that a 24-hour incubation of minced rabbit muscle, brain, kidney or liver indicated that all were capable of metabolising pentobarbitone. Subsequent criticism [14,57] on the degree of uncertainty in these

experiments, since no mention was made of conditions and the possible role of contaminating microorganisms, has cast considerable doubt on the validity of the results.

Dorfman and Goldbaum [14] found that rabbit liver slices rapidly degraded pentobarbitone, but that kidney slices had only a slight effect. Muscle and brain brei did not destroy pentobarbitone. It is interesting to note that these investigators considered that the ring structure of the barbiturate was broken down during metabolism.

Shideman [57] rejected the use of minces (less reactive than slices) and slices (not easily prepared for large scale experiments) for a special system using homogenates. He used this system in studies on thiopentone.

Brodie et al., [58] showed that rabbit liver homogenates metabolised pentobarbitone. Microsomes isolated from the homogenates were found to be incapable of metabolising pentobarbitone. Direct evidence was given that TPNH (reduced triphosphopyridine nucleotide) and oxygen were also required. Drug enzyme systems in various other rabbit tissues were examined but none were able to metabolise the barbiturate.

Cooper and Brodie, [59] in a continuation of the above work, showed that pentobarbitone was oxidised by an enzyme system or systems localised in the microsomes of liver cells of rabbits and rats. Microsomes from kidney, brain, heart and muscle were demonstrated to be incapable of facilitating oxidation.

These investigators described a system in which liver homogenates can be used to convert pentobarbitone to its metabolites. In such a system, the production of the alcoholic metabolites was confirmed by paper chromatography with a reference standard. Since no standard carboxylic acid metabolite was available, its presence was considered proved by R_f determination (paper chromatography) and pK_a determination using a distribution coefficient technique.[61] About equal amounts of the mixed alcohols and acid were formed in the system.

It was suggested that more than one enzyme was involved in the oxidation of pentobarbitone since a C-3 alcohol and a C-4 acid were formed. It was also concluded that splitting of the barbiturate ring was only a very minor role in metabolism.

Kuntzman et al.,[60] compared the relative activity of human and rat liver enzymes using whole homogenised liver and C^{14}-2 labeled pentobarbitone. By extracting the unchanged pentobarbitone with petroleum ether and the metabolites with ethyl acetate a double check was made on the rate of metabolic conversion. The relative amounts of acid and mixed alcoholic metabolites was computed by measuring the radioactivity at the corresponding points on a paper chromatogram of the appropriate extract.

Human liver enzymes were shown to be of low activity and metabolised pentobarbitone more slowly than rat liver enzymes. A sex difference was noted with rats, the male rat liver

metabolising pentobarbitone more rapidly than the female. The female rat liver metabolised the barbiturate at a rate more comparable to man. In comparison with the rat, man produced negligible amounts of acid metabolite.

From the results obtained by the various investigators above, it may be concluded that the sites of metabolism for pentobarbitone are the enzymes on the liver microsomes. No other organ or tissue appears to be involved to any appreciable extent although the kidney may play a very minor role.

Pentobarbitone blood clearance rates

The rate of disappearance of pentobarbitone from the blood has been measured in many animals receiving varying doses of the drug. Results of such experiments, which have been extracted from the literature, are summarised in Table VI.

From the results quoted in Table VI two points emerge. The rate of clearance varies greatly with the species, the mouse having the greatest and man the least clearance rate. Generally, for any one species, the lower the dose the greater the clearance rate.

Pentobarbitone urinary excretion rates

Little work has been done on the urinary excretion rates of pentobarbitone, or rather its metabolites, although the rates of excretion might be expected to be a function of blood

clearance rates.

Kahn,[66] using C^{14} labeled pentobarbitone, showed that of the total administered activity rats excreted 30% after 4 hours, 50% after 8 hours and 90% after 24 hours following a dose of 60 mg/kg. Titus and Weiss [44] have shown that C^{14}, when administered as pentobarbitone-2-C^{14}, was almost completely excreted after 15 to 20 hours in the urine of dogs.

Maynert and van Dyke [39] have shown that dogs excreted an average of 82% of N^{15} labeled pentobarbitone after receiving a dose of 50 mg/kg. Later Maynert [76] carried out quantitative determinations on N^{15} labeled pentobarbitone excreted in urine with a mass spectrometer. It was shown that humans who had received from 100 to 500 mg of the drug gradually eliminated it over a period of 5 days. From 7 to 8% and 40 to 43% of the urinary isotope, derived from a 500 mg dose of pentobarbitone, was accounted for as the dextrorotatory and laevorotatory diastereoisomeric alcoholic metabolites respectively.

The principal cause of the lack of data on the urinary excretion of pentobarbitone is doubtless the difficulty in assessing the excretion rate because there is no suitable method available for quantitatively determining the metabolites other than by isotopic labeling.

Table VI. Pentobarbitone clearance rates, extracted from the literature.

Species	Number used	Dose mg/kg	Clearance Rate %/hour	Analysis	Ref.
Mouse	9	50	I37		
	30	80	78 ± I5	Whole mouse	68
	I0	I00	64	homogenate	
	8	I20	5I		
	40	50	80*	Whole blood	69
	?	80	78	Whole body	70
Rabbit	I6	24	59*	Whole blood	75
	?	ca.33	3I*	Plasma	33
	6	30	24.7 mg/kg/hr	Biological	7I
	I0	40			
	5	25	35 ± I0	Plasma	68
	4	20	35	Plasma	72
	?	25	35	Plasma	73
Rat	I6	60	30*	Whole blood (C^{I4})	66
	I2	30	46*	Plasma	62
Sheep	6	20	49	Plasma	55

Table VI. Continued :-

Species	Number used	Dose mg/kg	Clearance Rate %/hour	Analysis	Ref.
Dog	4	?	I5	Plasma	46
	6	25	I5 ± 2	"	68
	?	25	I5	"	73
	2	I00	I5	"	67
	2	30	27 ± I*	"	74
Man	6	I2	4	"	46
	6	0.8 to Ig each	I.5	"	3I

* These results were calculated from the data given.

Pentobarbitone sex effects

Though a sex effect in rats affects the period of sleep following pentobarbitone administration, no *in vivo* attempts appear to have been made to establish whether this is due to differing metabolic rates or to a greater susceptibility of one sex. In the examples of clearance rates quoted in Table VI no mention of the sex of the animals was made.

Bester and Nelson [62] showed that, following a dose of 27.5 mg/kg, the recovery time for female rats was 45.2 ± 4.8 minutes compared with 26.0 ± 3.5 minutes for males, the rats being three to five months old. In a more detailed study Moir [63] showed that this effect was complicated by an age factor. Very young female rats were found to be more resistant to pentobarbitone than the corresponding males, slightly older females and males reacted similarly and mature females were definitely more resistant than the corresponding males.

Kuntzman et al., [60] using rat liver homogenates, showed that the male rat liver degraded pentobarbitone more rapidly than the female rat liver. This experiment would suggest that the sex difference is probably due to a metabolic rate difference rather than a comparative susceptibility difference.

Kato and Gillette [77] studied the hydroxylation activity of NADPH-dependent enzymes in rat liver microsomes with respect to pentobarbitone and other drugs. Normal rat enzymes showed that the male rat enzymes metabolised pentobarbitone about

three times faster than the corresponding female rat enzymes. Enzymes from rats starved for 72 hours showed that male and female rat liver enzymes had similar metabolic rates. However, compared with normal rats, the female rat liver enzymes had almost doubled their activity whereas the male rat liver enzymes had decreased in activity by about one-half.

It has been reported that a sex difference exists in rabbits to the action of pentobarbitone.[75] Females are claimed to be more susceptible to pentobarbitone depression than are males.

Tolerance to pentobarbitone

Using the criterion of sleeping time it has been shown that animals develope a tolerance to pentobarbitone [63,64,65] but whether or not this is due to increased rate of metabolism has not been established. The effectiveness of the drug as a hypnotic returns when repeated administration ceases. The tolerance of cats to pentobarbitone and the subsequent withdrawl symtoms has been described by Jaffe and Sharpless.[78] A quantitative study of tolerance and posttolerance hypersensitivity of the rat to pentobarbitone has been reported.[79]

Distribution of pentobarbitone

The distribution of pentobarbitone in various animal tissues, following administration of the drug, has been

investigated by many research workers, often in concurrence with clearance rate studies. The general conclusion reached by all was that pentobarbitone did not have a noticeably high concentration in any single organ or tissue in rats,[66] rabbits,[33,53] dogs [46,53,67] and sheep.[55]

Among the various organs and tissues tested were, blood, cerebral cortex, liver, sub-cortical brain tissue, spleen, heart, kidney, lung, diaphragm, intestine, muscle, plasma, brain and fat. Different routes of administration were also tried and proved to have no effect on distribution.[67]

Koppanyi et al.,[53] showed that the rapid intravenous injection of pentobarbitone led to a lower concentration of the drug in the cerebrum than in the medulla, and this was proposed as a reason for the greater toxicity of pentobarbitone relative to phenobarbitone which has a higher concentration in the cerebrum than in the medulla following rapid intravenous injection.

Goldbaum [33] showed that there was a noticeably higher concentration of the drug in the liver relative to other organs just following injection than after 99 minutes at which time equilibrium had been reached. The relative concentration in the liver remained slightly higher than in other organs, after equilibrium had been reached, until clearance was complete.[55,66]

A REVIEW OF BARBITURATE SALIVARY SECRETION

Many investigators have reported work concerning various aspects of salivary secretion involving principally eletrolytes and also non-electrolytes including drugs. Relatively little work has been published specifically dealing with barbiturate salivary secretion.

Amberson and Hober,[80] in a study on non-electrolyte secretion, gave a good introductory review of the position concerning electrolyte secretion up to I932. These workers went on to show that, in the cat submaxillary gland, non-electrolytes of low molecular volume and relatively high lipoid solubility penetrated into saliva easily. An increase in the molecular volume or a decrease in the lipoid solubility decreased the salivary secretion of non-electrolytes.

Burgen [8I] carried the work of Amberson and Hober a stage further in his investigation of the secretion of non-electrolytes in the parotid saliva of dogs. The ten substances investigated were classified into two groups depending on the effect of the rate of saliva flow on their secretion. With one group, the saliva-barbiturate concentration decreased with an increase in the saliva secretion rate, and with the other the concentration first decreased, passed through a minimum, and then rose again with a further increase in the saliva

secretion rate. The concentration of the substances in the saliva were shown to be lower than in the plasma although the concentration in the gland itself was the same as in the plasma.

Some of the first experiments, directly involving barbiturates and salivary secretion, showed that amylobarbitone[82] and hexobarbitone [83] inhibited salivary flow induced by stimulation of the chorda tympani or by the injection of choline or acetylcholine. Physostigmine [82] was found to antagonise barbiturate salivary inhibition.

In a single experiment on a dog, using pilocarpine to stimulate salivary flow, Koppanyi et al.,[35] showed that the concentration of barbitone in the saliva approximated to that in the plasma.

More recently, a comprehensive study of the degree of barbiturate salivary secretory inhibition and of antagonists to this action has been carried out by Guimaris et al.,[84] using dogs. Salivary flow was provoked by stimulation of the chorda tympani or by the continuous infusion of solutions of pilocarpine or adrenaline. The barbiturates studied depressed salivary flow to widely differing degrees. In decreasing order of depressive effectiveness they were hexobarbitone, eunarcon, thiopentone, amylobarbitone, pentobarbitone and phenobarbitone. Sodium fumarate and sodium succinate were shown to be antagonistic to the action of the barbiturates. It was concluded that there were two sites at which the barbiturates acted, the

ganglionic synapse and the salivary gland itself. The work of Kewitz and Reinhert [85] and Exley, [86] on the depression of autonomic ganglia by barbiturates in cats, was quoted in support of their conclusion that the ganglionic synapse was a site of barbiturate action. Some barbiturates showed an action which was predominantly due to action at one site only.

Rasmussen [87] studied quantitatively the parotid secretion of various barbiturates in cows and goats. The blood-barbiturate concentration was held almost constant by the administration of small supplementary doses at intervals following the initial dose. It was found that the concentration of phenobarbitone and pentobarbitone in the alkaline saliva was higher than the concentration in the ultrafiltrate of the plasma. The actual concentration in the saliva depended on the pK_a values and lipid solubility of the drugs. The non-protein bound and the unionised forms of the barbiturates tended to establish a diffusion equilibrium across the epithelium of the salivary gland. Limited variations in the saliva secretion rate did not affect the saliva/plasma ultrafiltrate concentration ratio, although fast flow rates tended to result in a decrease in the above ratio. The effects of secretion rate on saliva-barbiturate concentrations were less marked than those described by Burgen, [81] for non-electrolytes.

A REVIEW OF BARBITURATE PLASMA PROTEIN BINDING

Determinations of the degree of plasma protein binding of barbiturates have frequently been carried out at the same time as blood clearance determinations. The results have been used to calculate the amount of 'free' barbiturate in the blood at awakening time and also the concentration of 'free' barbiturate available for transfer across, for example the blood brain barrier.

Suspicion has recently been cast on the validity of some of these calculations by the work of Campion and North [88] who have shown that for secobarbitone, amylobarbitone and phenobarbitone, the concentration and type of the barbiturate determine the degree of protein binding. Thus for phenobarbitone in human serum, the degree of binding to the albumin was found to be 25% and 60% for barbiturate concentrations of 75 and 15 µg/ml respectively. The two other barbiturates reacted similarly in showing a rapid increase in the degree of binding with a rise in concentration. If this is the case, protein binding determinations would have to be carried out with barbiturate concentrations similar to those which are going to be used in subsequent calculations. An example of failure to meet this requirement was given by Rae [55] who determined pentobarbitone and thiopentone plasma protein binding using solutions of concentration 50 µg/ml and applied the results to plasma

barbiturate concentrations of I5.2 and 30 µg/ml respectively.

Results given by Rasmussen [87] for protein binding in goat plasma do not concur with those of Campion and North.[88] Pentobarbitone was found to be bound to the extent of 66 to 67% when present in concentrations of 5I and II5 µg/ml; phenobarbitone figures were 68% and 58% for concentrations of I07 and 86, and 28 µg/ml respectively; barbitone was bound to the extent of II, 2I and 50% at concentrations of 269, 2I3 and 6I µg/ml; and reposal was bound to the extent of 69 and 55% for concentrations of 90 and 5I µg/ml. Hence, with the exception of barbitone, these findings indicate the opposite effect to that reported by Campion and North.[88] Rasmussen appears to be the only person who has investigated saliva protein binding of barbiturates. No evidence of binding was found.

Waddell and Butler,[25] in an investigation on phenobarbitone, found the drug-serum albumin binding figure to be constant at 40% over the range 20 to I00 µg/ml when a 4 g/I00 ml solution of bovine albumin was used. A decrease in albumin concentration resulted in a decrease in binding. Binding to human serum did not differ significantly from that to bovine albumin. Binding in dog and human plasma was found to be approximately the same as in a solution of pure albumin of the same concentration as the plasma albumin. pH was shown to have only a minor effect on the degree of protein binding using a solution of about 75 µg/ml in solutions of 4 g of bovine serum

albumin in 100 ml phosphate buffer. These authors considered that the assumption of a constant degree of protein binding of phenobarbitone, regardless of concentration, was sufficiently accurate to apply to the calculation of renal clearances. This is in direct confliction with the findings of Campion and North.[88]

The conclusions that must be drawn from these results are that the position regarding plasma protein binding is uncertain and that the comparison of the results of various investigators on protein binding is unadvisable. The reason for the differences in the reported results may lie in the wide range of methods used to determine protein binding. A further variable is the time required for the various determinations which varies from 3[89] to 24[25] hours.

A REVIEW OF METHODS FOR THE DETECTION AND DETERMINATION OF BARBITURATES

During the last thirty to forty years an ever increasing number of methods has been advocated for barbiturate analysis in toxicological analysis. That no method has achieved universal usage must surely be some measure of the dissatisfaction felt among analysts about these methods. One of the main causes of this dissatisfaction is doubtless due to the difficulties in eliminating non-barbiturate material which will either react positively to the method of analysis or cause interference with that method.

A brief summary of some of the more popular methods of analysis which have been used over the years now follows. Some indication of their sensitivity and their applicability is also included.

Gravimetric and allied techniques

Gravimetric analysis, as first used in the determination of barbitone,[90] is the oldest analytical method. The size of samples required, greater than 10 mg, has now largely relegated this method to use on autopsy material.[91,92] Since the identification of a barbiturate, isolated in a gravimetric determination, can depend on a melting point determination the

accuracy of the method for identifying a specific barbiturate is doubtful as several barbiturates can exist in polymorphic forms [93,94] of different melting points. Similarly, microscopic identification of crystalline barbiturates,[93,94,95] obtained by sublimation,[96,95] is difficult.

Recently, Lobanov [97] has described microcrystalloscopic reactions for detecting barbituric acid derivatives using the reaction between barbiturates and copper salts in alkaline media.

A complete scheme for the identification of common barbiturates involving the techniques of sublimation, crystal tests, melting point determinations and colour reaction tests has been given by Turfitt.[98]

Complexometric methods

Many complexometric methods have been described for both the detection and determination of barbiturates. These methods are more sensitive than the gravimetric method, but have a lack of specificity as has been shown by Riley et al.,[I02] who investigated compounds, other than barbiturates, which reacted with the copper-isopropylamine reagent of Koppanyi.[99]

One of the earliest complexometric methods involved the use of the cobaltamine reaction. Thus, Koppanyi et al.,[35,99,I00] using the blue colour formed by barbiturates and cobalt chloride in alkaline methanolic solution for identification and determination, claimed a sensitivity of I0 μg/ml.

A summary of some of the earlier complexometric methods, and the problems involved, has been given by Maynert and van Dyke.[101] These methods have been replaced, however, by the more sensitive procedure of extracting the barbiturate-mercuric complex from aqueous solution using chloroform, and then determining the extracted mercury by a suitable colorimetric reaction.

For qualitative results the mercury complex is extracted from water with chloroform and, on the addition of dithizone, an immediate orange colour indicates the presence of barbiturate.[103] This procedure may be made quantitative by titrating the extracted mercury with standard dithizone.[104] Alternatively the violet colour, extractable with chloroform from a solution of barbiturate, diphenylcarbazone and mercuric sulphate, may have its optical density at 550 mμ measured to give quantitative results.[105] Zaar and Gronwall [106] described a micro method requiring I ml of serum. This is similar to the previous method but uses dithizone in place of diphenylcarbazone. The absorbance reading is made at 605 mμ. For solutions of amylobarbitone the accuracy was quoted as ± 40% at I μg/ml, ± 7% at I0 μg/ml and ± 5% at 20 μg/ml.

Pfeil and Goldbach [107] described a micro determination for barbiturates involving mercuric salt precipitation on filter paper followed by treatment with dithizone and subsequent colorimetric measurement of the eluted mercury. An accuracy of ± 7% was claimed. A micro technique has been described by

Wallenius et al.,[108] who used a continuous chloroform flow system which required 0.2 ml serum or urine. The method is very rapid, being designed for use in emergency poisoning cases. σ, in the range 20 to 50 µg/ml, was I.0 µg/ml (n = 26). The urine blank was found to be variable i.e. up to 5 µg/ml.

Bjorling et al.,[109] investigated the possibility of certain compounds interfering with the mercury-dithizone barbiturate determinations. Compounds found to interfere were bromide, iodide, cyanide, EDTA, stearic acid, methylthiouracil, propylthiouracil, phenazone and hydantoins. Phosphate, carbonate and borate did not interfere nor did nitrate, acetate, chloride or perchlorate the mercuric salts of which were used as a source of mercury.

The nature of some of the barbiturate-copper-pyridine complexes has been studied. Zwikker[110] was able to show that the barbitone complex had the formula, $(\text{barbitone})_2.\text{Cu}.(\text{pyridine})_2$. Using infrared spectroscopy, Levi and Hubley[111] verified Zwikker's formula to be general for a series of clinically important barbiturates, and devised a general method for preparing pure crystalline samples of the complexes. These complexes were all readily decomposed by dilute mineral acid.

Paper chromatography

One of the most valuable tools in barbiturate analysis is

paper chromatography. This technique allows the identification of several barbiturates in the same sample, which is of great importance especially with the introduction of mixtures of barbiturates into pharmaceutical preparations. In studies involving the metabolic excretion products of barbiturates, paper chromatography has provided an easy means of detecting different metabolites of the same barbiturate and also, when carried out on a large scale, a means of separating the individual metabolites, as may be typified by pentobarbitone [42, 44, II2] and phenobarbitone.[2, 3, II3]

Algeri and Walker [II4] carried out an extensive investigation of solvents for use in the paper chromatography of the common barbiturates. They found the most suitable system to be n-butanol saturated with 5N ammonia. Alternative proportions for the reagents have since been proposed. These solvents are not particularly stable and require renewal every 4 to 5 days.[II5] Other solvent systems have been described, including a highly stable system using petroleum ether and methanol.[II6] Collected characteristic R_f or $R_{standard}$ values of many of the common barbiturates have been listed in the literature.[II4, II7, II8, II9] A short list of the solvent systems which have been used are given in Table VII.

Barbiturate spots on a completed chromatogram may be detected by several means. After exposing the chromatogram to ammonia vapour, or spraying with 0.5N sodium hydroxide, the

Table VII. Solvent systems for the paper chromatography of barbiturates

Solvent	Reference
n-butanol saturated with 5N NH_3	II4
n-butanol:25% NH_3:water = 84:8:8	I23
n-butanol:water:NH_3(0.880) = 340:57:3	II9
n-butanol:NH_3(0.880) = 3:I	II9,I24
n-butanol:NH_3(0.880) = 90:I0	I22
Amyl alcohol:NH_3(0.880) = 9:I	II9,I24
Amyl alcohol:NH_3(0.880):ethylene glycol = I0:I0:I	I25
Amyl alcohol:n-butanol(I:I) saturated with NH_3	I22
Hexyl alcohol saturated with NH_3	I26
Ethanol:NH_3(0.880) = 95:5	I22
Isopropanol:NH_3(0.880) = 95:5	I22
Petroleum ether in methanol vapour	II6
Chloroform in NH_3(0.880) vapour	II9

barbiturates appear as dark spots against a white fluorescent backgroud when the chromatogram is viewed under a low pressure mercury lamp,[II9] or as dark spots when viewed through a fluorescent screen.[I20] A permanent record may be obtained by contact printing using Ilford Reflex No. 50 [II7,I2I] (or corresponding paper) and an ultra-violet light source. This method is not specific as any compound capable of absorbing ultra-violet light will react in a positive manner.

On exposing a chromatogram to piperidine vapour, after spraying with I% alcoholic cobalt nitrate and heating at 80° for one to two minutes, barbiturates will show up as violet spots.[I22] This process can be simplified to immersing the chromatogram in I% cobalt chloride in acetone, drying, and exposing to ammonia when the barbiturates show up as violet spots.[II9,I22] The sensitivity of this method is slightly less than that using ultra-violet light but has a greater degree of specificity.

The formation of mercury or silver barbiturate complexes followed by location of the metal using diphenylcarbazone has been described.[II4]

Unsaturated barbiturates can be detected by spraying the chromatogram with 0.I% aqueous potassium permanganate, when reduction of the permanganate will result in light coloured spots against a pink background. The rate of this decolourising reaction is, however, dependent on the barbiturate and can give

some indication of the type of barbiturate involved.[127] Fully saturated thiobarbiturates also give this reaction.

Thiobarbiturates may be detected under ultra-violet light as dark spots without prior exposure of the chromatogram to ammonia or spraying with sodium hydroxide.[127] This allows them to be differentiated from the 5,5- and I,5,5- barbiturates.

Bromobarbiturates react with fluorescein, as described by Curry,[128] to give pink spots which are more easily seen in ultra-violet light.

Location of barbiturates on a chromatogram and their quantitative determination can be achieved by eluting the chromatogram, section by section with alkali, and carrying out quantitative ultra-violet determinations on the eluates.[112] By using densitometry [129] the barbiturate spots need not be eluted and R_f values may be accurately pinpointed.

Barbiturates with similar R_f values may often be identified by treating the barbiturate mixture with concentrated sulphuric acid, before chromatography, when dealkylation will result in a change in the R_f values.[119,130,131,132] Stevens [133] has described a method in which mixtures of barbiturates were separated by first heating them with solutions of potassium dichromate in sulphuric acid of varying strengths. The oxidised and unoxidised barbiturates were then extracted and subjected to paper chromatography. By comparing the resulting chromatogram with known standards, identification of the unknown was

facilitated.

Modifications of the basic technique of paper chromatography include the use of buffered papers [I34,I35] and high temperature operation.[I36,I37] This latter method has reduced the time involved from I2 to I8 hours to about one hour. This reduction in time also results in higher resolution of the barbiturate spots as less time is allowed for spreading. A systematic identification of barbiturates in blood from emergency cases has been described.[I38] The whole procedure takes about one hour and involves high temperature chromatography, concentrated sulphuric acid treatment and ultra-violet measurements. The accuracy claimed is better than ± I0% and the concentration limit is below 5 µg/ml

Thin layer chromatography

Thin layer chromatography provides a rapid, inexpensive method for identifying barbiturates. Several techniques have been described in which barbiturates in blood can be detected and identified with qualitative accuracy suitable for clinical purposes.[I39-I44] Cochin and Daly [I45] have identified I6 commercially available barbiturates after their extraction from urine with methylene chloride.

The time involved in such analysis is in the range one to two hours. Detection of the barbiturates on the thin layer plates is achieved in a similar fashion to those on paper

chromatograms.

Though the quantitative nature of the method is low, due to adsorbent impurities,[I46] attempts have been made in this direction with some success when the quantities are in the range 50 to 250 μg.[I47]

Thin layer chromatography has been used in metabolic studies with methohexitone for the detection of the metabolites.[I48]

Gas-liquid chromatography

Several approaches have been made to the gas chromatographic detection and determination of barbiturates. The first method used involved qualitative identification based on the thermal degredation patterns of the sodium salts or of the free acids.[I49,I50,I5I] An improvement on this technique was to use the free acids and chromatograph them without pyrolysis.[I48,I52-57] A more recent method [I58] involved the methylation of barbiturates before chromatography. This was reported as resulting in reduced peak tailing and consequently in better quantitative results. The same technique has been reported by Cook et al.[I59]

Column packings are normally of a low weight per cent loading (I to 5%), although I0 to I5% loadings have been used,[I59] and have involved many different stationary phases. Brochmann-Hanssen and Svendson [I54] have investigated the relative retention times of 2I barbiturates on six columns of varying polarities. The actual column may be of glass,[I48,I53,I54,I56,I60,I6I]

stainless steel,[I57,I62] or of aluminium.[I55] The possibility of catalytic decomposition on metallic surfaces has been suggested.[I56] Operational temperatures reported vary considerably from I37° to 230°.

Detectors used have been hydrogen flame ionisation,[I55,I56, I57,I60,I62] electron capture [I53,I56] and argon ionisation detector.[I54,I6I,I59] The electron capture detector is suitable for concentrations down to 0.I to 0.8 µg/µl,[I56] although I to IO µg/µl is more usual. Braddock and Marec [I60] investigated by gas chromatography pentobarbitone and thiopentone, using barbitone as an internal standard, in the concentration range 0.005 to 0.I µg/µl. They claimed a sensitivity of 0.003 µg/µl for pentobarbitone, 0.005 µg/µl for thiopentone and 0.002 µg/µl for barbitone when using chloroform solutions of the pure compounds and a flame ionisation detector. Replacement of air by oxygen in the flame ionisation detector has been shown to increase the detector sensitivity by a factor of four.[I57]

As many as I3 barbiturates can be separated on a single column.[I55] By a suitable choice of column it has been found possible to separate barbiturates which would not separate on other columns.[I54] Though not necessarily affecting the separation of the other barbiturates, this latter process can result in a change in the order of elution from the column.[I54] Since low level stationary phases are normally used the barbiturate peaks tend to tail because of the partial adsorption on the solid

suport of the barbiturates. Cieplinski [I63] has shown that a hexamethyldisilizane treated stationary phase removed peak tailing for seven barbiturates which he investigated.

A general study of gas-liquid chromatography of sub-microgram amounts of drugs, including barbiturates, has been carried out by McMartin and Street.[I64] Many of the variable factors involved in such work were considered and the preparation and results of an SE30 column were described.

Extraction procedures have been described for the determination of barbiturates in blood [I53,I55,I57] and in urine.[I65] Determinations can be carried out on blood samples of as little as 50 µl.[I57] No interference is encountered from normal blood constituents.

Metabolites are either not extracted or, if so, appear separated from the original drug.[I57] Metabolic excretion in the urine of man has been studied using phenobarbitone, metharbital, mephobarbital, aprobarbital and barbital.[I65] Care must be made in the choice of column if the metabolites are to be determined. The hydroxy- metabolite of amylobarbitone has been determined by converting it to its silyl-ether derivative, using hexamethyl-disilizane, before gas chromatography. Methyl stearate was used as an internal standard.[I66]

The clearance of methohexitone from blood has been studied using gas chromatography [I48] and would appear to be the first application of the technique to a clearance rate

determination.

Gas chromatography possesses several advantages over any other single method for barbiturate analysis. Sample size is reduced; interfering naturally occuring compounds are not encountered in blood samples; the method is rapid and allows not only measurement of the quantity but also the identification of the barbiturate involved; it is possible to detect the presence and amount of more than one barbiturate simultaneously (of importance now that several barbiturates may be dispensed together).

One major disadvantage of gas chromatography is that unless a barbiturate is known or suspected to exist in a sample, the appearance of a peak on a chromatogram at a point coincident with that normally occupied by a particular barbiturate, does not prove the presence of that barbiturate. It has also been noted [I56,I60] that the retention time of some barbiturates increases with a decrease in concentration of the solution injected into the chromatograph, indicating that caution must be exercised in the use of retention times for qualitative analysis. Care must also be taken with respect to ghost peaks which can be produced, at random, by the release of adsorbed material from previous runs.

Ultra-violet spectrophotometry

Ultra-violet spectrophotometry provides the most often used method of quantitatively determining barbiturates. It was first used on biological samples by Hellman et al.,[167] for the determination of thiopentone in blood. Refinements in the method since then, plus rigorously controlled extraction procedures, have greatly increased the specificity of the method.[168-171]

Methods in use now for 5,5-disubstituted barbiturates make use of differential ultra-violet spectrophotometry. These depend on the changes in the ultra-violet spectra of the compounds that occur at different pH values due to the various stages of enolisation in the barbituric acid ring.[170,171] Determinations use either the optical density difference between solutions at pH 13-13.5 and pH 10-10.4 at 260 mμ,[172-175] or between solutions at pH 10-10.4 and pH 2 at 240 mu.[27,170,176,177]

Thiobarbiturates can be determined by optical density measurements at 305 mμ using a solution at pH 13, and at 290 mμ with a solution at pH 2.[167]

The determination of 1,5,5-substituted barbiturates has been carried out by measuring optical density at 248 mμ in 2.5N sodium hydroxide.[178]

The effect of the various stages involved in the ultra-violet determination of serum barbiturate have been considered. These included the stability of barbiturates in alkaline solution, solvents and pH for extraction, and interfering

compounds (sulphonamides and salicylic acid).[166]

Interfering naturally occuring compounds provide a major difficulty in this type of analysis and impose limits on its ultimate accuracy. Thus the variation in the normal blank value of blood from 16 post mortem samples was found to be 0.05 ± 0.12 mg% of apparent pentobarbitone when n-butyl ether was used for direct extraction.[172] Attempts to reduce this interference have included tungstic acid protein precipitation,[99,117,139,170,179,180] bicarbonate[119,170] or phosphate[173] buffer washes and preliminary extraction of chromogens from alkaline samples.[171] Gould and Hine,[181] in their determination of serum barbiturate by continuous ether extraction followed by ultra-violet measurements of the extract at pH 9.5, produced a method for overcoming variable blank values which they considered more accurate than that of assuming an "average" blank. This method involved optical density measurements at 225, 240 and 265 mμ. By considering the blank optical density of the serum to be a linear relationship between 225 and 265 mμ, a value was computed for 240 mμ. This latter value was subtracted from the actual reading at 240 mμ, the point of peak absorption of the barbiturate, and the resulting figure taken as a measure of the barbiturate concentration.

Serious interference to the ultra-violet technique under certain conditions[182,183] has been attributed to breakdown products of tyrosine[182] which produce similar absorption curves

to the 5,5-dialkylbarbiturates. They are, however, distinguishable from the barbiturates by an absorption peak at 292 mμ which the barbiturates do not possess. This demonstrates the necessity for recording spectra over a range of wavelengths rather than just at the wavelength required for quantitative measurement. The interfering compounds are thought to be various hydroxyphenyl aliphatic acids.[I82]

Also reported as an interfering naturally occuring compound is p-hydroxybenzaldehyde which was said to appear only in unusual circumstances.[I84] Since then this compound has been isolated from normal human liver by Kaempé,[I85,I86] who also isolated from the same source p-hydroxyphenylacetic acid, p-hydroxybenzoic acid and p-hydroxyphenylethanol. The latter compound corresponded closely in its behaviour to the substance detected previously in liver extracts by Curry.[I83] All these substances possessed spectra which would greatly interfere with the ultra-violet determination of barbiturate.

Apart from naturally occuring chromogens, there are also certain drugs which can cause interference. β-methyl-β-ethyl glutarimide (Bemegride) has an ultra-violet spectrum which can give serious interference in barbiturate determinations.[I87] It also gives a positive cobaltamine reaction. However, since the compound undergoes ready hydrolysis in alkali it can be removed in this fashion leaving the barbiturate material almost unaffected.[I73] α-methyl-α-phenylglutarimide (Doriden) also

interferes in a similar way and may be removed by the same treatment.

Salicylate also causes interference [I88] and a method for its removal, involving magnesia-silica gel column chromatography, has been described. This process also serves to remove much of the natural colouring material from the urine extracts.

p-hydroxynirvanol [I90] and p-hydroxyphenytoin [I9I] (hydroxy metabolites of Nirvanol and Phenytoin respectively) have ultra-violet spectra which are pH dependent and appear in the weak acid fraction of extraction procedures.

Attempts have been made to make the ultra-violet technique qualitative as well as quantitative. Maher and Puckett [I92] used the ratio of the optical density difference to the maximum positive optical density difference between solutions at pH I3 and pH I0 over a range of wavelengths. This was an extension of a previous method by Golbaum.[I74] The variable factors in this determination have been treated by Broughton.[I73]

Curry [I32] using acid hydrolysis, and Broughton [I73] using alkaline hydrolysis, were able to characterise classes of barbiturates rather than single barbiturates. Paper chromatography was required for complete identification. A modification of Broughtons's method has been published and also involves treatment of the barbiturate with hot alkaline solution.[I93]

By using the partition of barbiturates between a borax buffer and butyl ether, barbiturate material may be classed

according to its degree of polarity and partial identification achieved.[I72] The method has been made more exact by the introduction of potassium permanganate oxidation and alkaline hydrolysis stages.[I94]

The determination of pK_a values by ultra-violet spectrophotometry [I95] has been described as a means of barbiturate identification, but can only be applied to single barbiturates.

Infrared spectrometry

Infrared spectrometry is comparitively little used in barbiturate analysis probably due to the quantity (greater than I00 μg) and purity of sample required, although Bonnichsen et al.,[I96] and Alha and Tamminen [I97] have described how infrared spectra of barbiturates may be obtained with as little as 5 μg of barbiturate using a spectrometer with a microscope attachment. It has been claimed that the slight differences in the spectra of barbiturates coupled with other analytical constants will lead to identification.[I98,I99] The relationship between infrared spectra and pharmacological action of the 5,5-disubstituted barbiturates has been studied.[200]

Levi and Hubley [III] have suggested the use of the infra-red spectra of barbiturate-copper-pyridine complexes as affording a method with a high degree of specificity for

detecting and characterising these drugs. The spectra of twelve clinically important barbiturates and their complexes were given.

Spectrophotofluorimetry

Recently a spectrophotofluorometric method for the determination of low concentrations of amylobarbitone in plasma has been described.[201] The procedure has, however, been found to be unsuitable for determinations in urine due to a high and variable "blank".[202] Udenfriend [203] and Udenfriend et al.,[204] have given details of excitation and fluorescent wavelengths of some barbiturates and quote a practical sensitivity of about 0.5 µg/ml.

Other methods

X-ray diffraction is a regularly used tool in toxicological analysis and complete imformation is available for many pure barbiturates.[205,206,207]

A nephthelometric method has been described by Kalinowski and Baran.[208] Kalinowski [209] has also described a turbidimetric determination involving titration with alkaline silver nitrate to permenent turbidity. A similar technique has been described by Budde.[210]

Isotopic and radioactive labeling has been extensively used in the study of barbiturate metabolism and may be typified

in the cases of pentobarbitone [39,41,44] and phenobarbitone.[7,8,9,13]

An automated colorimetric method for determining phenobarbitone has been described [211] and involves alkaline hydrolysis and subsequent determination of the urea formed by conversion to a coloured triazine derivative.

Both aqueous and nonaqueous titrimetry have been used in barbiturate determinations. The aqueous techniques have used the mercury-barbiturate complexes. Curry [104] extracted the complexed mercury with chloroform and titrated it with dithizone. Pedley,[212] after adding a known excess of mercury perchlorate, filtered off the complex and determined the remaining mercury with ammonium thiocyanate. The nonaqueous method is an acid-base titration using potassium hydroxide, the end-point being detected by visual or potentiometric means. Various solvent systems have been described.[213-216] Unsaturated barbiturates may readily be determined by quantitative reaction with a standard bromine solution.[217,218]

EXTRACTION PROCEDURES

Various systems have been used for extracting drugs from body fluids so that they are, if present, separated up into distinct groups depending on their chemical and physical properties. These group extracts may then be tested for the presence of specific substances. A typical extraction scheme has

been outlined by Curry.[219]

Many extraction procedures have been expounded solely for the identification and determination of barbiturates, and a summary of some of these applicable to blood and urine samples now follows, although other sample sources have been considered.

Blood

Several methods have been used including, Soxhlet extraction of blood after treatment with anhydrous sodium sulphate,[220,221] calcium sulphate,[99] or sodium phosphate,[222] direct extraction of whole blood with chloroform [9,168] or n-butyl ether,[172] and extraction of plasma or serum with ether,[181] chloroform,[162,168,171] ethylene dichloride,[33] or petroleum ether.[169] Extracts of a high degree of purity can be obtained using tungstic acid protein precipitation followed by ether extraction [99,117,139,170,179,180] but with a considerable loss of barbiturate. Koppanyi et al.,[35] used copper hydroxide precipitation followed by chloroform extraction to remove interfering material prior to colorimetric determination of barbiturate.

Wright and Johns,[27] who used chloroform extraction of whole blood, pointed out that only pure heparin should be used as an anticoagulant for blood samples to be used for barbiturate analysis since heparin for intramuscular use contains phenolic

preservatives which would interfere in any subsequent ultra-violet determination.

For analysis by ultra-violet spectrophotometry, the method of McCallum gives perhaps the cleanest extraction with a high recovery.[171] The basified blood is first extracted with chloroform, then acidified and the barbiturate material extracted with chloroform. The barbiturate is then washed with dilute sulphuric acid and finally returned to aqueous solution by extracting with dilute ammonia. The optical density of the ammoniacal solution at 240 mu is then measured against a standard blank. The use of ammonia in place of sodium hydroxide gives an almost constant low blank reading for serum samples (about 0.050) and, furthermore, the alkaline solutions are sufficiently stable to be left overnight. The recovery is about 90% which is superior to the tungstic acid method.

A micro extraction method, involving the ether extraction of I ml of serum absorbed on filter paper, has been described.[106]

The treatment of decomposing samples of blood has been dealt with by Turner,[223] and by Teppo and Alha [224] who improved Turner's method by introducing thin layer chromatography to eliminate interfering material present in the blood.

The use of buffer washes to remove chromogens involves careful attention to pH. Walker et al.,[170] have pointed out that serious losses of barbiturate material can result from using

washes of too high a pH. It has been shown that the pH of the commonly used bicarbonate buffer rises with the length of time it is shaken with the extract.[223]

Special techniques for gas chromatographic samples have been described by Parker and Kirk,[153] Jain et al.,[157] and Reith et al.[155]

Paper chromatography provides a useful means of purifying extracts but is not often used with fresh blood samples for this sole purpose. Its main use is in separating and identifying mixtures of barbiturates.

Kahn [69] used alumina column chromatography to purify chloroform extracts of mouse blood before ultra-violet spectrophotometric determination of barbiturate.

Urine

Since the metabolic degredation products of many barbiturates are excreted in the urine the extraction procedure must be known either to extract both metabolites and parent drug, or only unchanged barbiturate. Thus, chloroform extracts from acid urine principally the unchanged drug. This has been shown for pentobarbitone [41] and phenobarbitone,[2] whereas ether will extract some if not all of the metabolic material as well as the parent drug. Thiopentone and its carboxylic acid metabolite have been determined separately in urine by first extracting

the thiopentone with petroleum ether and then the metabolite with ethylene dichloride.[I69] Pentobarbitone metabolites have been extracted using amyl alcohol.[46]

The use of 'salting out' with ammonium sulphate has been shown to be an efficient method for allowing quantitative ether extraction of metabolites.[I24]

Zwikker [II0] described a technique in which barbiturate derivatives were separated from crude extracts by precipitation with copper salts and pyridine. Decomposition of the precipitated complex with acid allowed the isolation of barbiturate pure enough for a melting point determination.

The use of a bicarbonate wash to remove chromogens must be carefully considered since some metabolites have a high water solubility. Also carboxylic acid metabolites [44,I69] would be readily lost in such a wash.

Most extraction techniques described in the literature deal solely with the extraction of the parent barbiturate. All involve extraction of the acidified urine with a suitable organic solvent. Among the solvents which have been used are ether,[II9,I89] petroleum ether,[I69] and chloroform.[99] McCallum [I7I] used a technique adapted from that which he used for blood samples with chloroform as the extracting agent.

With urine, organic solvent extraction often results in emulsion formation. This can be overcome by centrifugation or by

the addition of large amounts of ammonium sulphate,[II9] but the use of a wetting agent has been advised as a more convenient method.[225] Mechanical rolling instead of shaking also prevents emulsion formation.

Natural colouring material in urine hinders analytical investigations and various techniques have been used to overcome this problem. For coloured or concentrated urines Koppanyi et al.,[35,99] advocated copper hydroxide precipitation as a means of removing colouring matter. The use of Lloyd's reagent (a purified siliceous earth) has been claimed highly efficient and does not remove barbiturate material.[226] Merley [225] used charcoal decolourisation after ether extraction of the barbiturates. Silica gel,[I89] celite,[I35] magnesium oxide,[222,227] alumina [223,228] column chromatography have all been used as a means of purification.

In the present investigation on the fate of pento-barbitone and phenobarbitone in the horse use was made of many of the methods indicated in the foregoing review. The principal techniques used were paper chromatography, ultra-violet spectrophotometry, complexometry and gas chromatography. Extracting solvents used were ether, chloroform and benzene with either ammonium sulphate or mechanical rolling being used, when necessary, to prevent emulsion formation.

METHODS

Analytical

(a) Blood analysis Whole blood samples were analysed for barbiturate as soon as possible after collection by the method of Goldbaum,[33] modified by Brackett and Finkle,[229] which is as follows:

Reagents :- Chloroform (M & B analytical reagent or B.D.H. AnalaR).

0.45N sodium hydroxide (B.D.H. AnalaR) prepared by dissolving I.8 g in I00 ml distilled water.

Ethylamine buffer prepared from 32.8 g ethylamine hydrochloride (B.D.H. laboratory reagent), I0.6 g ammonium chloride (B.D.H. AnalaR) and distilled water to make I00 ml. 0.5 ml of this solution when added to 3.0 ml of 0.45N sodium hydroxide should produce a pH of I0.3 to I0.6.

Spectrophotometer :- Unicam SP.500.

Since a sample blank was always available, the chloroform used for extracting the blood was not further purified. However, for any one series of samples the same batch of chloroform was used to counteract any difference in the properties of different batches.

Samples of blood were analysed in duplicate, using 6 ml portions of whole blood. Each portion was shaken, in a

separatory funnel, for three minutes with 50 ml of chloroform. The mixture was then filtered through Whatman No. 4 paper which was found to give a more rapid filtration than the corresponding No. I paper. 40 ml of the filtrate, measured in a 50 ml measuring cylinder as was the original 50 ml of chloroform, was then added to 5 ml of 0.45N sodium hydroxide and shaken for three minutes. After allowing the layers to separate, the alkaline layer was separated off and centrifuged to remove droplets of chloroform. 3 ml of the alkaline solution were transferred to I0 mm silica cuvette and the optical density of the solution measured at 260 mμ against 0.45N sodium hydroxide as a reference solution. 0.5 ml of ethylamine buffer was then added to both solutions and the new optical density measured after thorough stirring to ensure homogeneity.

The barbiturate concentration was calculated from the figure obtained by subtracting from the first optical density reading the second, after multiplying the latter by 7/6 to account for dilution by the buffer. By comparing this difference value (ΔOD) with those obtained from a series of standard barbiturate solutions in 0.45N sodium hydroxide a measure of the barbiturate concentration in the blood was found. The true concentration was determined by adding known amounts of the appropriate barbiturate to blood and analysing the sample as described previously. This allowed the recovery of the

extraction process to be calculated and the suitable factor to be applied to the result of the unknown sample.

In this method an 'average' 5,5-disubstituted barbiturate gave an optical density difference of about 0.02 at a concentration of I μg/ml in the 0.45N sodium hydroxide solution. From this it was obvious that, if the actual barbiturate was unknown, the blood barbiturate concentration was given by the following formula which assumes a 100% extraction process -

$$\text{concentration} = \frac{I}{0.02} \cdot \Delta OD \cdot 5 \cdot \frac{50}{40} \cdot \frac{I}{6} \ \mu g \text{ barbiturate / ml}$$

However, using standard solutions of phenobarbitone and pentobarbitone and applying the appropriate extraction recovery factor, a graph was constructed which allowed the blood barbiturate concentration to be found directly from the optical density difference value without performing a calculation each time.

The final figure, taken as the value for the blood barbiturate concentration, was the mean of the duplicate analysis less the blank value obtained by analysing a sample of normal blood taken just before drug administration. All analytical results were quoted in terms of the free acid form of the barbiturate per ml of whole blood, and it was assumed that only the parent barbiturates and not metabolites were being determined.

The period of time, from the alkaline extraction of the chloroform solution of the barbiturates until the optical density measurements were made, had to be kept to a minimum to prevent the formation of carbon monoxide bubbles [172] which interfered with the optical density determinations.

(b) Saliva analysis The saliva samples were analysed in a similar way to that described for blood samples, only the method used for extracting the samples differed. A saliva sample was rolled mechanically, at a rate of approximately one revolution per second, with an equal volume of ether for 15 minutes in a 500 ml stoppered bottle. This procedure overcame the tendency of the ether and saliva to form an emulsion. The ether layer was then separated from the saliva which was extracted a further time with an equal volume of fresh ether. On the completion of the second extraction the two ether extracts were combined, centrifuged to remove any emulsified saliva, and extracted with 10 ml of 0.45N sodium hydroxide by shaking for 3 minutes. The aqueous layer was then separated off, centrifuged if necessary to break up any emulsion, and 3 ml pipetted into a 10 mm silica cuvette ready for ultra-violet absorption analysis as described for blood samples. The concentration of the barbiturate in the original saliva was readily calculated from the optical density difference value in a fashion similar to

that described for blood samples. The true concentration was calculated by applying a recovery factor for the incompleteness of the extraction process which was found by analysing samples of saliva containing known amounts of the barbiturate.

The ether used was a technical grade (MacFarlan, Smith Ltd., B.S.S. 579) and was not further purified. As different batches of ether gave small, but significant, differences in the value for apparent barbiturate in normal saliva, a sample of normal saliva was always analysed at the start of a series of analyses to establish the value of this blank. As the same batch of ether was always used for the analysis of a series of samples, this blank remained constant for each sample in the series.

(c) Determination of protein binding The method used to determine barbiturate plasma and saliva protein binding was essentially that of Goldstein and Aronow.[89] 0.5 ml of a standard solution of barbiturate was added to I5 ml of plasma or saliva to give a barbiturate concentration of about 9 µg/ml. The resulting solution was placed in a 'Visking' dialysis bag and subjected to ultrafiltration for 5 hours at 37° as described by Goldstein and Aronow. I5 ml of normal plasma or saliva was carried through the same procedure. A 5 ml portion of each ultrafiltrate, after acidification with 0.5 ml of 2N hydrochloric acid, was then analysed for barbiturate using the method

already described for the analysis of whole blood. The difference between the two values obtained was taken as a measure of the ultrafiltered barbiturate. This process was repeated several times and the mean value for the apparent ultrafiltered barbiturate calculated (A%).

To determine the efficiency of the analytical technique, and also to overcome any error introduced by decomposition of the barbiturate during ultrafiltration, the above determinations were repeated using pH 7.4 phosphate buffer in place of plasma or saliva. The mean value of apparent ultrafiltered barbiturate from several determinations was again calculated (B%).

The final figure for the degree of plasma or saliva protein binding, which was the value of (B - A)%, was the percentage of barbiturate retained within the dialysis bag by virtue of its binding to protein molecules.

(d) Extraction techniques for urine The method used for extracting barbiturate material from urine depended on what was being sought. In all cases the urine was first subjected to the clean-up procedure described by Green et al.[226] The urine was acidified with one-quarter of its volume of 5% v/v sulphuric acid and shaken with Lloyd's reagent (Fuller's earth for adsorption purposes; B.D.H.), using 2 g per 25 ml of acidified urine, for 10 minutes. The mixture was then filtered

and the filtrate used for extraction.

For the extraction of phenobarbitone the filtrate was first saturated with ammonium sulphate, to prevent emulsion formation, and then extracted with benzene. The phenobarbitone metabolite, which was isolated, was not extracted by this procedure but, if present, could then be extracted with ether. The benzene extract of phenobarbitone was normally shaken with dilute sodium hydroxide to remove the phenobarbitone from the benzene. After acidification of the alkaline solution, the phenobarbitone was extracted from it using ether. This latter stage left behind some of the water soluble material which had been 'salted-out' of the original urine by the ammonium sulphate. The ether extract was then dried over a mixture of sodium sulphate and sodium bicarbonate (2:1 by bulk) and finally evaporated to dryness. The use of solid sodium bicarbonate was found to be superior to a buffer wash. There was no loss of barbiturate and no risk of pH changes incurred with the bicarbonate buffer wash.[223] The same process was used to dry the ether extract of the phenobarbitone metabolite before its evaporation to dryness.

For pentobarbitone metabolites all extractions required saturation of the acidified urine with ammonium sulphate, to 'salt-out' the metabolites, followed by ether extraction. Neither buffer washes nor the solid sodium bicarbonate treatment

could be used with these ether extracts as one of the metabolites was acidic in nature and was only readily extracted from acid solution by ether.

(e) Paper chromatography of urine extracts The principal paper chromatographic system used was the n-butanol/ammonia (0.880) system (BuAm) described by Jackson [II9] but with Whatman No. 3MM paper. Qualitative chromatograms were normally run with a descending solvent, and large scale separations with an ascending solvent purely as a matter of convenience. Other solvent systems used were amyl alcohol/ammonia (0.880) [I24] (AmAm) and the isopropyl alcohol/ammonia solvent (IPrAm) described by Smith [230] for phenolic acids and which was found to be suitable for metabolites. Chromatograms were usually developed overnight.

Barbiturates were detected on chromatograms by ultra-violet light [II9] (Hanovia "Chromatolite"), by the cobalt chloride/ammonia reagent [II9] or by eluting the suspected areas with 0.45N sodium hydroxide and subsequently recording the ultra-violet absorption spectra of the eluted material at different pH values.

(f) Quantitative determination of phenobarbitone in urine

As the concentration of phenobarbitone in urine was low (maximum concentration about I2 µg/ml) its determination, using

ultra-violet spectrophotometry, proved impossible due to the presence, in the extracts, of interfering urinary constituents. These interfering compounds were not easily removed by the use of different extracting solvents, buffer washes, charcoaling or by paper chromatography. For this reason the following procedure was adopted which is a combination of the Lloyd's reagent [226] purification which has been described and the complexometric method of Lubran.[105]

10 ml portions of the filtrate from the Lloyd's reagent purification (at least 2) were saturated with ammonium sulphate (M & B, analytical reagent) and extracted with 50 ml of benzene (B.D.H. AnalaR) by shaking for 3 minutes. The resultant mixture was centrifuged to give a good separation of the two phases, and the aqueous layer discarded. The benzene was then extracted with 10 ml of 0.45N sodium hydroxide and the aqueous layer, after separation, acidified with 6 ml of 2N hydrochloric acid. The aqueous solution was then extracted with two 25 ml portions of ether (MacFarlan, Smith Ltd., B.S.S. 579) and the combined ether extracts dried over a suitable quantity of sodium sulphate/ sodium bicarbonate (both B.D.H. AnalaR)(the amount of this mixed reagent did not appear to matter as long as sufficient was present to dry the ether). The ether was then reduced in bulk and finally evaporated to dryness in a 30 ml stoppered test-tube on a boiling water bath. A jet of air was used to prevent the

ether from frothing out of the tube.

To the residue were added 3 ml of chloroform (B.D.H. AnalaR), Iml of 0.0I% w/v diphenylcarbazone (B.D.H. laboratory reagent) in chloroform (B.D.H. AnalaR) and 0.5 ml of mercuric sulphate solution (5 g mercuric oxide, B.D.H. AnalaR, in 20 ml of concentrated sulphuric acid, B.D.H. AnalaR, and water to 250 ml). After immediate shaking the phases were separated by centrifugation and the optical density of the chloroform layer determined as soon as possible at 550 mμ against a reference blank prepared by using chloroform in place of the chloroform solution of the urine extract. Optical density measurements were made on a Unicam SP.500.

(g) Quantitative determination of pentobarbitone metabolite A in urine The procedure used to determine metabolite A was similar to that already described for phenobarbitone. I0 ml portions of the filtrate, obtained from the Lloyd's reagent purification, were saturated with ammonium sulphate and extracted with two 25 ml portions of ether. The combined ether extracts were dried over the sodium sulphate/sodium bicarbonate mixture which also served to remove metabolite B. The dried ether was then evaporated to dryness and the residue dissolved in 3 ml of ethyl acetate (M & B, analytical Grade) since the metabolic material had only limited solubility

in chloroform. The ethyl acetate solution was then filtered through a small plug of cotton wool and I ml of the filtrate used for the colour reaction which involved adding to it 2 ml of chloroform, I ml of the diphenylcarbazone reagent and 0.5 ml of the mercuric sulphate reagent as described previously. The optical density of the organic layer was then recorded at 550 mμ in a I0 mm cuvette against a corresponding reagent blank.

(h) Gas-liquid chromatography of pentobarbitone and its metabolite A The column and packing used were as described by Jain et al.,[157] and the conditions used were as follows;

	Pentobarbitone	Metabolite A
Chromatograph	Aerograph 600-C	
Recorder	0 to I0 mv Honeywell	0 to I mv
Oven temperature	I75°	222°
Injector temperature	220°	240°
Detector	flame ionisation	electron capture
Nitrogen carrier gas	inlet pressure I0 lb/sq.in.	40 ml/min.
Hydrogen	Inlet pressure 25 lb/sq.in.	-
Sample volume	I to 2 μl	

Oxygen was not used to replace the air supply for the flame ionisation detector as recommended by Jain et al.[157]

100 ml of urine were extracted by mechanically rolling with an equal volume of ether which, after separation from the aqueous layer, was washed with 25 ml of a pH 8 buffer (5% sodium bicarbonate saturated with sodium chloride). After drying over sodium sulphate, the ether was evaporated to dryness and the residue taken up in 200 ul of ethanol. Portions of this solution were then injected into the chromatograph.

With metabolite A, the pure compound was dissolved in anhydrous methanol and portions of the solution injected into the chromatograph.

(i) Partition coefficient of phenobarbitone The partition coefficient of material thought to be phenobarbitone in urine, between a pH 7 phosphate buffer and benzene, was determined as follows; six portions of urine containing suspected phenobarbitone were subjected to the quantitative determination. Two completed the process normally. Four were stopped before the addition of reactants to the solid residue from the extraction procedure. Of the four residues, two were shaken with 10 ml of pH 7 phosphate buffer and 10 ml of benzene, and two were shaken with 5 ml buffer and 15 ml benzene. In each case 6 ml of the benzene layer was then evaporated to dryness

and the determination repeated normally on the residue. From the resultant optical densities the partition coefficient of the barbiturate material was calculated.

(j) β-glucuronidase hydrolysis To determine if the metabolite of phenobarbitone, detected in horse urine, was conjugated with glucuronic acid the following procedure, based on that described by Butler,[6] was used. A sample of the urine was adjusted to pH 4.5 with acetic acid and extracted with an equal volume of ether. Air was then bubbled through the separated aqueous layer to remove dissolved ether. To 10 ml of the extracted urine were added 10,000 Fishman units of β-glucuronidase (Sigma, bovine liver type B-3) and the mixture was incubated at 37° for 48 hours. The urine was then saturated with ammonium sulphate and extracted with 50 ml of ether. The ether in turn was extracted with 10 ml of dilute sodium hydroxide and the separated alkaline layer acidified with dilute hydrochloric acid. After two extractions with 50 ml of benzene, the acidified aqueous solution was extracted with 50 ml of ether and the ether dried over sodium sulphate/sodium bicarbonate mixture containing a little decolourising charcoal. The ether was then evaporated and the residue paper chromatographed in the BuAm solvent system. It was assumed that there would be sufficient phosphate present in the urine to inhibit any

sulphatase present in the β-glucuronidase.

To determine if pentobarbitone metabolite A was conjugated with glucuronic acid, the following procedure was adopted. Two 10 ml portions of the purified urine were analysed as described previously for metabolite A. A further two 10 ml portions were made slightly alkaline by the dropwise addition of 5N sodium hydroxide. The pH was then adjusted to 4.5 with 2N acetic acid and 10,000 Fishmann units of β-glucuronidase added to each portion before incubating at 37° for 48 hours. The incubated urines were then acidified with dilute hydrochloric acid and analysed for the metabolite as normal. The difference between the two analytical figures for hydrolysed and non-hydrolysed urine was a measure of the amount of conjugated A.

(k) Infrared analysis The infrared spectra of phenobarbitone, pentobarbitone and their metabolites were recorded on a Unicam SP.200 spectrophotometer using the potassium bromide disc technique.

Biological

(a) Animals Six ponies were used and ranged in weight from 140 to 308 kg. Two ponies were stallions (VI and VII), three were geldings (II, III, and V) and one was a mare (IV). Two of the ponies (II and III) had permanent parotid fistulae

details concerning which have been published by Alexander.[231]

(b) Drug administration

(I) Blood clearance experiments :- It was found that the maximum amount of sodium pentobarbitone able to be given to a pony, without incurring the risk of respiratory arrest, was 10 mg/kg. This was the dosage value used in all blood clearance experiments with pentobarbitone. The appropriate quantity of sodium pentobarbitone (M & B) was dissolved in 20 ml of water, just before administration, and injected rapidly, i.e., in about 15 seconds, into a jugular vein the skin around which had previously been treated with a local anaesthetic. After about a further 15 seconds the pony involved fell to the ground unconscious. The time required for the animal to regain consciousness varied from a few minutes to more than a quarter of an hour, but this time was not accurately determined. As with certain ponies the return to consciousness resulted in the animal going into an excitement phase it was necessary, in some cases, to apply a minimum form of restraint which took the form of light halothane or chloroform anaesthesia until this phase passed. The pony was then allowed to recover normally and was usually able to stand unaided about one hour after the original injection.

Although the ponies were found to be less sensitive to phenobarbitone, compared to pentobarbitone on a mg/kg basis,it

was considered unwise to give sufficient phenobarbitone to cause the ponies to lose consciousness because the clearance rate of the drug was much slower than with pentobarbitone. Consequently the risk of complications arising while the ponies were unconscious was greater as the period of unconsciousness would, in all probability be much longer than with pentobarbitone. The dose rate finally decided on was 8 mg/kg of sodium phenobarbitone (B.D.H.) given by stomach-tube. Apart from slight incoordination during the first hour the ponies appeared to suffer no serious effects under these conditions.

(2) Saliva clearance experiments :- A limit was placed on the amount of the barbiturates able to be given to the ponies by the fact that the animals after dosage had to remain conscious and able to eat readily so as to produce saliva. As the barbiturates were secreted in low concentrations in the saliva it was desired that dosage values should be such that the resulting concentrations were sufficiently high to be readily measurable. With pentobarbitone the optimum dose was found to be 4 mg/kg given by intravenous injection. This route of administration was chosen to allow comparison of the results with those obtained for blood clearance rates in which this form of administration was used. Results were obtained also following oral administration of the drug. The lesser hypnotic activity of phenobarbitone allowed a range of dosage values to be used.

Those used were 3.7, 4.4, 6.2 and 6.6 mg/kg, given by stomach-tube to allow comparison of the results with those obtained for blood clearance rates. All dosage values are as mg/kg of the sodium salt of the appropriate barbiturate.

(3) Urine excretion experiments :- Sodium pentobarbitone was administered by stomach-tube. In the excretion rate determinations the dose used was 5 mg/kg. For the experiments involving the isolation of pentobarbitone metabolites the normal stomach-tube technique was modified to allow frequent dosage. A fine polythene tube was used, onto the end of which had been fused a bulb prepared from a tube of slightly larger bore (see Figure I). The bulbous end of the tube was passed into the pony's stomach as usual and the other end made secure by fastening it to a head-stall. The bulbous end was found to retain the stomach-tube in position for as long as required. Sodium pentobarbitone was then dissolved in a small volume of water and the solution pumped down the stomach-tube with a syringe. This solution was followed by sufficient water to ensure complete passage of the drug down the tube. When this method was used an initial dose of 4.4 mg/kg was given followed by hourly supplementary doses of 2.2 mg/kg. All solutions were prepared immediately prior to use.

Sodium phenobarbitone was administered by normal stomach-tube at various dosage rates. 6 mg/kg was used in the phenobarbitone urinary excretion rate determinations. Higher doses

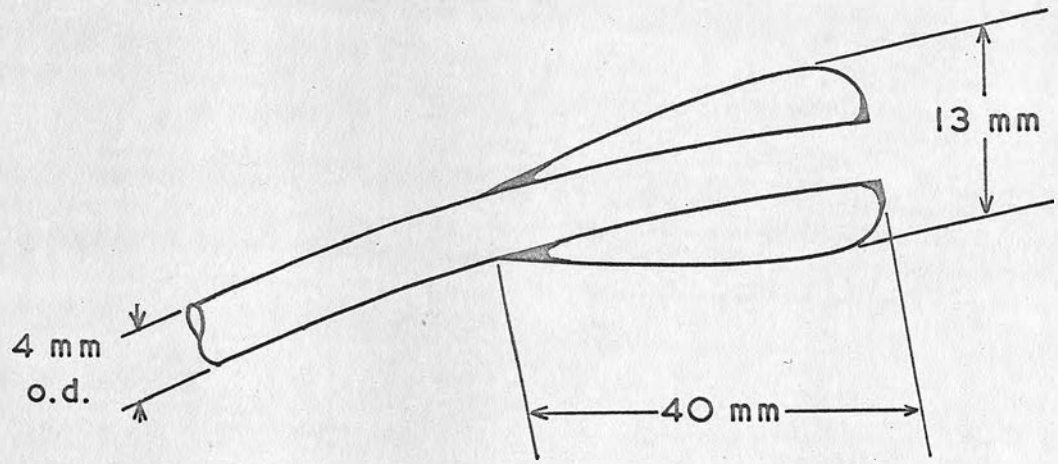

Figure I. Modified stomach-tube. Not to scale.

were used in attempts to isolate the metabolite of phenobarbitone.

(c) Sample collection

(I) Blood :- In all cases I5 ml blood samples were collected in evacuated tubes containing lithium oxalate from a jugular vein. Before each experiment a sample of normal blood was taken to act as a reference blank. Subsequent samples were normally taken at I5, 45, 75, I20, I80 and 240 minutes after pentobarbitone administration, and at I, 2, 4 and 6.5 hours after phenobarbitone administration.

(2) Saliva :- Saliva was collected from a polythene cannula inserted into the parotid duct as described by Alexander.[23I] For the determination of pentobarbitone I00 ml samples of saliva were collected in I00 ml volumetric flasks. The samples were then acidified with 2N hydrochloric acid and set aside to await analysis. The interval between drug administration and the beginning of each sample collection, and the total time for collection were noted. The concentration of barbiturate found in the sample was taken as the concentration of the barbiturate in the saliva half way through the collection.

The higher concentrations of phenobarbitone in the saliva allowed the use of 50 ml samples of saliva to determine the barbiturate's concentration. Otherwise the procedure was as described for pentobarbitone. With both barbiturates, a sample

of saliva was collected before drug administration and used to establish the 'blank' value for apparent barbiturate in the saliva.

The rate of saliva flow varied but it was usual to collect 50 ml of saliva in from 50 to 250 seconds. Slower flow rates were encountered infrequently. It was not uncommon for cessation of salivary flow to occur at any time during an experiment, when no amount of eating of hay or oats by the pony would restore flow. The flow of saliva normally recommenced after a time of an hour or more when samples were again collected. For this reason it was not always possible to collect samples when required, so that sample collection times were in general 'when convenient'.

(3) Urine :- Urine was collected using the apparatus of Warwick [232] which had been modified. The modification consisted of a paddle inserted through the tube at the base of the funnel. When urine flowed into the funnel and down this tube the paddle was tilted downwards, and in so doing closed an electrical circiut containing a relay operating a pointer. As this pointer touched a smoked drum which rotated once every 24-hours a permanent record was obtained of the times at which urine samples were voided. This allowed urine samples of known age to be obtained easily.

During phenobarbitone excretion rate determinations the

urine voided at intervals over about 72 hours was collected. During this time the ponies were exercised usually just after after the passage of a urine sample.

During pentobarbitone metabolite A excretion rate determinations urine samples were collected and analysed as they were voided over about 24 hours after drug administration. Only Ponies VI and VII provided sufficient samples during the time of metabolite excretion to be of use in clearance rate determinations. The other ponies were used to provide samples for the determination of the total amount of metabolite excreted.

No urine preservative was used and the urine was analysed as soon as possible after collection.

RESULTS

Analytical

(a) Determination of barbiturates in blood

(I) Barbiturate blood blanks :- Since the method for analysing blood samples for barbiturate gave either a slight positive or a slight negative value for apparent barbiturate in normal blood samples, it was necessary to establish the value of this blank. For this reason values of the blank were investigated in various samples i.e., random blood samples; samples which had been taken within a short time of each other; Samples which had waited some time before analysis; blood samples whose chloroform extracts were allowed to stand for some time before completing the analysis.

From a series of blank determinations carried out on normal fresh blood samples randomly taken over several months a collection of 32 blank values was made and is presented in Table VII as µg/ml of apparent pentobarbitone and phenobarbitone in normal blood.

As can be seen from Table VIII the barbiturate blood blank was subject to considerable fluctuations. To determine if this variation was as great during a shorter period of time between sampling, two blood samples were taken from each of

three ponies at intervals of not greater than 24 hours. These samples were analysed immediately after they were taken and the results, given in Table IX, showed that blanks were capable of appreciable variation even within 24 hours.

Table VIII. Barbiturate blood blanks. Results from 32 normal blood samples.

	Apparent barbiturate (µg/ml)	S.D.
As pentobarbitone	-0.05	I.53
As phenobarbitone	-0.05	I.46

Table IX. Short time variation in barbiturate blood blanks.

Pony	Time of blood sampling (hours)	Blank (µg/ml of pentobarbitone)
IV	0	2.6
	6	0.4
II	0	I.8
	20	I.6
III	0	2.2
	20	-0.6

To establish what effect ageing had on the blank value sufficient fresh blood was collected to allow eight blank determinations to be made at intervals over two days. While awaiting analysis the blood was allowed to stand in a closed vessel at laboratory temperature. The results thus obtained are given in Table X. Again considerable fluctuations were observed in the value of the blank.

Table X. Effect of sample ageing on barbiturate blood blanks.

Age of sample	Blank (µg/ml of pentobarbitone)
10 min.	-1.5
1 hr. 10 min.	-1.0
2 hr. 40 min.	-0.6
4 hr. 40 min.	0.0
6 hr. 40 min.	+0.4
9 hr. 40 min.	-1.0
23 hr. 40 min.	-0.3
48 hr.	-0.6

In pentobarbitone blood clearance experiments insufficient time existed between the first few sampling times

to allow the analysis of a sample to be completed before the next had been collected. In consequence, this next sample had to stand for a short time before analysis. It was thought that if each analysis was carried only as far as the chloroform extraction stage each blood sample could be extracted with chloroform as it was taken, and the extract allowed to stand until completion of the sampling. Each analysis could then be carried to its conclusion and, if no change occurred in the chloroform extract on standing, the variation in the blood blank due to the sample standing before being analysed could be counteracted. However, as can be seen from Table XI, the blank value did change in the chloroform extract of a series of normal fresh blood samples so nullifying this postulation.

Table XI. Variation of the barbiturate blood blank with standing of the chloroform extract of blood.

Sample	Age of $CHCl_3$ extract (hours)	Blank (µg/ml of pentobarbitone)
I	0	I.4
I	5	I.9
I	22	-0.2
2	0	0.9
2	5	0.3
2	22	0.6

In most clearance experiments, which did not normally cover a period of more than 6.5 hours, it was found that the blank did not deviate sufficiently far from the value originally established, from the blood sample taken before drug administration, to prevent a clearance curve being plotted. An example of an instance in which the blood blank did alter appreciably during a clearance experiment is recorded in Figure II along with a normal clearance curve. The blank in this case had a value of -I.5 µg/ml so that any change in this value would be expected to be towards a more positive value and hence result in a false rise in the barbiturate concentration. Such a change was usually indicated in the analysis by a disproportionate change in one of the optical density readings, either at pH I3 or pH I0.4, relative to the value for previous samples.

(2) Qualitative aspects :- Since the ultra-violet absorption curves of barbiturates in solutions at pH I3 and I0.4 may be used to qualitatively identify barbiturates [I73] this aspect of blood analysis was investigated. The ultra-violet absorption spectra of several extracts of normal blood samples were recorded at pH I3 and I0.4 and the pairs of curves were found to be of similar shapes in all cases. A typical example is shown in Figure III. To establish how the presence of barbiturate affected these curves, the absorption curves of an

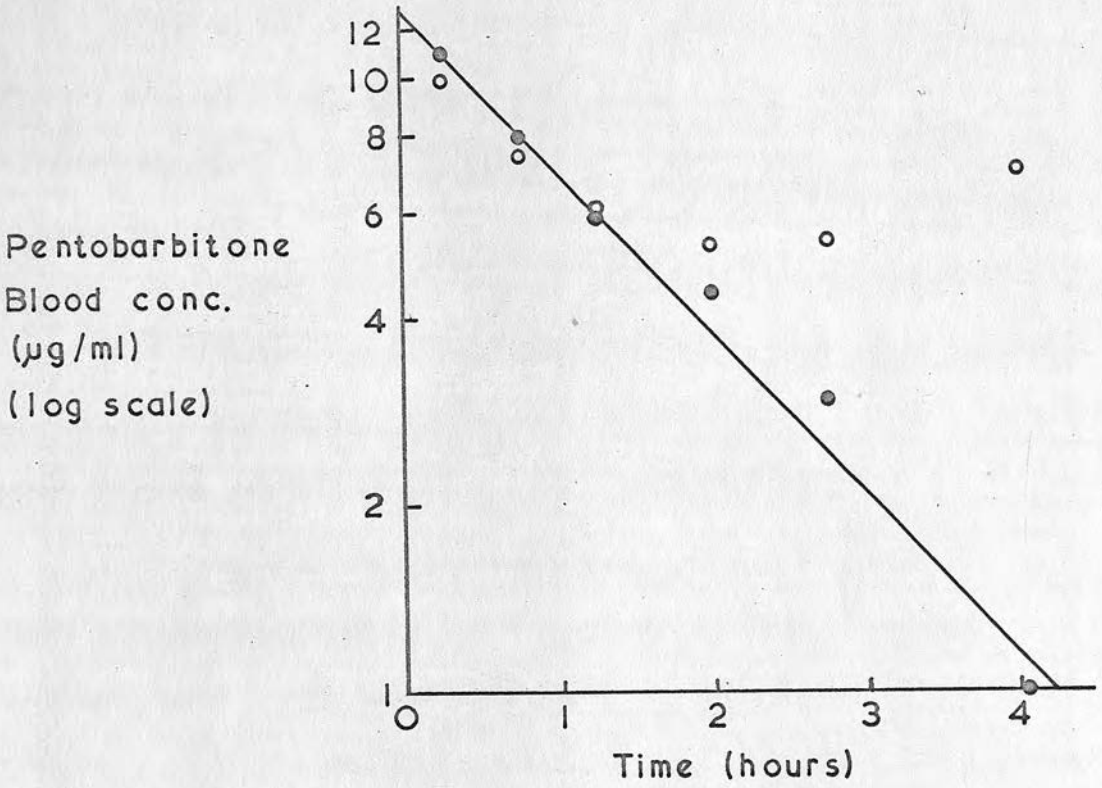

Figure II. Pentobarbitone blood clearance:
(●) normal
(o) affected by a change in the blood blank.
Pony I V.
Dose 10 mg/kg.

aqueous solution of pentobarbitone at pH 13 and pH 10.4, corresponding to an extract from blood containing 10 µg/ml, were superimposed on the curves from Figure III and the result is shown in Figure IV. As can be seen the typical barbiturate spectra are almost masked and could not be used with confidence as proof of the presence of barbiturate in a blood sample from an unknown source. Since barbiturate concentrations encountered in this investigation were, except soon after drug administration, lower than 10 µg/ml it must follow that the qualitative accuracy of the spectrophotometric analysis would be absent in all samples taken some time after drug administration.

(3) Recovery of the extraction process :- In order to apply a correction factor, to the figure obtained for the apparent blood barbiturate concentration at the end of the analysis, to take into account incomplete extraction and transfer losses of barbiturate material, the recovery of the whole extraction process was determined. Standard solutions of the two barbiturates were prepared and 0.5 ml portions added to 6 ml samples of blood which were then analysed normally. Two portions of treated blood and two portions of normal blood were analysed simultaneously. The mean of each pair of results was calculated and the difference between the two means taken as the barbiturate recovered. By comparing this figure with the amount actually added, the percentage recovery was calculated. The results

are quoted in Table XII. The actual concentration of barbiturate in the blood lay in the range 7.5 to 10 µg/ml.

Table XII. Recovery of pentobarbitone and phenobarbitone from blood.

Barbiturate	Recovery (%)	S.D.	Number of analysis
Pentobarbitone	95	6	9
Phenobarbitone	77	5	7

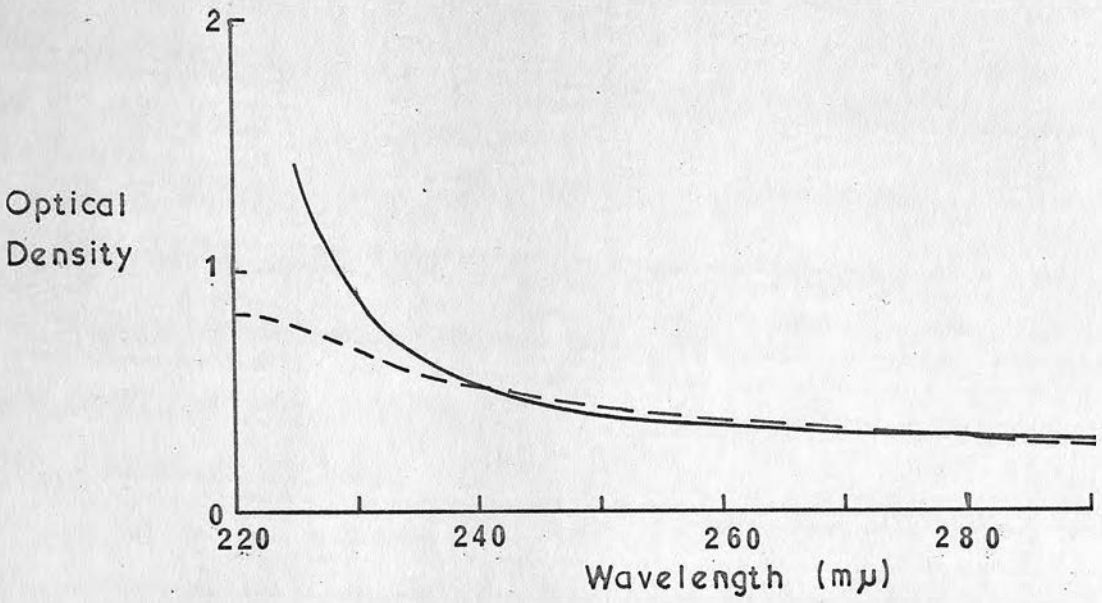

Figure III. Absorption spectra of a chloroform extract of normal blood at pH 13 (——) and pH 10.4 (— — — —).

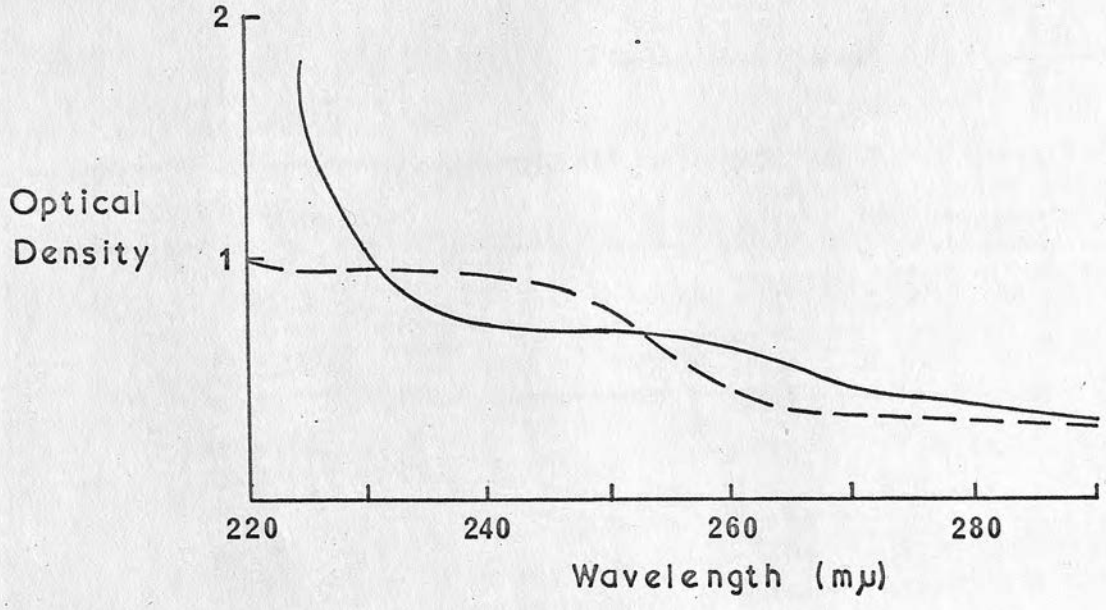

Figure IV. Absorption spectra of a chloroform extract of blood containing 10μg/ml of pentobarbitone at pH 13 (——) and pH 10.4 (— — — —).

(b) Determination of barbiturates in saliva

(I) Barbiturate saliva blanks :- As with the barbiturate blood blank it was necessary to investigate thoroughly the value of the corresponding saliva blank. The conditions under which the blank was investigated were in random samples and in samples collected at different flow rates. In both cases the same batch of ether was used to allow comparison of the individual results. I7 saliva samples were collected randomly from Pony III over a three day period and analysed as for phenobarbitone. The samples were divided into two groups which were, fast saliva flow (50 ml/45 seconds) and slow saliva flow (less than 50 ml/I25 seconds), and the results obtained are quoted in Table XIII.

Table XIII. Barbiturate saliva blanks for different saliva flow rates.

Saliva flow rate	Blank (µg/ml)*	S.D.	n
Slow	-0.09	0.09	8
Fast	-0.I0	0.I0	9

* As phenobarbitone

As can be seen from Table XIII there was no significant difference in the blank due to alterations in the saliva flow rate. Furthermore, the variation of the blank among the samples was low in comparison with blood blanks, and was therefore considered to be negligible.

To demonstrate the variation in the blank due to the use of different batches of ether nine saliva samples were collected on eight separate occasions and analysed for pentobarbitone. The samples were divided into three groups of 3, 5 and I, and each group analysed with a different batch of ether. The results are given in Table XIV.

Table XIV. Variation of barbiturate saliva blank with different batches of ether.

Sample group	Number in group	Blank (µg/ml)*	S.D.
I	3	0.0I	0.0I
2	5	0.I2	0.04
3	I	0.27	-

* As pentobarbitone

(2) Qualitative aspects :- The ultra-violet absorption spectra of a series of saliva sample extracts were recorded at pH 13 and pH 10.4 and were found to be of very similar shape of which a typical example is shown in Figure V(a).

To demonstrate the effect of the presence of barbiturate on the normal absorption spectra of the saliva extract the appropriate absorption curves for pure pentobarbitone, corresponding to a concentration in the saliva of 2 µg/ml, were superimposed on the curves in Figure V(a). The result is shown in Figure V(b). As with the corresponding extracts of blood, the natural chromogens distorted the characteristic barbiturate curves to the extent of destroying the qualitative properties of the analytical technique.

(3) Recovery of the extraction process :- To correct the figure obtained by the previously described analytical technique, for transfer losses and incomplete extraction, the recovery of the process was determined by adding known amounts of the two barbiturates to saliva samples and then analysing them. The results, expressed as a percentage of added barbiturate recovered in the final alkaline extract, are given in Table XV.

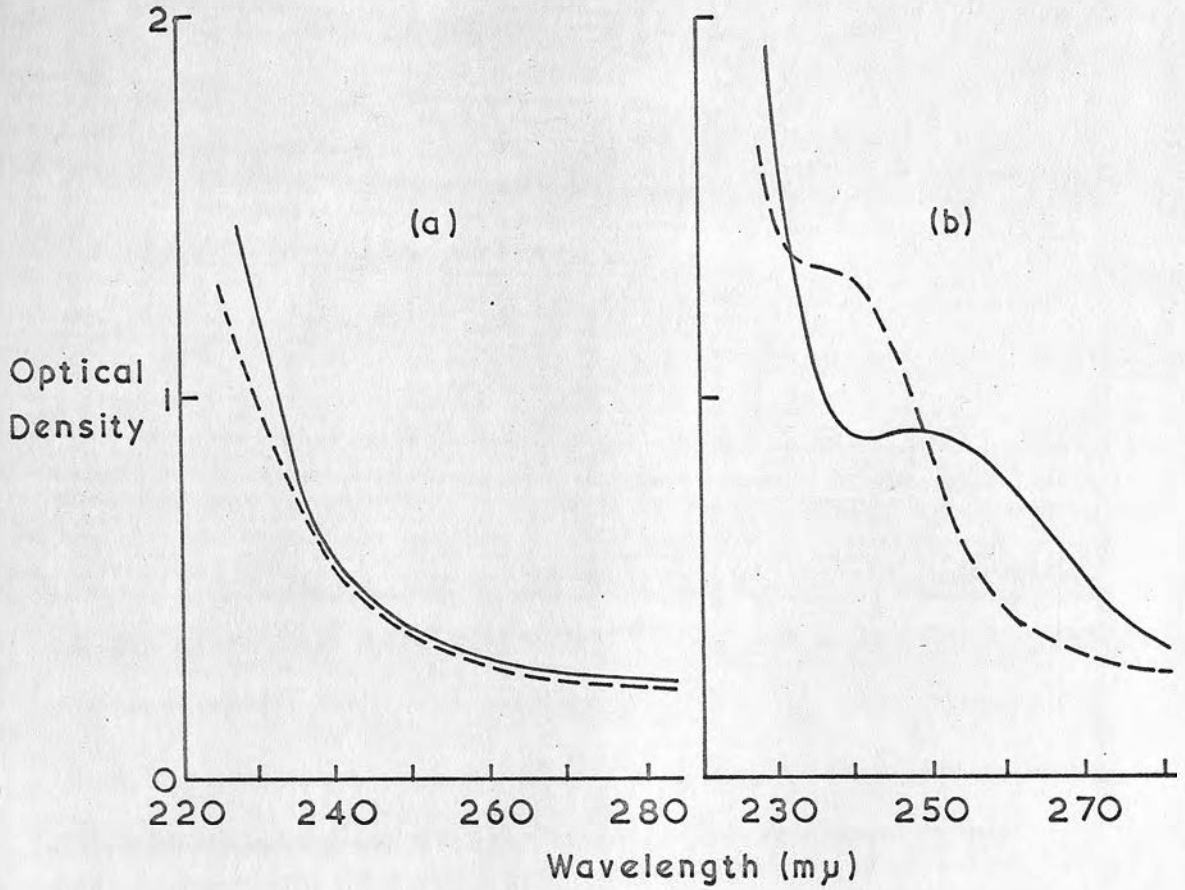

Figure V. Ultra-violet absorption spectra of an ether extract of saliva analysed as for pentobarbitone.
(a) Normal saliva.
(b) Saliva containing 2 μg/ml of pentobarbitone.
———— pH 13: — — — — — — pH 10.4.

Table XV. Recovery of phenobarbitone and pentobarbitone from saliva.

Barbiturate	Recovery (%)	S.D.	n
Phenobarbitone*	92	2.6	8
Pentobarbitone*	80	3.9	7

* Barbiturate concentrations were -

phenobarbitone 3.02 µg/ml

pentobarbitone 0.95 to I.03 µg/ml

(c) Protein binding determinations The efficiency of the analytical technique used to determine plasma and saliva protein binding was found by using pH 7.4 phosphate buffer in place of plasma or saliva but otherwise carrying out the method as described in the methods section. The recovery of the barbiturate, in the final alkaline solution, was expressed as a percentage of the original amount of barbiturate added to the buffer which was equivalent to a concentration of about 9 µg/ml of buffer. Results obtained by this method are given in Table XVI.

Table XVI. Recovery of barbiturate from pH 7.4 buffer in protein binding experiments.

Barbiturate	Recovery (%)	S.D.	n
Pentobarbitone	I07	8	5
Phenobarbitone	9I	3	5

(d) Determination of phenobarbitone in urine Before using the described analytical technique to determine excretion rates it was necessary to establish its limitations and accuracy. The aspects examined were urine 'blanks', recovery and the stability of the colour formed.

(I) Blank values of normal urine :- Ten separate urine samples obtained on different occasions were analysed identically and the value for the apparent barbiturate found. A mean value of 0.9 µg/ml (S.D. 0.3 µg/ml) was attributed to apparent phenobarbitone in normal urine when the appropriate recovery factor was applied.

(2) Recovery of phenobarbitone from urine :- Sodium phenobarbitone was added in known amounts to samples of normal urine and the treated urine analysed for phenobarbitone as well as the untreated urine. From the difference in the two sets of results the recovery of phenobarbitone was calculated. In seven

such recoveries a mean value of 70% of the added barbiturate was recovered (S.D. 4%, concentration range 4.49 to 4.83 µg/ml). In the absence of ammonium sulphate the recoveries were not precise and were therefore of no value.

(3) Stability of colour :- The colour formed by carrying out the quantitative reaction with reactants only or with added phenobarbitone was found to be stable for some considerable time as was described by Lubran. However it was observed that when urine extracts were used considerable fading of the colour occurred regardless of whether the extract contained barbiturate or not. It was in fact possible for the optical density value of the blank urine extract colour to fall below the corresponding blank value for the reagents, relative to water, as can be seen in Figure VI.

The error introduced by this fading, whose rate and degree varied with the urine sample, was minimised by carrying out the addition of the reagents and optical density measurements on a standardised time schedule.

(e) Determination of pentobarbitone metabolite A in urine

Before the analytical method was used to determine the urinary excretion rates and amounts of A the following factors were investigated; (a) The effect of ethyl acetate on the intensity of the colour developed in the complexometric

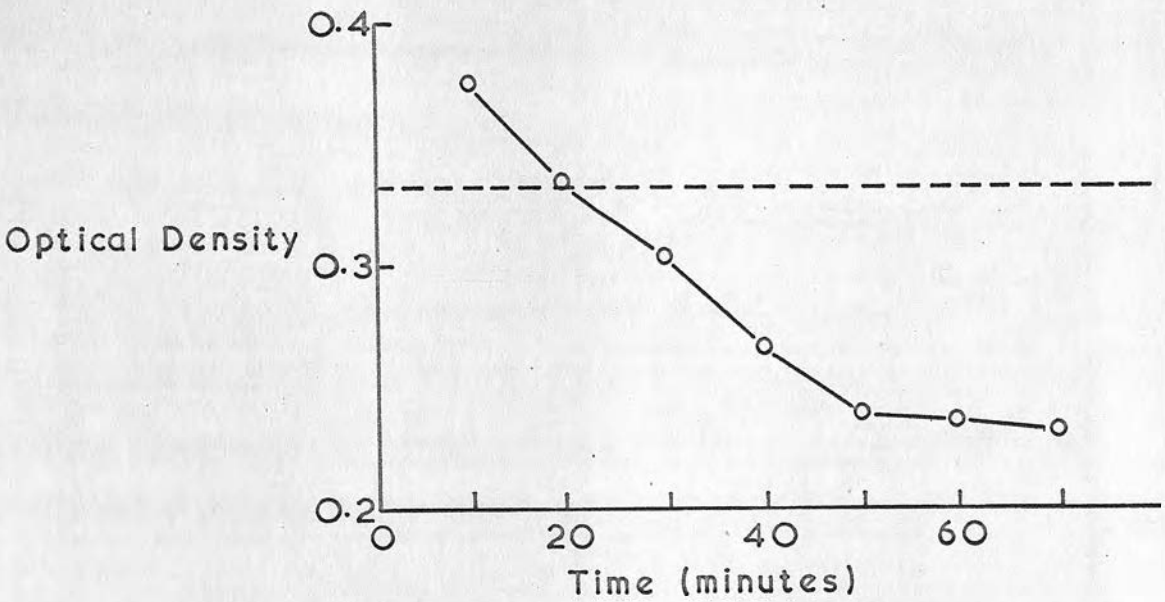

Figure VI. Fading of a urine blank in the quantitative determination of phenobarbitone.

– – – – – Reagent blank.

——————— Urine blank.

determination. (b) The concentration range over which a linear relationship existed between the optical density of the colour formed and the concentration of A. (c) The recovery of A from urine. (d) The normal urine blank values.

The presence of ethyl acetate significantly decreased the intensity of the colour developed in the normal Lubran method. To determine the degree to which the colour intensity was affected the reaction was carried out on several equal amounts of A, but with varying amounts of ethyl acetate present and replacing an equal volume of chloroform in the normal reagents used. A plot of optical density, relative to the appropriate blank, against the amount of ethyl acetate present is shown in Figure VII.

As was later found the range of urinary concentrations of A varied considerably and the use of chloroform alone would have resulted in very high optical density readings when A was excreted in high concentration. By using the conditions stated for the method, (i.e. equivalent to I ml of added ethyl acetate) readings ranged from 0.765 to 0.I00. For chloroform alone this would correspond to a range of I.6I6 to 0.2II plus a reagent blank of about 0.5 relative to water. This would mean that the optical densities obtained at high concentrations of A could not be read with the same degree of accuracy as could be achieved by decreasing the optical density using ethyl acetate.

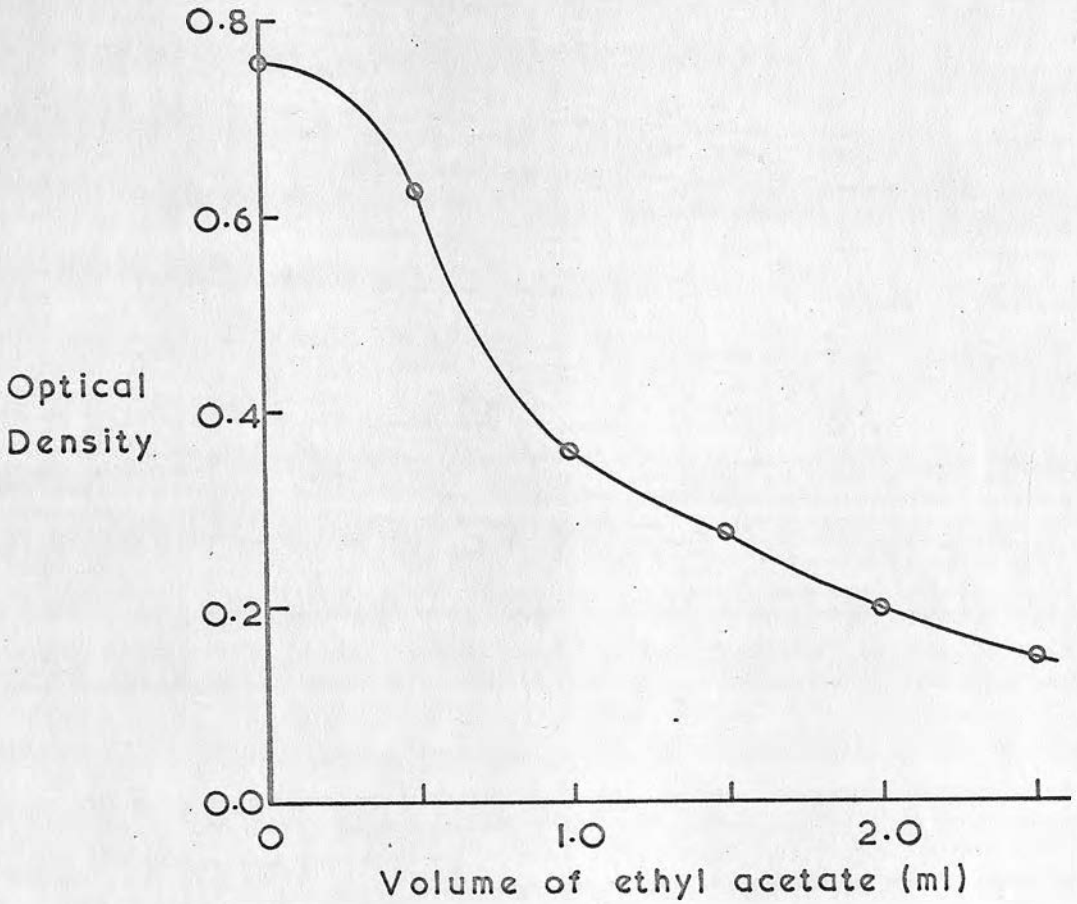

Figure VII. The effect of increasing amounts of ethyl acetate on the colour developed in the quantitative determination of pentobarbitone metabolite A.

The concentration range, expressed as µg of A present in the final test solution, overwhich a linear relationship existed with optical density was found. This range extended up to not less than 1500 µg. 500 µg was found to give an optical density of 0.476.

The recovery of the extraction procedure was determined by adding known amounts of A to urine and analysing both treated and untreated urine for barbiturate. From the difference in the two values the recovery of A was calculated. In eight such experiments a mean recovery of 63% (S.D. 3.5%) was achieved at a concentration of 115 µg/ml urine.

A small positive reaction was observed with normal urine to the analytical procedure. To determine the magnitude of this blank 17 separate urine samples were collected on different occasions and analysed. A mean value of 30 µg of A per ml of urine was obtained (S.D. 10 µg). The variation was sufficiently small to be of no great significance and the mean blank value was subtracted from all quantitative results.

Biological

(a) Blood clearance rates Individual clearance rates for pentobarbitone in six experiments on four ponies are quoted in Table XVII. The mean clearance rate was 46%/hour (S.D. 8%/hour). A mean clearance curve calculated from the six sets of

results is shown in Figure VIII.

As the individual clearance rate experiments with phenobarbitone did not appear to give a logarithmic clearance a mean clearance curve was constructed with the results from eleven experiments on five ponies (Figure IX). Calculation of the lines of best fit for all the experimental points and subsequent analysis of variance [233] clearly demonstrated that the clearance was not logarithmic. The calculated best fitting quadratic equation matched very closely the mean clearance curve shown in Figure IX. To pursue clearance determinations beyond 6.5 hours was considered pointless, since changing blanks and low barbiturate concentrations made accurate determination of the phenobarbitone concentrations impossible.

Figure XVII. Rate of barbiturate blood clearance from ponies receiving 10 mg/kg of sodium pentobarbitone.

Pony	Weight (Kg)	Type	Clearance Rate (%/hour)	Blood Barbiturate at t=0* (µg/ml)	Restraint
IV	308	mare	45	13.0	halothane
			39	11.0	halothane
V	140	gelding	61	11.0	chloroform
			44	11.0	chloroform
VII	144	stallion	46	11.8	very light $CHCl_3$
VI	140	stallion	39	15.5	none

* time after injection.

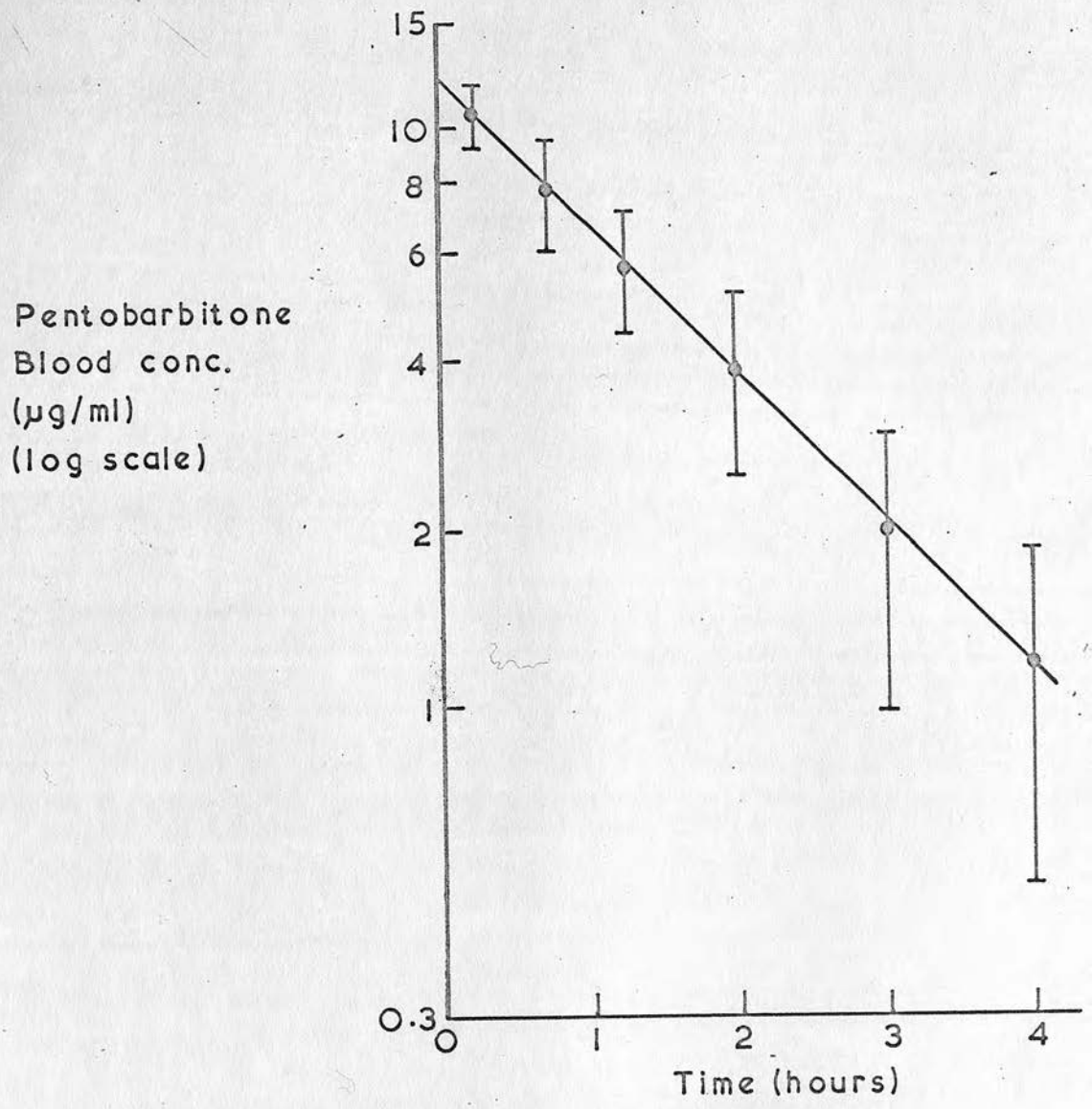

Figure VIII. Pentobarbitone blood clearance. Plot of mean values (±S.D.) obtained from six experiments on four ponies. Dose, 10 mg/kg of sodium pentobarbitone.

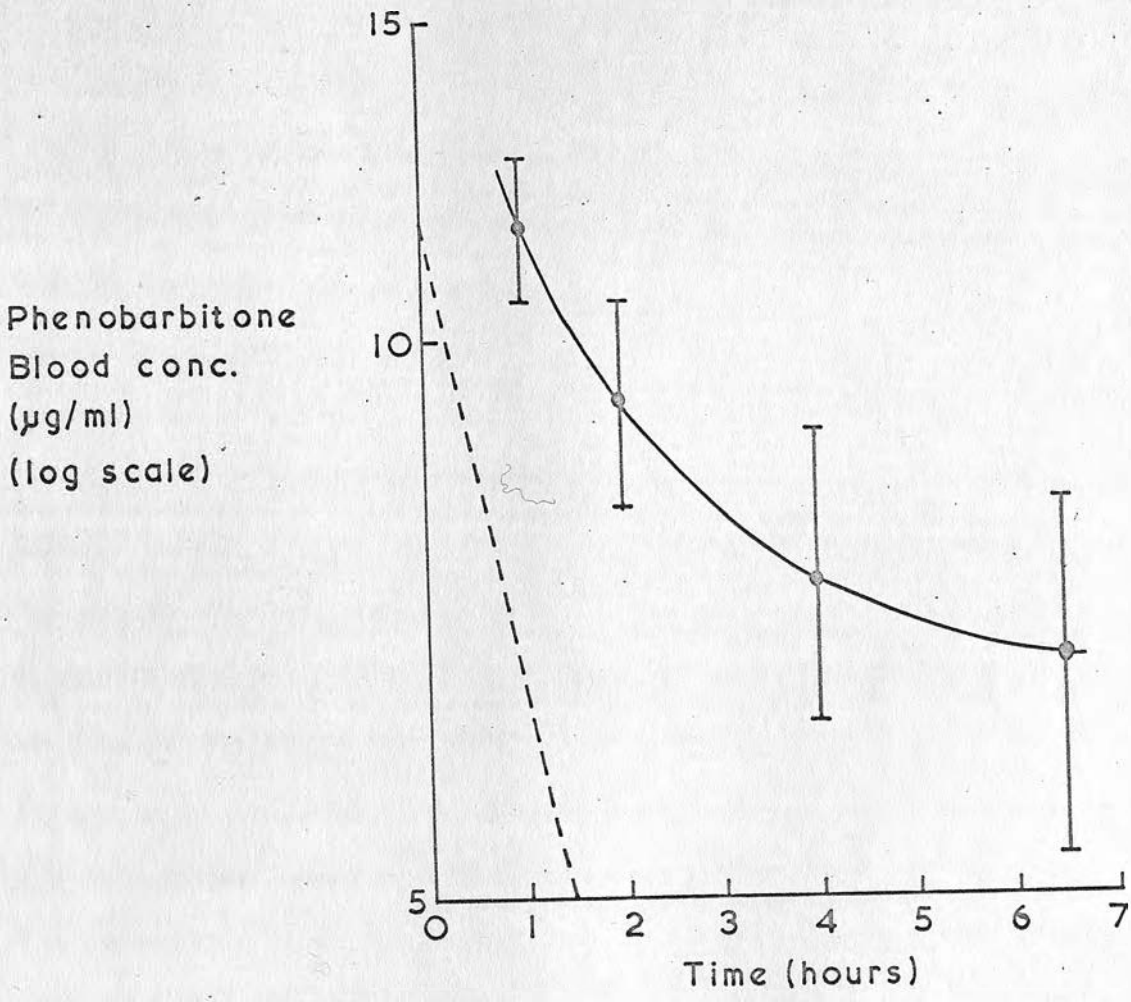

Figure IX. Phenobarbitone blood clearance. Plot of mean values (±S.D.) obtained from eleven experiments on five ponies. Dose, 8 mg/kg of sodium phenobarbitone.

---- Pentobarbitone clearance from Figure IV.

(b) Saliva clearance rates

(I) Phenobarbitone :- Three separate experiments were carried out at all but the lowest dosage rate with which four experiments were performed. Each experiment provided from 5 to 9 results for phenobarbitone-saliva concentrations during the period from I to 7 hours after dosing. It was found that the results from any single experiment were scattered to such an extent as to make any definite interpretation impossible. By grouping the results of each set of experiments, at the same dosage rate, together on a scatter diagram a general clearance pattern became obvious as can be seen from the results shown in Figures X, XI, XII, and XIII. In these plots it was convenient to divide the samples into three groups according to the rate of flow of saliva at the time of collection. The three groups chosen were (I) $<$I00 sec. (2) I00 to 200 sec. (3) $>$200 sec., the time given being that required to collect 50 ml of saliva. The calculated best straight line, in a plot of log(concentration) against time, was drawn through each set of points to allow an approximate value for the clearance rates in each case to be obtained.

Calculation of the lines of best fit for each set of results followed by analysis of variance [233] showed that the saliva clearances were definitely not logarithmic and that quadratic equations fitted the sets of points more favourably.

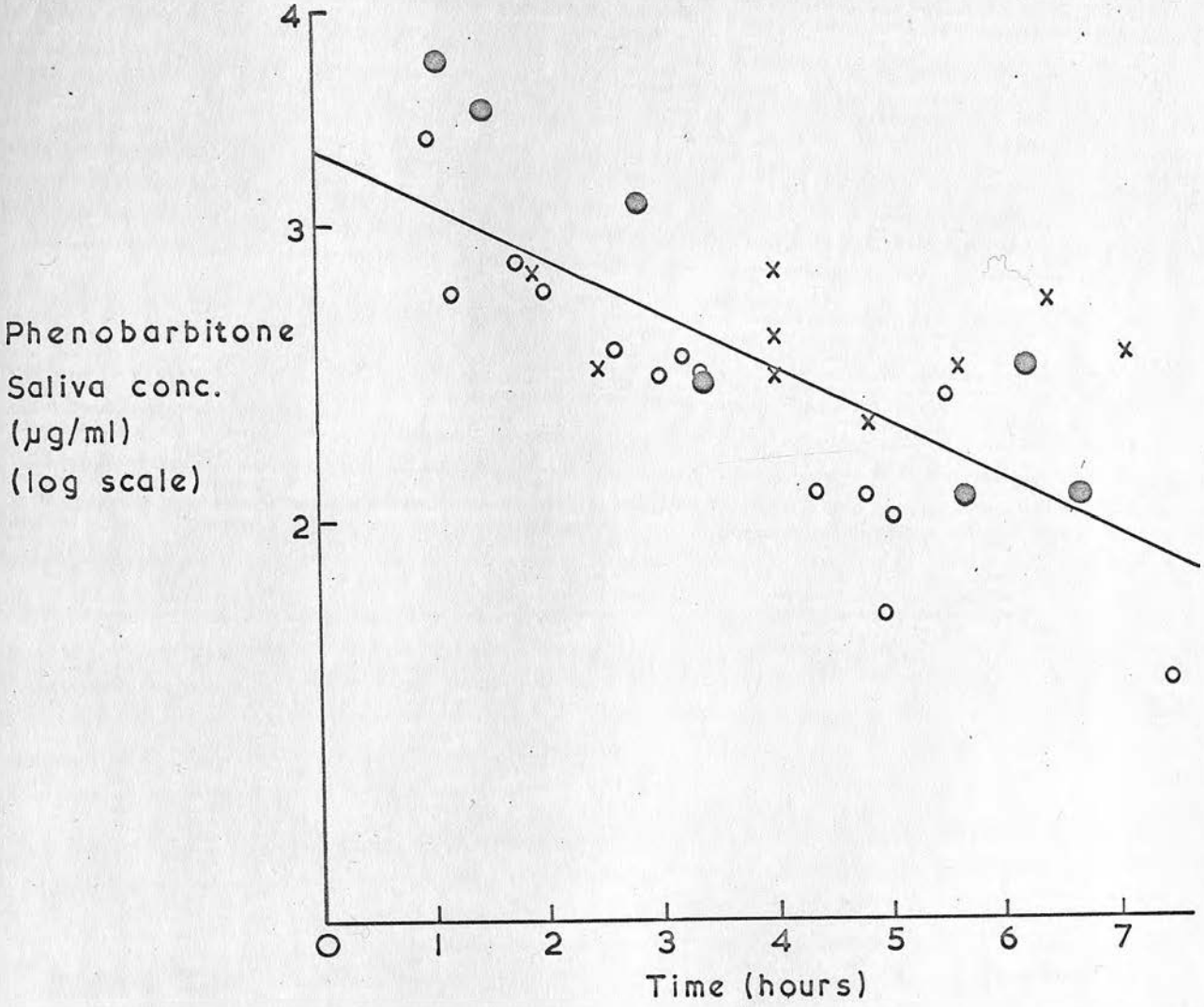

Figure X. Phenobarbitone saliva clearance. Results from four experiments on Pony III. Dose, 3.7 mg/kg of sodium phenobarbitone. Calculated clearance rate was 7.4%/hour. Saliva flow rates; (o) < 100, (x) 100 to 200 and (●) > 200 sec./50ml.

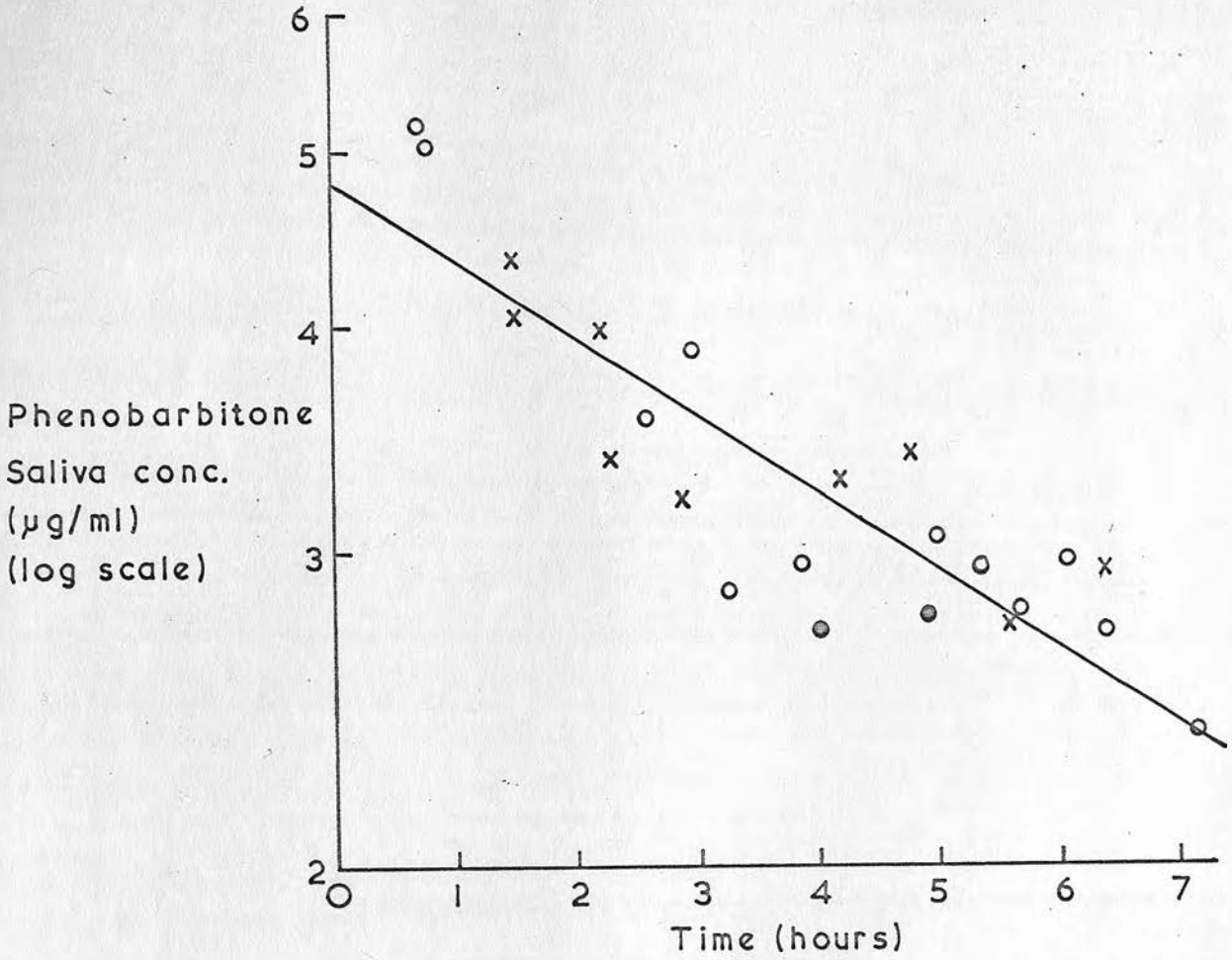

Figure XI. Phenobarbitone saliva clearance. Results from three experiments on Pony II. Dose, 4.4 mg/kg of sodium phenobarbitone. Calculated clearance rate was 9.4 %/hour. Saliva flow rates; (o) < 100, (x) 100 to 200 and (●) > 200 sec./50ml.

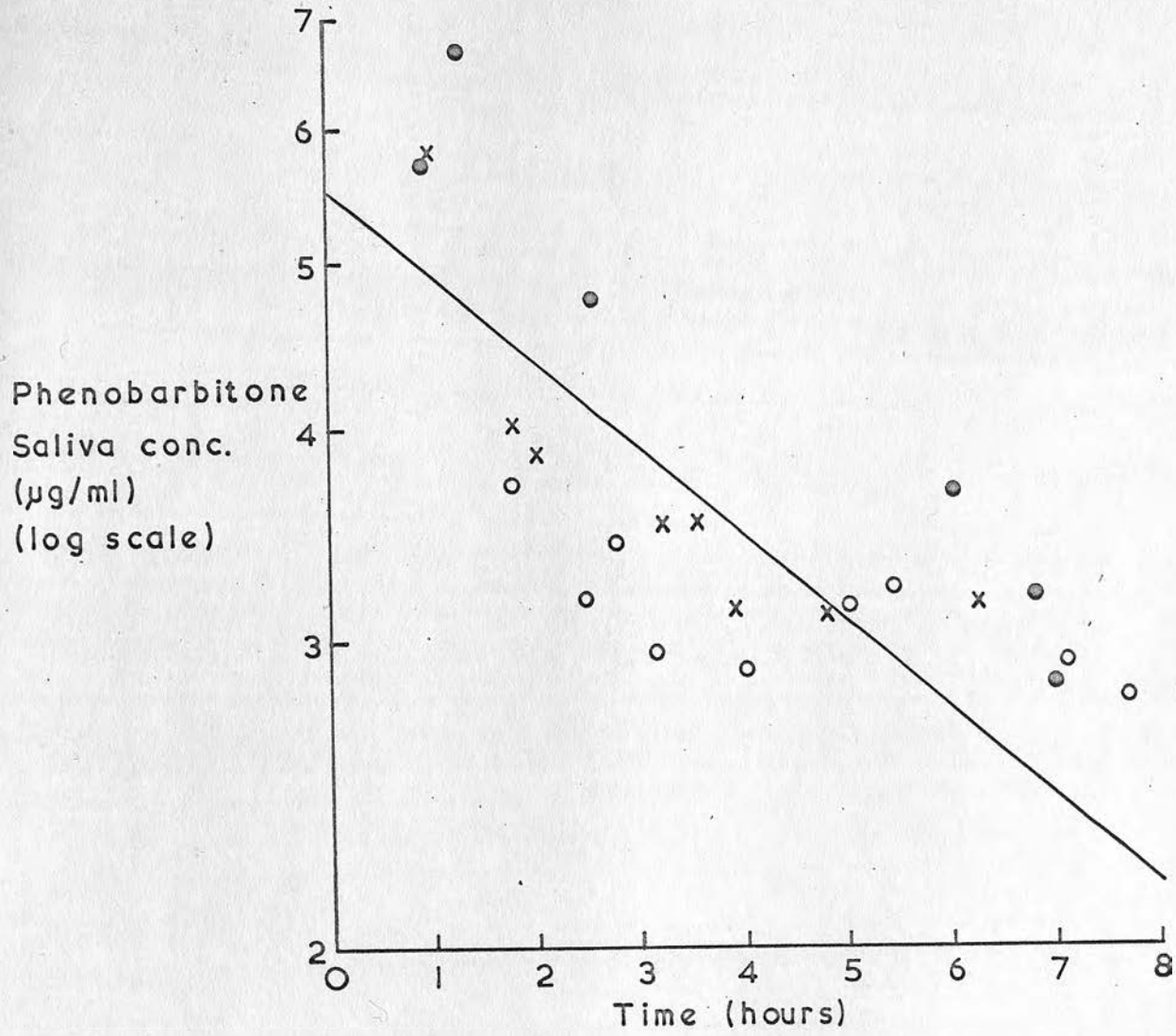

Figure XII. Phenobarbitone saliva clearance. Results from three experiments on Pony III. Dose, 6·2 mg/kg of sodium phenobarbitone. Calculated clearance rate was 14·8%/hour. Saliva flow rates; (o) < 100, (x) 100 to 200 and (●) > 200 sec./50ml.

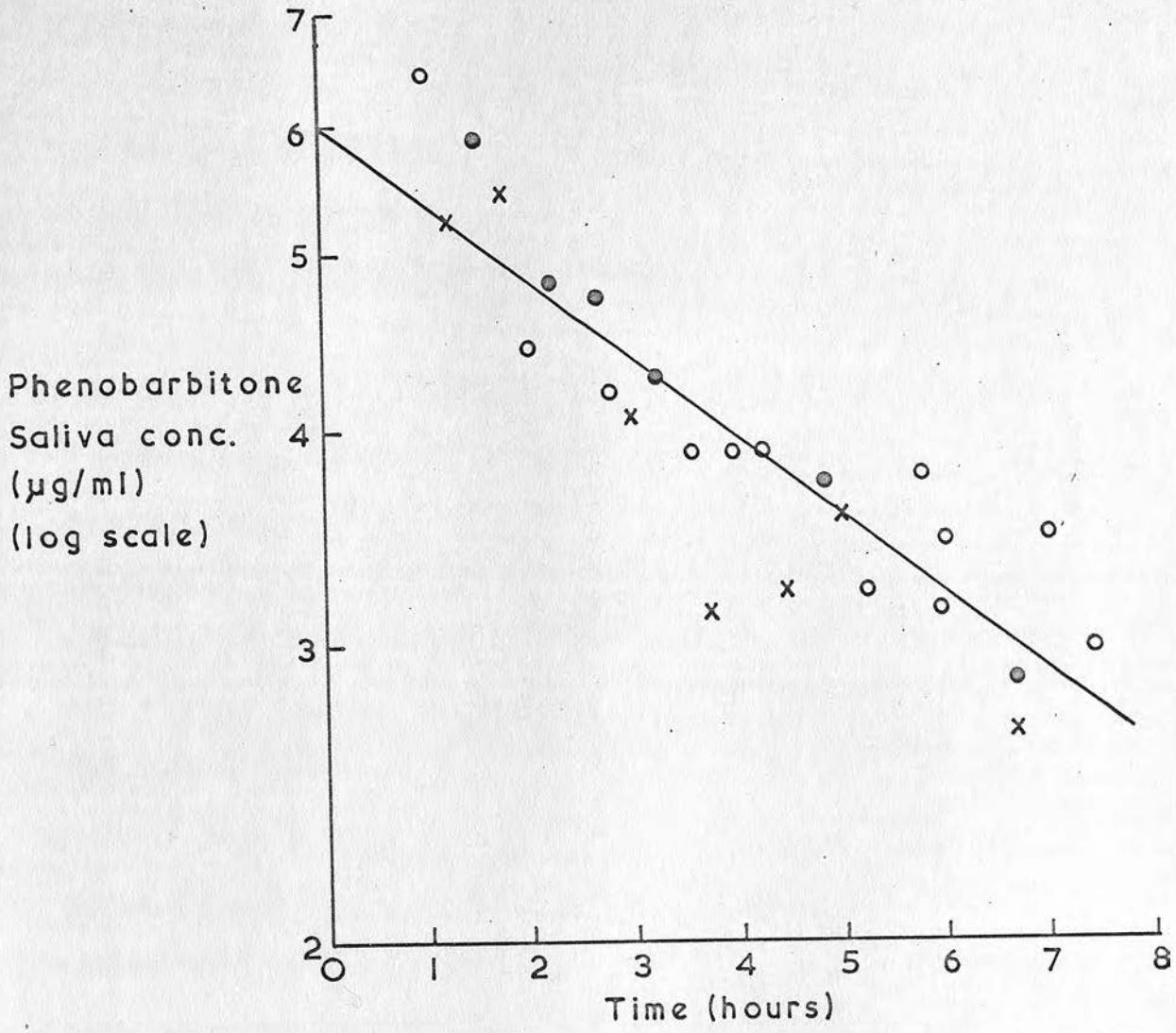

Figure XIII. Phenobarbitone saliva clearance. Results from three experiments on Pony II. Dose, 6·6 mg/kg of sodium phenobarbitone. Calculated clearance rate was 10%/hour. Saliva flow rates; (o) <100, (x)100 to 200 and (●) >200 sec./50ml.

From a study of the four phenobarbitone saliva clearance scatter diagrams several points became obvious. The rates of clearance of phenobarbitone closely paralleled the corresponding blood clearances and the relative concentrations of the drug in saliva and blood were estimated to be of similar magnitude for the same dose rate. As there was no obvious correlation between saliva-barbiturate concentration and saliva flow rate, as shown by grouping the results into three groups according to an arbitrary choice of flow rates, it was concluded that saliva-barbiturate concentrations were independant of saliva flow rates. The barbiturate was not observed to have any effect on saliva flow rate. The wide scatter of the points on each scatter diagram was not readily explained since the variation was much in excess of the possible analytical error.

A brief study was made of the salivary secretion of phenobarbitone during the first hour following drug administration. The methods used were the same, but samples were taken at closer intervals of time and the results of each experiment were plotted separately. The results are given in Figures XIV, XV and XVI. As can be seen, the maximum concentration of phenobarbitone in the saliva was reached in from I0 to 20 minutes after drug adminstration. In the experiment whose results are depicted in Figure XVI the saliva-barbiturate concentrations were followed for a longer period of

time and the scatter in the saliva-barbiturate concentrations became obvious. This, as has been described, was typical of all experiments carried out over a long period of time.

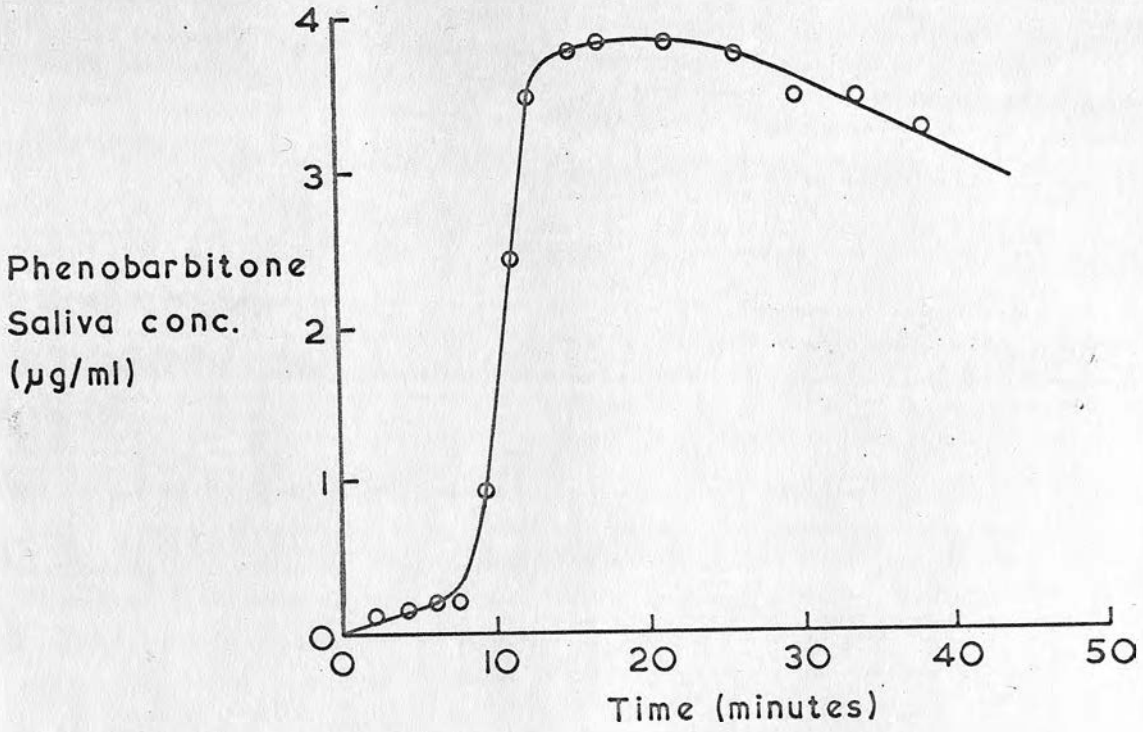

Figure XIV. Phenobarbitone saliva clearance. Results from a single experiment on Pony III. Dose, 3·1 mg/kg of sodium phenobarbitone.

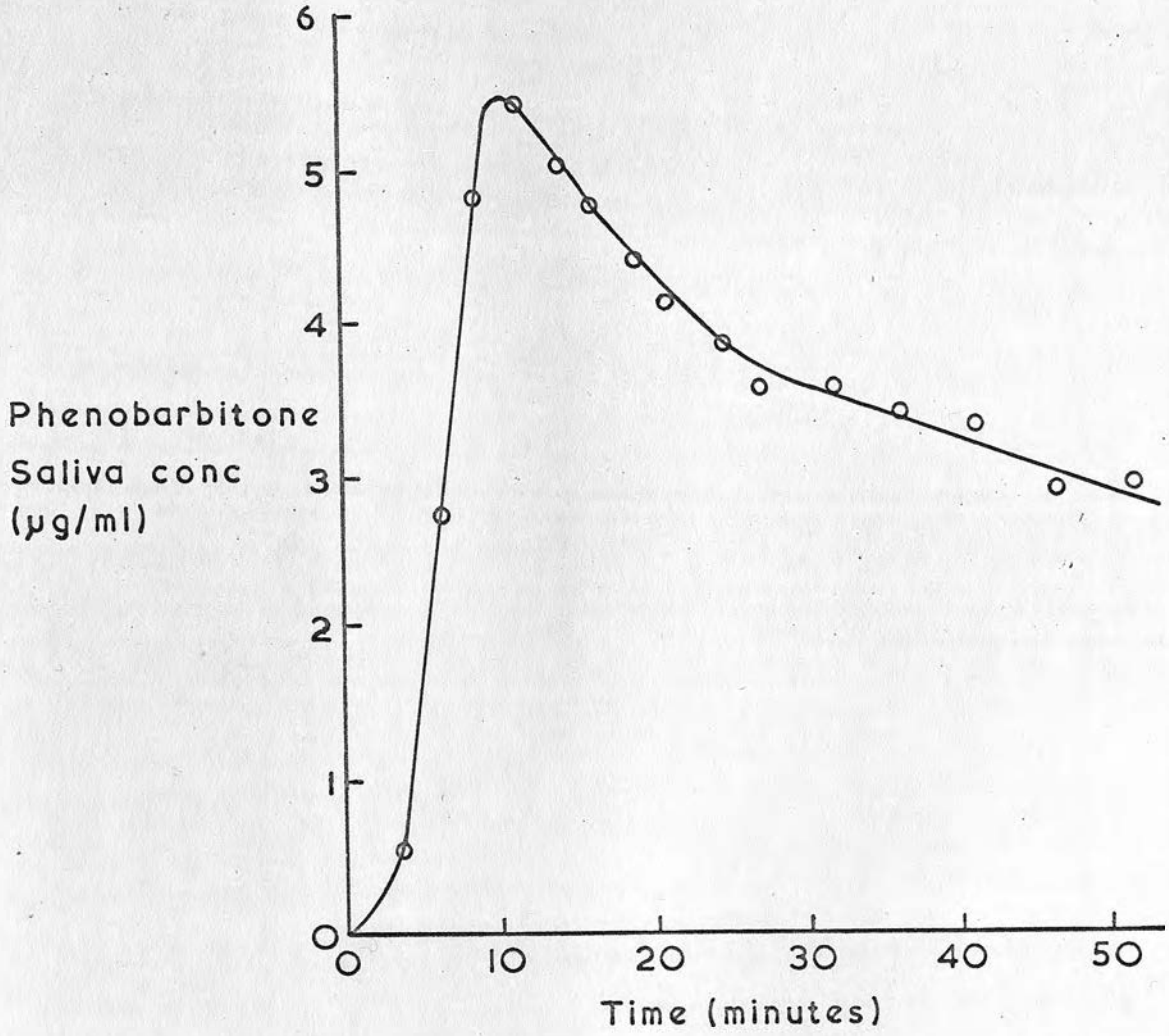

Figure XV. Phenobarbitone saliva clearance. Results from a single experiment on Pony III. Dose, 3·1 mg/kg of sodium phenobarbitone.

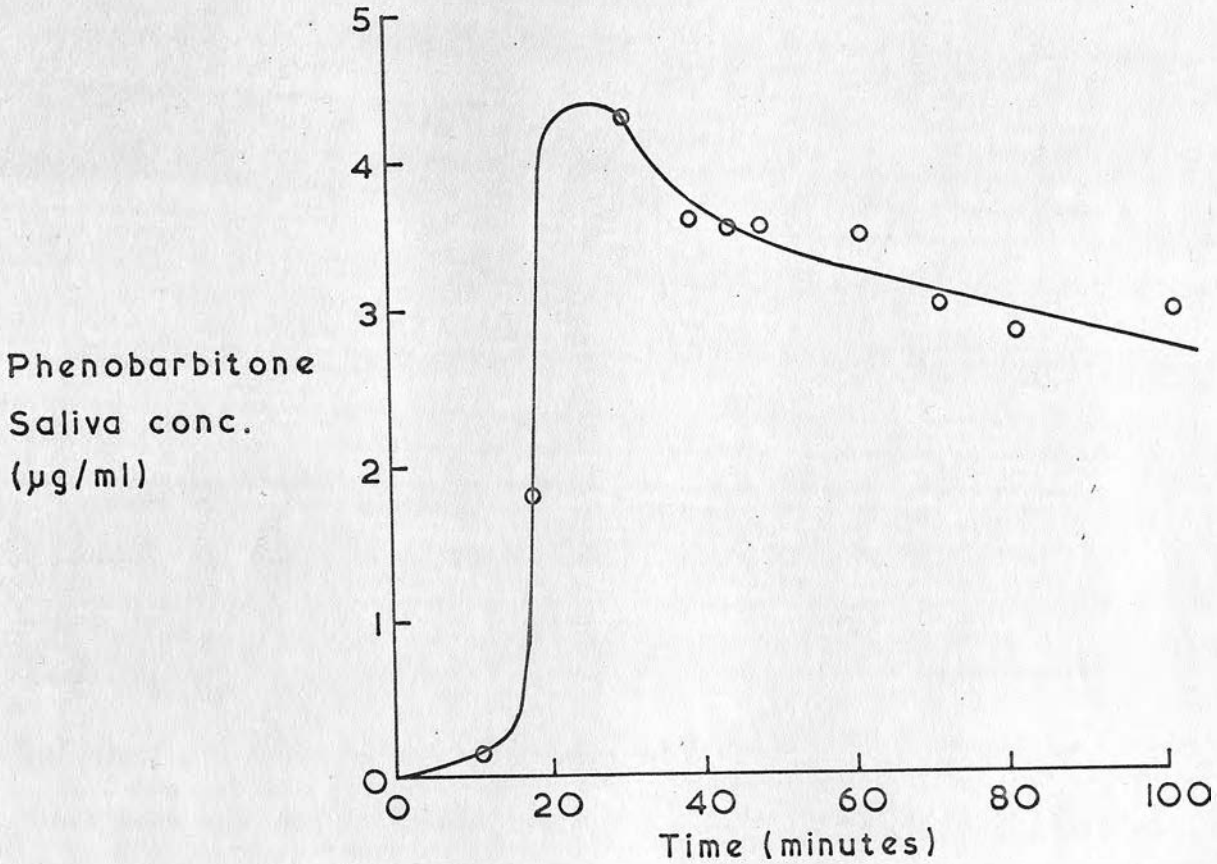

Figure XVI. Phenobarbitone saliva clearance. Results from a single experiment on Pony II. Dose, 2·8 mg/kg of sodium phenobarbitone.

(2) Pentobarbitone :- As the saliva clearance rate of pentobarbitone was in the order of 50%/hour it was necessary to collect a sufficient number of samples over the first three hours, after drug administration, to allow a clearance curve to be plotted. It was not always possible to obtain regular samples over the full three hours, but usually at least the first half of this period could be covered. In four experiments, two on each pony, it was found that the salivary secretion rate of pentobarbitone declined logarithmetically with time when the intravenous route of administration was used. The mean clearance rate was 49%/hour (S.D. 4%/hour) and a typical clearance curve, covering the first three hours after drug administration, is shown in Figure XVII. From Figure XVII it was clear that saliva flow rate had no effect on pentobarbitone-saliva concentrations, as the first four points were obtained from saliva with a low flow rate and the last four samples from saliva with a fast flow rate. Pentobarbitone produced no observable effect on saliva flow rate. The results of the four experiments are given in tabular form in Table XVIII.

Some results were obtained for the saliva clearance of pentobarbitone following oral administration and these are depicted in Figures XVIII and XIX. Both show that the final logarithmic decline in barbiturate concentrations did not commence until about 45 minutes after drug administration by

Table XVIII. Pentobarbitone saliva clearance rates.

Pony	Clearance rate (%/hour)	Concentration in saliva at zero time* (µg/ml)
II	5I	2.4I
	43	2.I2
III	52	2.25
	50	2.65

* Pentobarbitone concentration in saliva at time of dosage, found by interpolation.

which time the concentrations had fallen to low values. This was one of the reasons why the intravenous route of administration was preferred, since, as has been shown, a steady logarithmic decline was observed to start within a short time of dosage. In all three cases the clearance rates measured after 45 minutes were in close agreement with those obtained after intravenous administration.

Figure XVIII shows the effect of an increase in the dosage rate of pentobarbitone on its concentration in the saliva. Saliva collection during the first hour, after the higher dose rate, was not possible as the drug affected the pony to the extent of making it refuse to eat for that period of time.

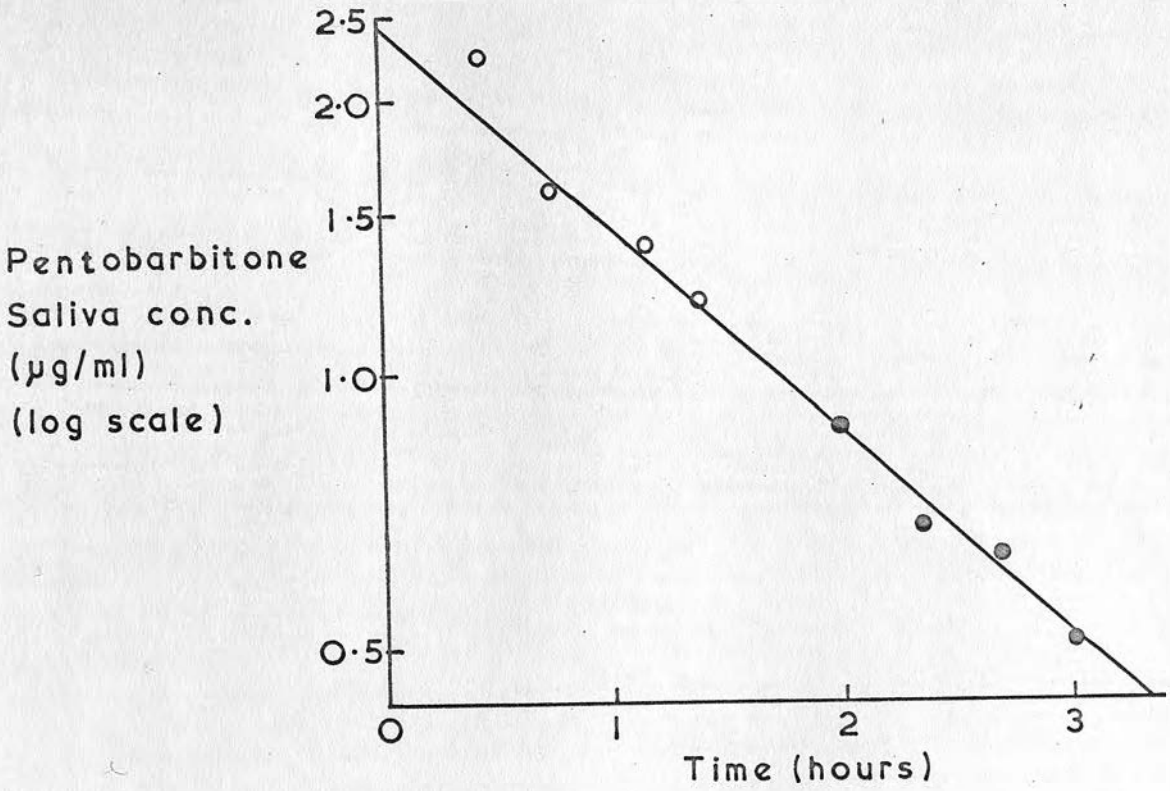

Figure XVII. Pentobarbitone saliva clearance. Results from a single experiment on Pony II. Dose, 4 mg/kg of sodium pentobarbitone. Calculated clearance rate was 51% /hour. Saliva flow rates were - (o) 100 ml/ 7 min. (●) 100 ml/ 2·5 min.

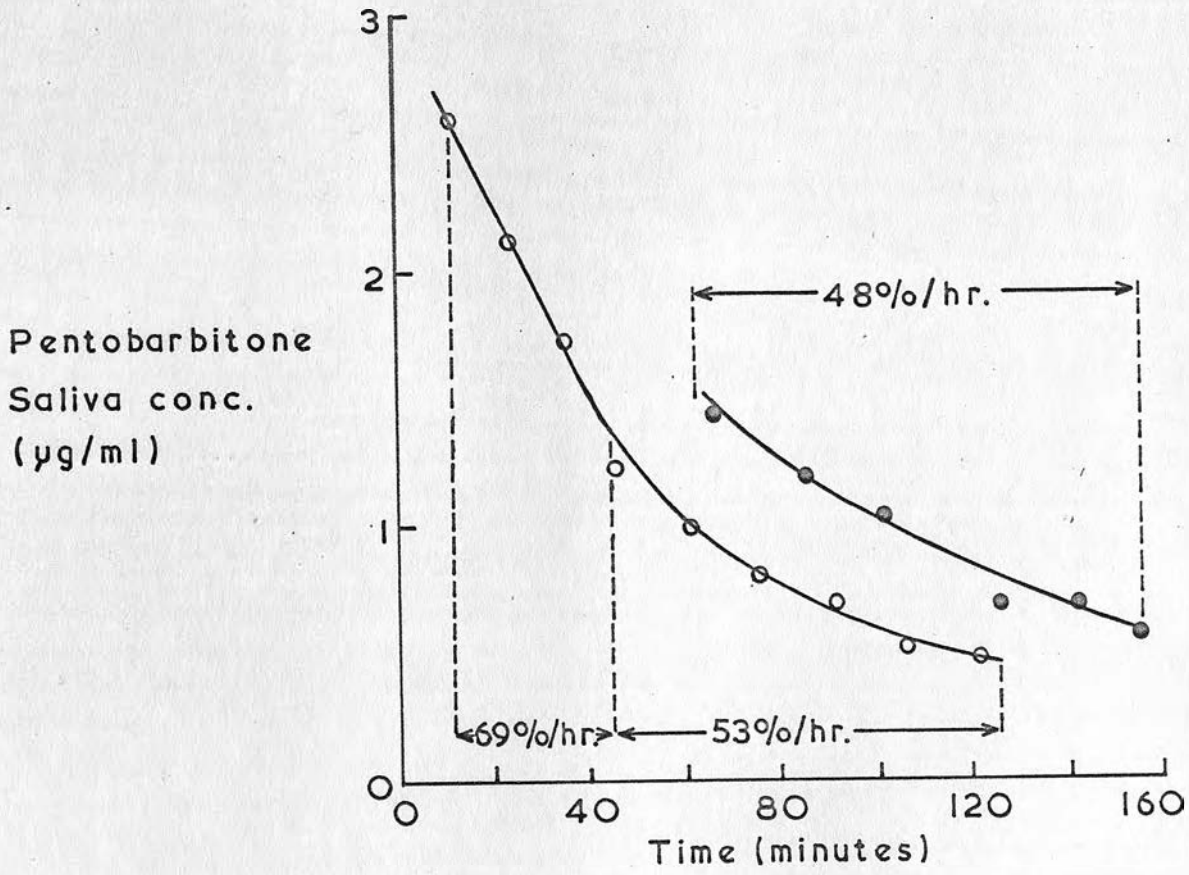

Figure XVIII. Pentobarbitone saliva clearances after oral administration to Pony III. Doses were (o) 3·1 mg/kg and 4·7 mg/kg.

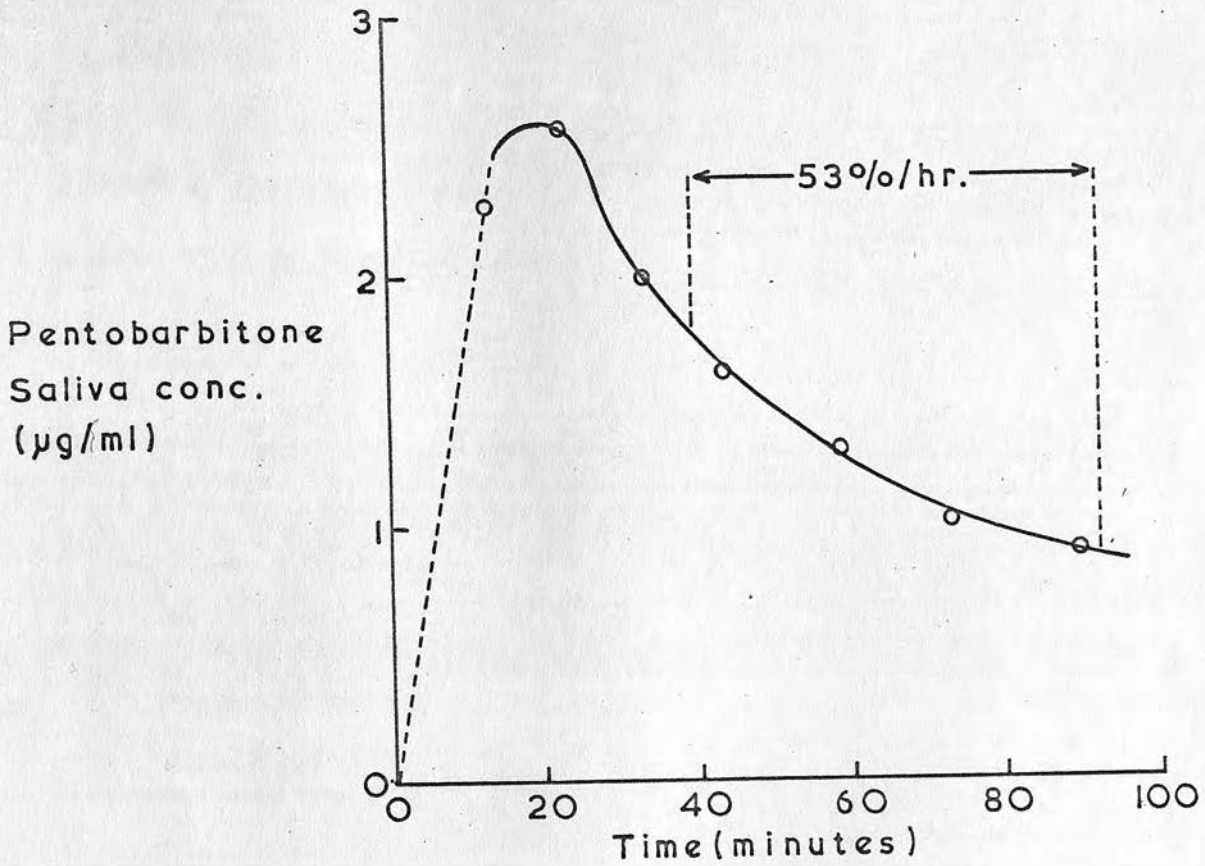

Figure XIX. Pentobarbitone saliva clearance after oral administration to Pony II. Dose, 2·8 mg/kg.

(c) Barbiturate protein binding

(I) Plasma protein binding :- The results given by the previously described analytical technique are described in Table XIX. When these values were subtracted from those in Table XVI, which were a measure of the efficiency of the analytical method, the true values for barbiturate plasma protein binding were obtained and are given in Table XX.

Table XIX. Recovery of barbiturates from plasma in protein binding experiments.

Barbiturate	Recovery (%)	S.D.	n
Pentobarbitone	63	5	3
Phenobarbitone	76	II	7

Table XX. Barbiturate plasma protein binding.

Barbiturate	Protein Binding (%)
Pentobarbitone	44
Phenobarbitone	I5

(2) Saliva protein binding :- The results of saliva protein binding experiments, obtained in an identical manner to that already described for plasma protein binding, are given in Tables XXI and XXII. Student's t-test was used to demonstrate that there was no significant difference between the recovery of pentobarbitone from saliva and from the buffer. It followed, therefore, that pentobarbitone was not protein bound in the saliva. The same test showed that there was a significant difference between the recoveries of phenobarbitone from the two media. The difference between the two mean recoveries indicated 7% protein binding of phenobarbitone in horse saliva.

Table XXI. Recovery of barbiturates from saliva in protein binding experiments.

Barbiturate	Recovery (%)	S.D.	n
Pentobarbitone	I09	4	6
Phenobarbitone	84	4	5

Table XXII. Barbiturate saliva protein binding.

Barbiturate	Protein Binding (%)
Pentobarbitone	0
Phenobarbitone	7

(d) Comparison of blood and saliva barbiturate concentrations Table XXIII shows the calculated concentrations of non-protein bound and unionised phenobarbitone and pentobarbitone in horse blood assuming a total concentration of I0 µg/ml of each barbiturate.

Table XXIII. Physical equilibria of phenobarbitone and pentobarbitone in horse blood.

Barbiturate	Concentration (µg/ml)	Protein Bound (%)	Unbound (µg/ml)	Unionised* (µg/ml)
Pentobarbitone	I0	44	5.6	4.5
Phenobarbitone	I0	I5	8.5	3.4

* Calculated assuming pK_a phenobarbitone = 7.2

pK_a pentobarbitone = 8.0

If it is assumed that the final concentration of barbiturate in the saliva was determined by an equilibrium between the unionised barbiturate in the blood and in the saliva, it follows, from the values in Table XXIII, that pentobarbitone should be present in a higher concentration in the saliva than phenobarbitone when the two barbiturates are present in equal concentrations in the blood. As has been shown this was not the

case. In fact the concentration of phenobarbitone in saliva was estimated to be relatively higher than that of pentobarbitone. The estimated concentrations approximated to the values in Table XXIII corresponding to unbound barbiturate in blood. A more accurate comparison was made from the estimated relative concentrations of the barbiturates present in the saliva at zero time following doses of 4 mg/kg of each drug. It was necessary to assume that the distributions of both barbiturates throughout the horse were similar. The results are given in Table XXIV.

Table XXIV. Comparison of the saliva concentrations of phenobarbitone and pentobarbitone following equal doses of the drugs.

Barbiturate	Dose mg/kg	Estimated conc. in saliva at zero time	$\frac{\text{Phenobarb. conc.}}{\text{Pentobarb. conc.}}$
Pentobarbitone	4	2.36 µg/ml*	I.69
Phenobarbitone	4	3.98 µg/ml**	

* Mean value from Table XVIII.

** Estimated from data in Figures X and XI.

When the phenobarbitone/pentobarbitone ratio of

unbound drugs was calculated from the values in Table XXIII a figure of I.52 was obtained. This value agreed very closely with the observed value of I.69 given in Table XXIV and supported the theory that the saliva-barbiturate concentration was dependant on the amount of non-protein bound barbiturate and not on the amount of unionised barbiturate in the blood.

(e) Urinary excretion of phenobarbitone

(I) Evidence for the excretion of phenobarbitone in urine :- Urine, obtained from ponies which had been given phenobarbitone, was extracted as has been described and the extract examined by paper chromatography. All three described methods of location failed to detect phenobarbitone on the developed chromatogram due to the presence of interfering material extracted from the urine. Attempts to purify the extract by dissolving it in chloroform and washing the solution with water, dilute hydrochloric acid or bicarbonate buffer failed to improve the situation. With all urine samples used the quantitative complexometric method indicated a concentration of about I0 µg/ml of barbiturate material, quoted as phenobarbitone.

A fresh sample of urine, after centrifugation, was acidified with concentrated acetic acid (5 ml/I00 ml urine) and I50 ml extracted with two equal volumes of ether. After drying the combined ether extracts over a mixture of sodium sulphate,

sodium bicarbonate and activated charcoal, the ether was evaporated and the residue chromatographed with the BuAm solvent. The region on the developed chromatogram, with R_f value corresponding to phenobarbitone, was eluted with dilute sodium hydroxide and the alkaline eluate acidified. After extracting the acidified eluate with two equal volumes of ether and evaporating the sodium sulphate dried ether to dryness the residue was rechromatographed with the IPrAm solvent. The developed chromatogram was treated exactly as was the first and the residue so obtained subjected to further chromatography in the AmAm solvent system.

Examination of the third chromatogram under ultra-violet light revealed no absorbing region. The region, corresponding to that at which phenobarbitone was expected to occur, was eluted with 0.45N sodium hydroxide and the ultra-violet spectra of the eluate recorded at pH I3 and pH I0.4. The spectra had a similar appearance to those already described for extracts of saliva containing small amounts of barbiturate. The differential spectrum was almost identical to those described by Stokes et al.,[I89] for barbiturates and when a true differential spectrum of phenobarbitone was superimposed a close match resulted (Figure XX).

When the complexometric reaction of Lubran [I05] was carried out on the ether extractable material present in the

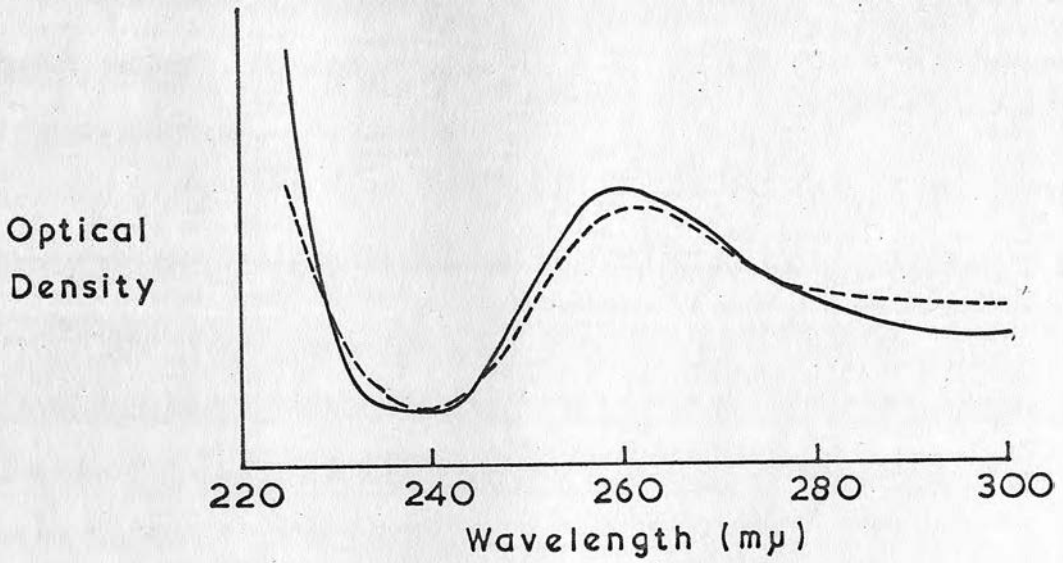

Figure XX. Differential ultra-violet spectra of phenobarbitone (-----) and of suspected phenobarbitone in urine (———).

acidified alkaline eluate from the chromatogram a strongly positive result was obtained. It was therefore concluded that the urine contained a barbiturate material which possessed the same R_f values as did phenobarbitone in three paper chromatography systems.

The partition coefficient of this barbiturate material between benzene and a pH 7 phosphate buffer was determined and a figure of 0.59 was obtained. This compared favourably with the value of 0.53 found for phenobarbitone, when the small amounts of interfering chromogens in the urine were considered.

When taken in conjunction with the knowledge that phenobarbitone is excreted in the urine of man, rat, dog, mouse, and rabbit, as has already been mentioned in the appropriate review section, the above evidence was considered sufficient for it to be assumed that phenobarbitone was excreted to some extent unchanged in the urine of horses.

(2) Excretion of phenobarbitone metabolites :- Under no circumstance was there any indication of the presence of phenobarbitone metabolites on paper chromatograms of urine extracts. As Butler [I,6] has shown, the p-hydroxy metabolite of phenobarbitone in the dog is conjugated in the urine. The same acid hydrolysis used by him was applied to a horse urine sample. Extraction of the hydrolysed urine and BuAm paper chromatography

of the extract resulted in the detection of an ultra-violet absorbing spot on the chromatogram at R_f 0.29 which reacted with the cobalt chloride/ammonia reagent. On the basis of this result a large scale isolation of the suspected metabolite was attempted.

I.3 g of sodium phenobarbitone was given to Pony III and the urine voided over the following 24 hours (4,000 ml) collected. The urine was hydrolysed by heating it with an equal volume of concentrated hydrochloric acid on a boiling water bath for three hours. After cooling, the urine was extracted with ether. By means of alkaline extracts from organic liquids, organic liquid extracts from acidified aqueous solutions and utilising the inability of benzene to extract the metabolite from water, a series of purification stages were carried out before a final large scale BuAm paper chromatography of the extract. Elution of the appropriate regions with dilute sodium hydroxide followed by ether extraction of the acidified alkaline eluate and evaporation of the dried ether gave a gummy brown residue. This was recrystallised several times by dissolving it in a minimum volume of acetone and diluting this with a large volume of benzene. On standing for several days the metabolite crystallised out. The final product was needle shaped crystals melting at 223-225°. The ultra-violet spectra of this material at pH I3, I0.4 and I were the typical characteristic spectra

of p-hydroxyphenobarbitone [3] (m.p. between 220 and 226° [I,2,6]). As further evidence of the identity of the isolated material its distribution coefficient between ether and water was determined and a value of 5.3 found. This was in good agreement with the literature value of 5.[I,6] When the infrared spectra of the metabolite and phenobarbitone were compared (Figures XXI and XXII) significant differences were observed. A band at 3450 cm^{-I} in the metabolite spectrum was characteristic of a hydroxyl group and this was further supported by bands at I360 and I220 cm^{-I}. Bands in the region 970 to I220 cm^{-I} were as would have been expected with I,4-substitution in the benzene ring and convincing evidence of this was presented by a strong absorption band at 830 cm^{-I}.

From the above evidence it was concluded that the metabolite of phenobarbitone which had been isolated was p-hydroxyphenobarbitone (i.e. 5-ethyl-5-(4-hydroxyphenyl)-barbituric acid. As no other metabolite was detected it was assumed that this was the principal metabolite of phenobarbitone in the horse.

(3) Conjugation of p-hydroxyphenobarbitone :- Incubation of different urine samples with and without β-glucuronidase gave evidence of the liberation of p-hydroxyphenobarbitone only when the enzyme was present. The metabolite was located at the appropriate position on paper chromatograms with either ultra-

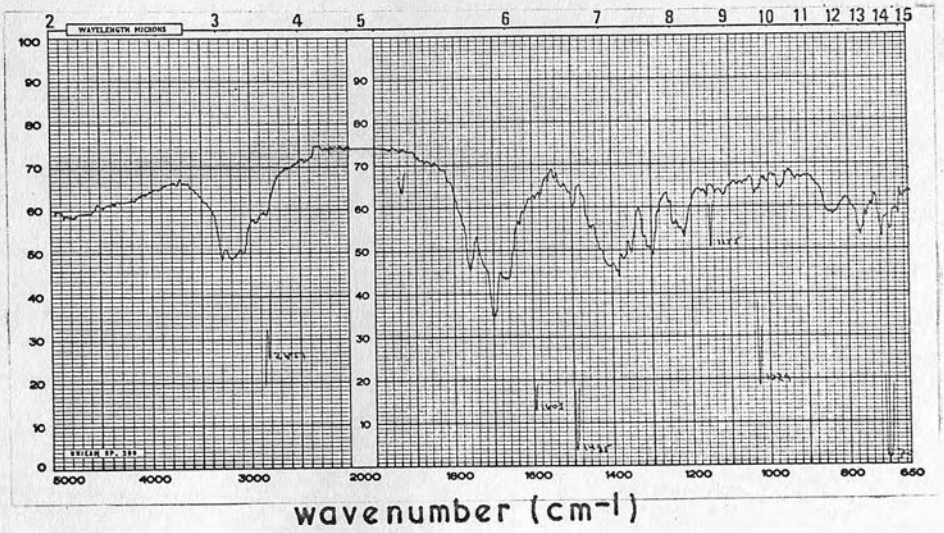

Figure XXI. Infrared spectrum of phenobarbitone.

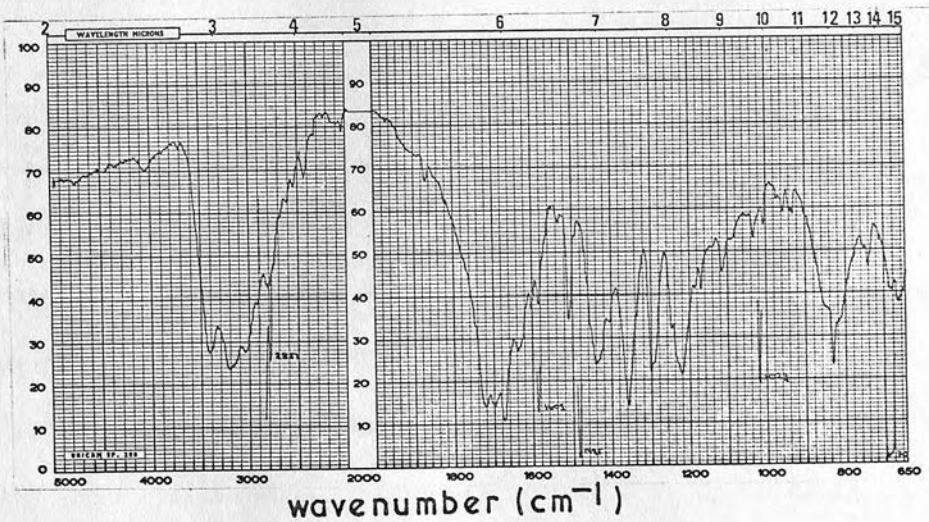

Figure XXII. Infrared spectrum of the phenobarbitone metabolite.

violet light or the cobalt chloride/ammonia reagent. No other ultra-violet absorbing regions were observed. Elution of the spot with 0.45N sodium hydroxide and measurement of the ultra-violet absorption spectra of the eluate at pH I3, I0.4 and I gave spectra identical to those of the previously isolated metabolite. The conclusion was therefore drawn that p-hydroxy-phenobarbitone was conjugated to a large extent with glucuronic acid in horse urine.

(4) Rate of urinary excretion of phenobarbitone :- The rate of excretion of phenobarbitone was followed in single experiments on five ponies over a period of 72 hours or until the concentration in the urine became too low for accurate measurement. The results obtained are reported in two forms in Figures XXIII and XXIV. Figure XXIII presents the individual excretion rates and Figure XXIV presents the mean clearance rate. As a check on whether or not the total volume of urine excreted had any effect on the amount of phenobarbitone excreted these two factors were compared as shown in Table XXV. No obvious interdependence was observed.

Table XXV. Comparison of the volume of urine and amount of phenobarbitone excreted by ponies.

Pony	Volume of Urine (ml)	Phenobarbitone Excreted (%)	Period of Observation (hours)
VII	4560	6.9	71.5
V	7600	4.1	46.25
VI	10420	12.7	71
II	17540	10.0	68
III	17590	11.6	72

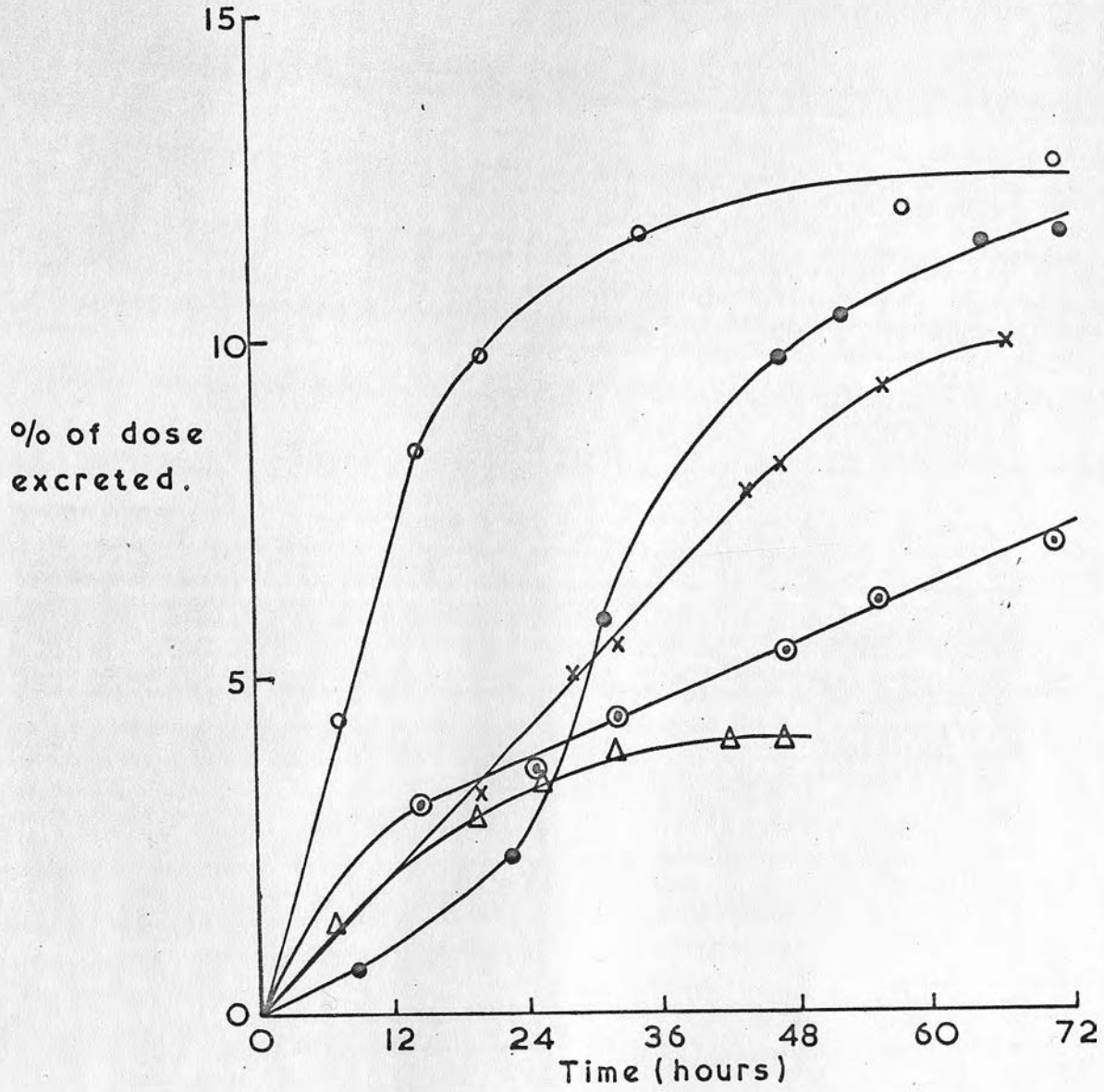

Figure XXIII. Rates of urinary excretion of phenobarbitone following a dose of 6 mg/kg of sodium phenobarbitone. (x) Pony II. (●) Pony III. (Δ) Pony V. (o) Pony VI. (◎) Pony VII.

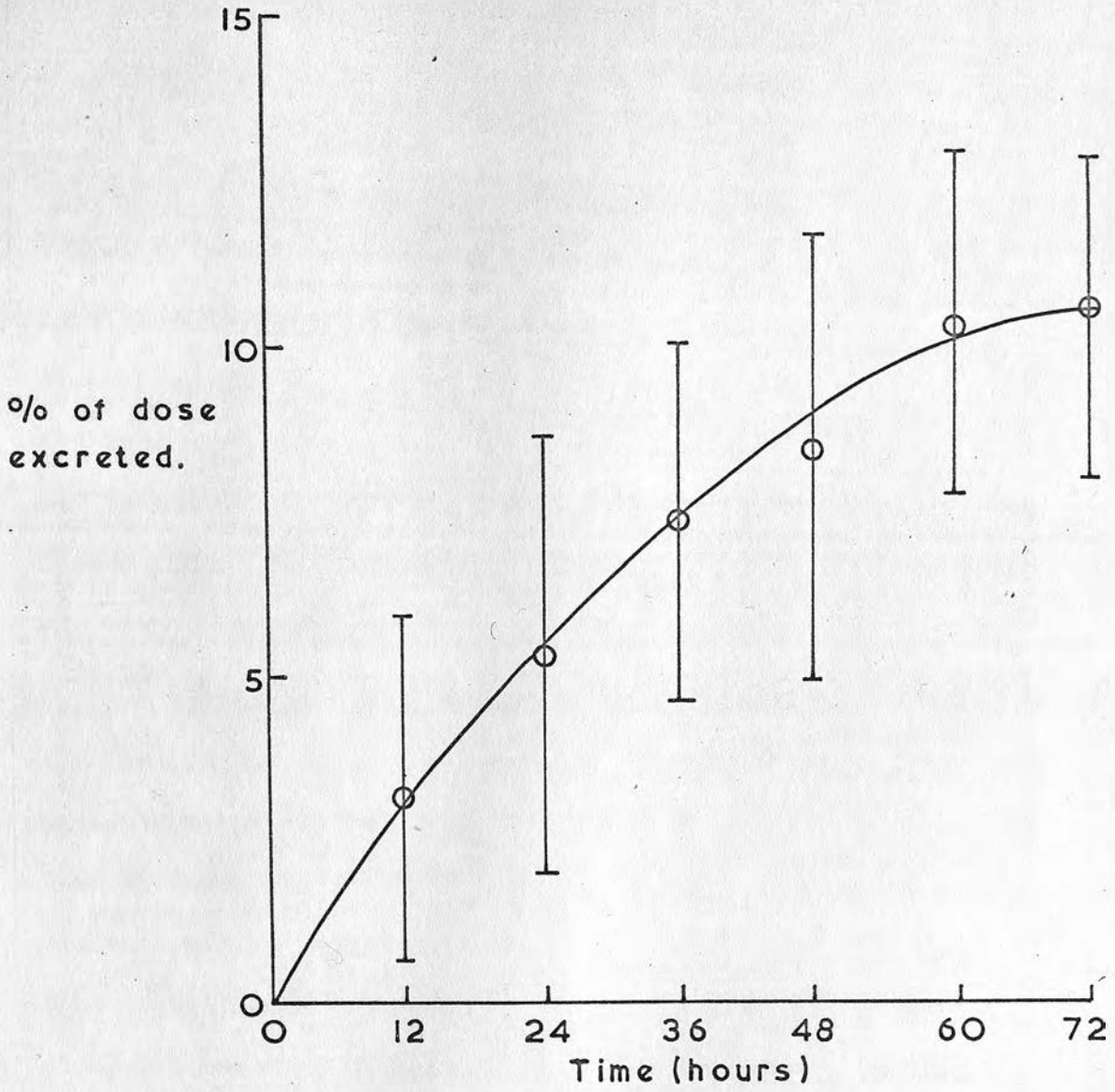

Figure XXIV. Mean rate of urinary excretion of phenobarbitone (±S.D.) obtained from the data in Figure XXIII.

(f) Urinary excretion of pentobarbitone

(I) Evidence for the urinary excretion of pentobarbitone:- Pentobarbitone was not detected, by paper chromatography, in the urine of ponies even after large doses of the drug. The same statement was true when gas-liquid chromatography was used. As a measure of the sensitivity of this latter technique, pentobarbitone was added to normal urine to give a concentration of about I µg/ml, i.e. equivalent to less than I% of the doses used in these experiments. Subsequent analysis revealed a peak corresponding to pentobarbitone on the GLC chromatogram (see Figure XXV). It was therefore concluded that any pentobarbitone excreted in horse urine was in negligible amounts.

(2) Detection of pentobarbitone metabolites :- Paper chromatography, using the BuAm solvent, of ethereal extracts of purified urine allowed ready detection of spots at R_f 0.50 and 0.08 by the cobalt chloride/ammonia reagent. The use of ultra-violet light to detect absorbing barbiturate regions, under these conditions, was found to be of no value due to the strongly absorbing properties of extracted natural urinary constituents, such as benzoic acid and hippuric acids. Elution of the regions at R_f 0.50 and 0.08 with 0.45N sodium hydroxide and measurement of the ultra-violet absorption spectra of the extracts at pH I3, I0.4 and I resulted in distorted barbiturate spectra for the R_f 0.50 spot and idefinite spectra for the

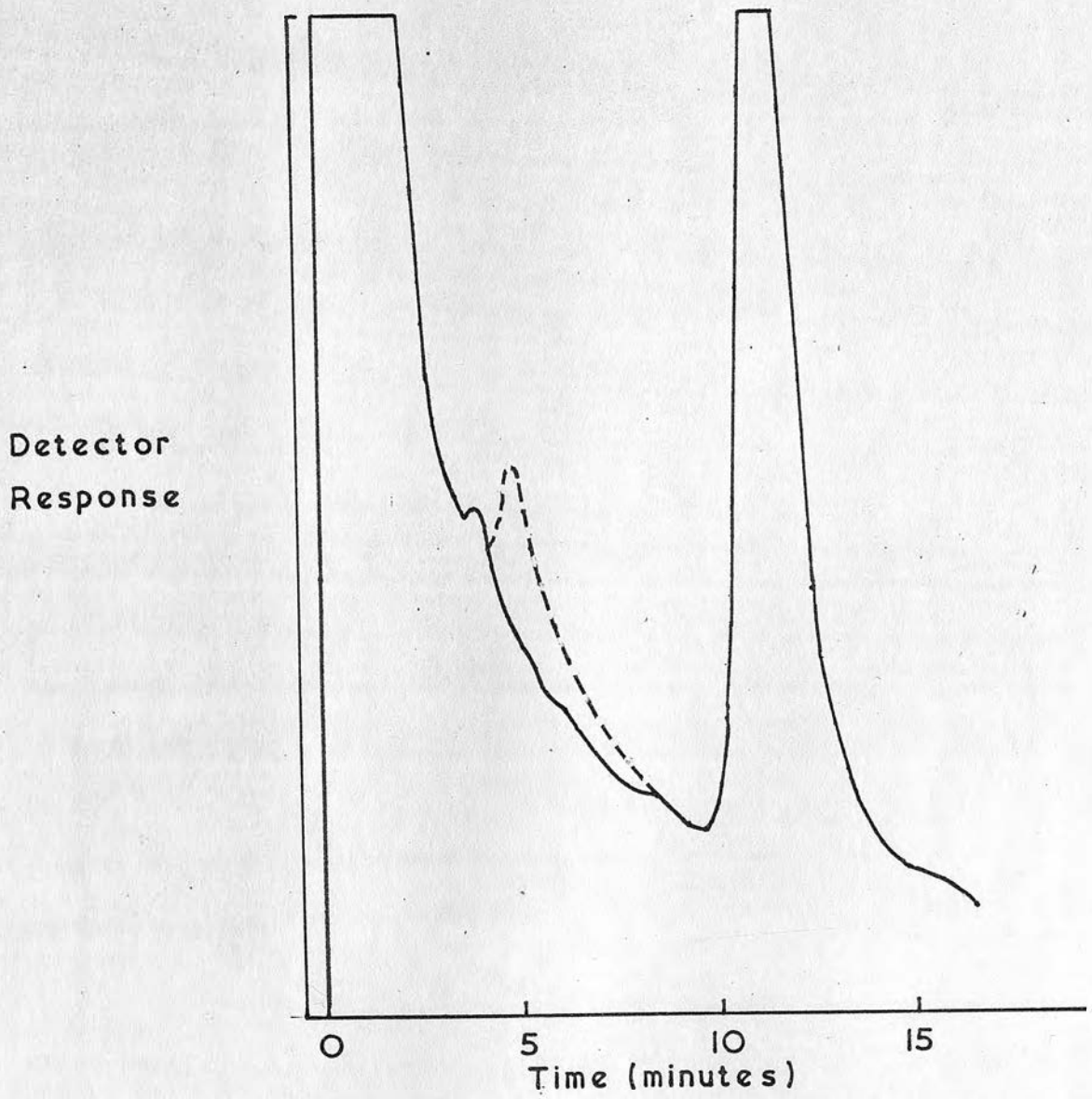

Figure XXV. GLC chromatogram of an ethereal extract of horse urine. (———) Normal urine. (– – – – –) Effect of pentobarbitone when present in urine to a concentration of 1 µg/ml.

R_f 0.08 spot.

When extracts were subjected to paper chromatography after purification, including charcoal column chromatography, the region at R_f 0.05 gave definite barbiturate ultra-violet spectra after elution with 0.45N sodium hydroxide. The spot at R_f 0.08 gave spectra which fitted the description given by Algeri and McBay [43] to a suspected impure pentobarbitone metabolite detected in human and dog urine.

It was of interest to note that the ultra-violet absorbing regions at R_f 0.28, 0.93 and 0.2I, which were consistently detected on these paper chromatograms, possessed the "pseudo-barbiturate" absorption spectra as described by Jackson and Finkle [I82] and would consequently present difficulties in the detection of barbiturates with similar R_f values. These regions did not react to the cobalt chloride/ ammonia reagent.

(3) Isolation of pentobarbitone metabolites :- As the above results indicated that at least two metabolites of pentobarbitone were excreted in horse urine a large scale isolation of them was attempted. For convenience the metabolites will be refered to as A (R_f 0.50) and B (0.08).

Initial attempts indicated that the isolation of pure A and B could not be achieved by simple paper chromatography

followed by recrystallisation, as had been used by previous investigators working with dog or human urine, due presumably to the concentrated nature of horse urine. The following procedure was therefore adopted.

7.0 g of sodium pentobarbitone was given to a pony over a period of 32 hours and the 48 hour urine, timed from the initial dose, collected. After Lloyd's reagent purification the urine was saturated with ammonium sulphate. A 500 ml portion was extracted with an equal volume of ether. The separated ether was then extracted with a small volume of dilute sodium hydroxide which was then acidified. The urine, before being discarded, was re-extracted with the same ether and then the ether was extracted with dilute sodium hydroxide as before. A further 500 ml of urine was then extracted with the original ether, made up to its original volume to take into account loss of ether by virtue of its solubility in water. By repeating this process several times a large volume of urine could be extracted with a relatively small volume of ether, before renewing the ether when it became heavily contaminated with material not extracted from it by the alkali.

When all the urine had been extracted, the acidified aqueous solution of the extract was filtered and the residue washed thoroughly with water. The combined filtrate and washings were saturated with ammonium sulphate and extracted with two

equal volumes of ether which were then dried over sodium sulphate/sodium bicarbonate. Evaporation of the ether, the solid from the drying process being retained, yielded a light brown residue which was extracted with two 50 ml portions of benzene to remove much of the colouring matter. The residue was then subjected to large scale paper chromatography using the BuAm solvent. Treatment of the developed chromatograms with the cobalt chloride/ammonia reagent revealed strong barbiturate reaction at R_f 0.50 and a very weak reaction at R_f 0.08. The two areas were cut out and kept separately for further treatment.

The sodium sulphate/sodium bicarbonate residue retained above was dissolved in dilute hydrochloric acid and the solution extracted with half its volume of chloroform which was then discarded. The aqueous solution was then extracted with two equal volumes of ether which were combined and dried over sodium sulphate. Evaporation of the ether gave a strongly coloured residue which was subjected to large scale paper chromatography as described above. Cobalt chloride/ammonia treatment of strips cut from the developed chromatograms gave a positive reaction only at R_f 0.08. These regions were cut out and added to the corresponding ones collected from the first batch of large scale chromatograms.

Isolation of metabolite A :- The regions with R_f 0.50 cut from the chromatograms were extracted several times with

water (continuous extraction of the paper with ether failed to remove A efficiently from the paper). The aqueous solution obtained was saturated with ammonium sulphate and extracted with two equal volumes of ether. After drying and evaporating the ether a gummy white residue resulted.

This residue assayed about 50% barbiturate (as pentobarbitone) when analysed by differential ultra-violet spectrophotometry. For this reason the whole of the material was rechromatographed using the IPrAm solvent. Examination of the chromatogram under ultra-violet light revealed absorbing spots at R_f 0.67 and 0.40 and fluorescent spots at R_f 0.55, 0.23 and 0.04. Only the spot at R_f 0.67 reacted positively to the cobalt chloride/ammonia reagent and gave typical barbiturate spectra after elution with 0.45N sodium hydroxide. The spectra of all the other regions were found to be such that would cause considerable interference in ultra-violet barbiturate analysis.

A was extracted from the IPrAm chromatograms in the same manner as from the BuAm chromatograms. The white residue thus obtained was dissolved in from 2 to 3 ml of acetone and about 100 ml of benzene added rapidly. After filtering through a cotton wool plug the solution was allowed to stand in a closed vessel for several days. The white needle shaped crystals thus obtained were recrystallised in a similar manner to give 200 mg

of A. A further recrystallisation was carried out by dissolving A in a large volume of hot benzene and allowing this to stand for several days. The crystals obtained melted sharply at I30° (uncorr.) and possessed ultra-violet spectra of a typical barbiturate at pH I3, I0.4 and I.

The infrared spectra of A, pentobarbitone and synthetic 5-ethyl-5-(3-hydroxy-I-methylbutyl)barbituric acid (kindly supplied by Y.C. Dickert of the Dow Chemical Co.) were compared, and are shown in Figures XXVI, XXVII and XXVIII. The spectra of A and C (the synthetic hydroxy-pentobarbitone) showed a band at 3520 cm^{-I} characteristic of a hydroxyl group. If the two compounds were considered to be identical the slight differences between their spectra could be considered due to differences in the relative amounts of the two diastereoisomers present in each specimen. Maynert and Dawson [4I] have shown that the infrared spectra of the two isomers do differ to some extent. That some difference existed between A and C was obvious from a comparison of their melting points (I87-I88° for C and I30° for A).

Both A and C possessed the R_f value of 0.45 in the n-butanol/ammonia paper chromatography system of Algeri and Walker.[II4]

The partition coefficients of A and C between ether and water were determined and values of 0.4 and 0.5 respectively

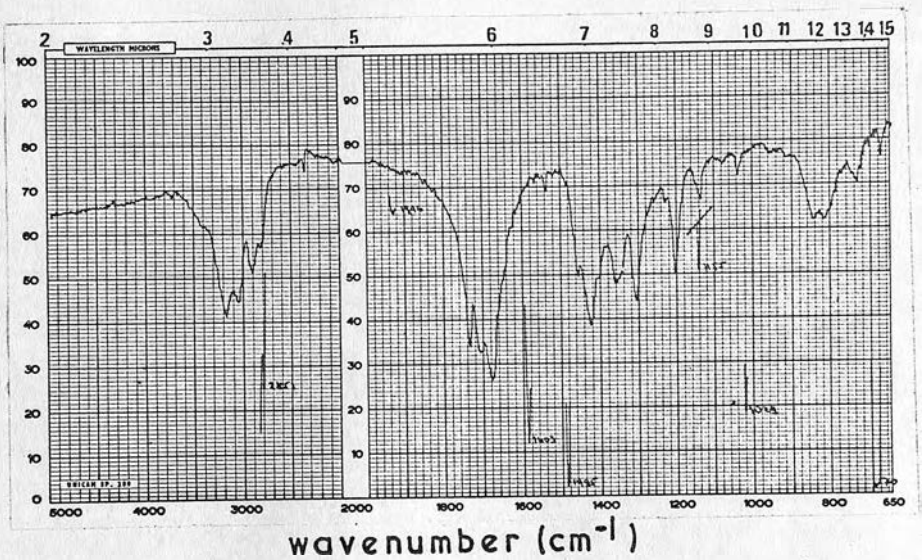

Figure XXVI. Infrared spectrum of pentobarbitone.

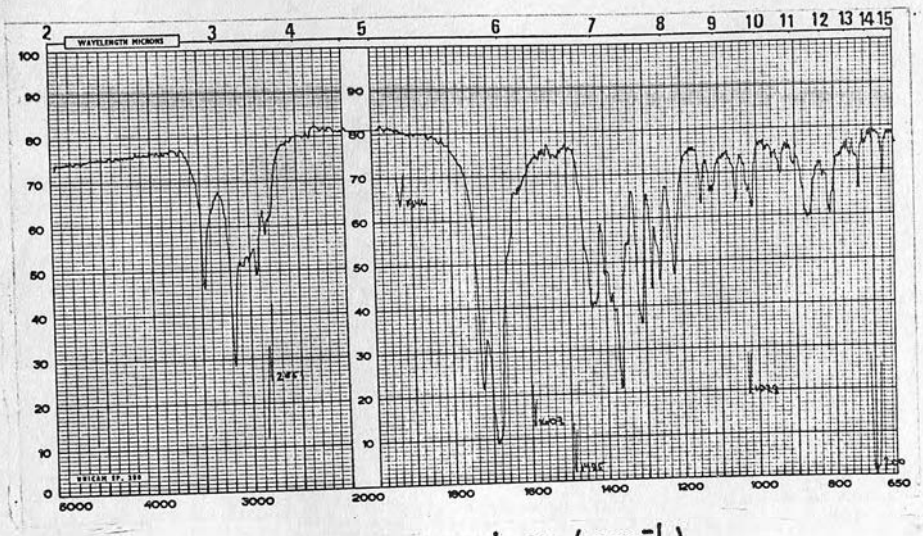

Figure XXVII. Infrared spectrum of 5-ethyl-5-(3-hydroxy-1-methylbutyl)barbituric acid.

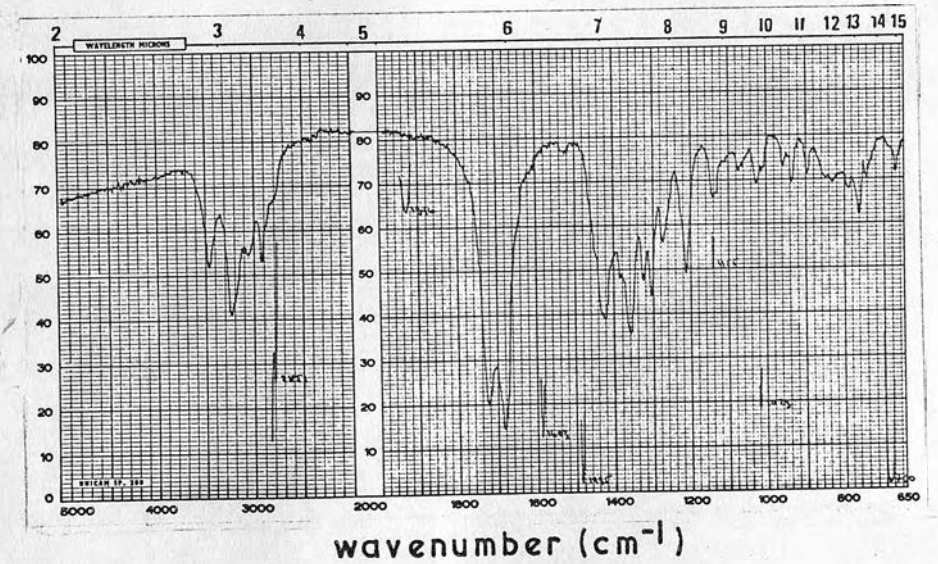

Figure XXVIII. Infrared spectrum of pentobarbitone metabolite A.

were obtained. A difference between the values was considered possible due to the differing solubilities of the diastereo-isomers in ether.[41] The ethyl acetate-water partition coefficients were 2.2 and 2.3 for A and C respectively.

Small amounts of A and C were dissolved in anhydrous methanol and subjected to gas-liquid chromatography. Identical chromatograms were obtained from each, which showed two separate peaks distinct from the solvent peak. The retention times were 17 and 57 seconds.

Assuming a molecular weight of 242.3 the extinction coefficient of A was found to be 7230 (10 mm, 0.5 sodium hydroxide) which was in reasonable agreement with the literature values. Insufficient of C existed for a corresponding measurement to be made on it.

It was therefore concluded that A was a mixture of the two diastereoisomeric alcohols 5-ethyl-5-(3-hydroxy-1-methyl-butyl)barbituric acid. No attempt was made to isolate either or both isomers from A.

Isolation of metabolite B :- The collected sections of paper chromatograms containing metabolite B were continuously extracted with ether for several hours. Evaporation of the ethereal extract and subsequent measurement of the ultra-violet absorption spectra of the residue revealed pH dependent spectra

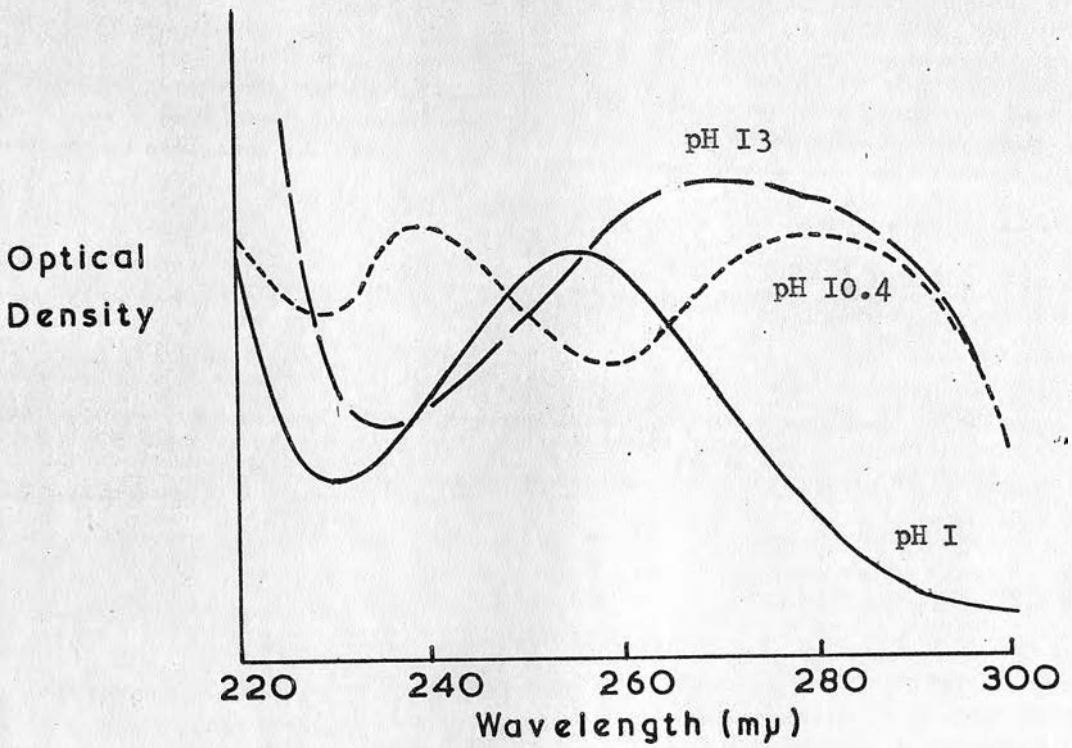

Figure XXIX. Ultra-violet absorption spectra of material separated from pentobarbitone metabolite B.

(Figure XXIX) which were not those of a barbiturate but which would cause considerable interference in an ultra-violet determination of barbiturate. Paper chromatography of this material in the IPrAm solvent gave rise to ultra-violet absorbing spots at R_f 0.II and 0.I5 neither of which reacted to the cobalt chloride/ammonia reagent. This material was not further characterised.

The extracted paper, on which it was assumed B had remained as its ammonium salt, was extracted with dilute ammonia. After acidification of the ammoniacal extract, it was extracted with ether which was then dried and evaporated to dryness. Paper chromatography of the residue with the IPrAm solvent resulted in ultra-violet light absorbing regions at R_f 0.I9 and 0.I0. A strongly fluorescent region, whose fluorescence was greatly increased on exposure to ammonia, was observed at R_f 0.29. Only the region at R_f 0.I9 reacted with the cobalt chloride/ammonia reagent and on elution with 0.45N sodium hydroxide gave typical barbiturate spectra at pH I3, I0.4 and I. B was eluted from the paper as described for the BuAm chromatograms and a gummy residue obtained. This was recrystallised in the same way as was A, but included one stage in which it was recrystallised from concentrated hydrochloric acid. A small amount of crystalline material was finally isolated which had a melting point of I93° (corr.).

The partition coefficients of B between (I) the two phases formed by mixing equal volumes of water, ether, methanol and chloroform, and between (2) ether and a pH 4.56 buffer (46 volumes of 0.2M Na_2HPO_4 and 54 volumes of 0.IM citric acid) were determined. Values of I and 0.9 were found.

B had an R_f value of 0.08 in the n-butanol/ammonia paper chromatography system of Algeri and Walker [II4] and was acidic in nature.

The above determined physical properties of B were consistent with it being 5-ethyl-5-(I-methyl-3-carboxypropyl)-barbituric acid, and this assumption was therefore made.

(4) Rate of urinary excretion of A :- The amount of A excreted by five ponies after dosage with 5 mg/kg of sodium pentobarbitone was found by analysing urine samples, as they were voided, until no further A could be detected. The results are given in Table XXVI. The mean amount of A excreted was 33% of the administered dose (n = 7, S.D. 7%).

As ponies VI and VII urinated frequently during the period of urinary excretion of A a curve was constructed relating the amount of A excreted at a given time to the total amount of A excreted. From this curve (Figure XXX) it was obvious that complete urinary excretion of A occurred within 2I hours after oral dosage.

Table XXVI. Total amount of metabolite A excreted by ponies.

Pony	Total urine collected (ml)	Original dose excreted as A (%)
VI	3915	40
VI	2805	30
VII	2660	35
VII	2575	37
V	2450	39
III	2500	20
II	3800	38

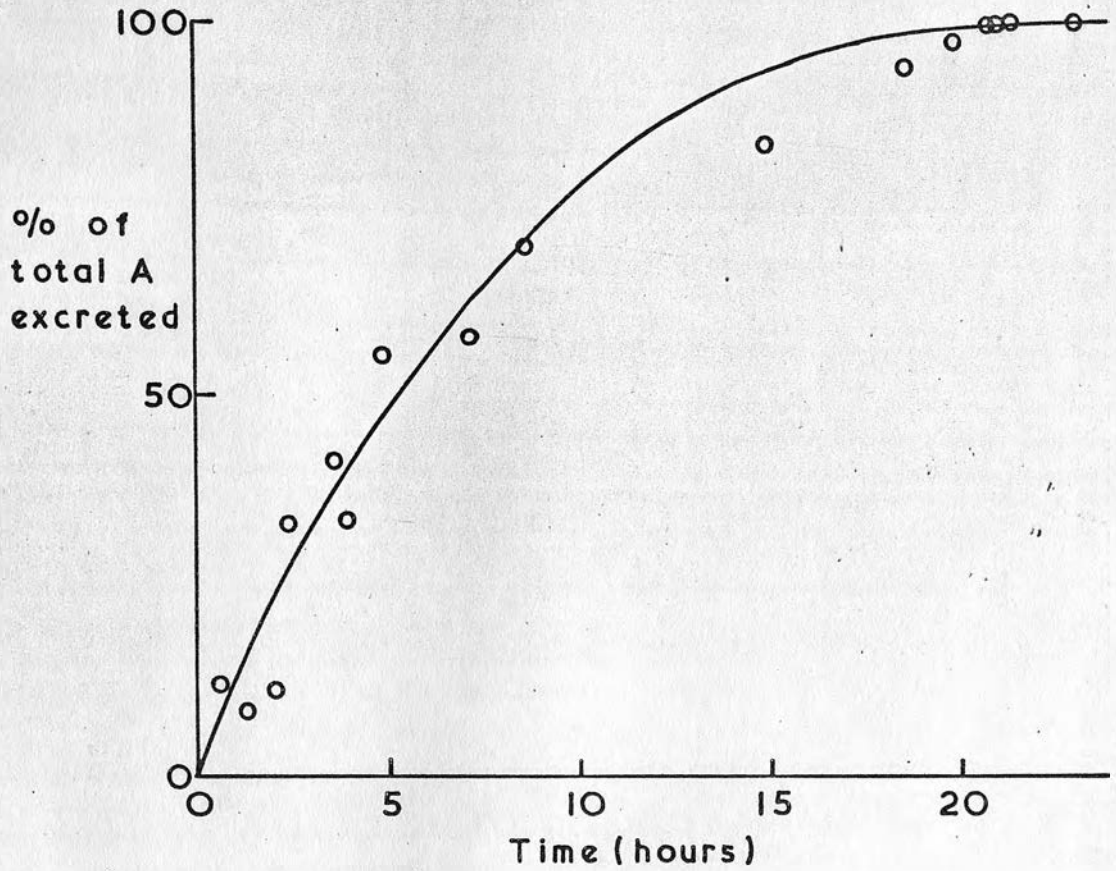

Figure XXX. Rate of urinary excretion of pentobarbitone. Results from four experiments on Ponies VI and VII in which metabolite A excretion was measured.

(5) Glucuronic acid conjugation with A :- Urine obtained from Ponies II, III, and V containing A were analysed normally and following the addition of β-glucuronidase. No significant difference was noted between the two sets of analytical figures from each urine. The conclusion was drawn that A was not conjugated with glucuronic acid in horse urine.

DISCUSSION

From the values obtained for the amounts of the two barbiturates able to be given, with safety, to the ponies it is obvious that the animals showed a greater susceptibility to barbiturates than members of other species. This point may be seen more clearly from the range of dosage values used in the clearance rate experiments, quoted under the appropriate review sections on the metabolism of phenobarbitone and pentobarbitone, given in Table XXVII. It is evident from the figures in Table XXVII that man is the only species mentioned whose dosage rates were similar to those found in the horse. All other species are able to tolerate greater amounts of the barbiturates.

The result of the low dosage rates was the production of relatively low blood concentrations of the drugs, as can be seen from the paper by Rae [55] who, in a study of the fate of pentobarbitone and thiopentone in the sheep, gave a diagram illustrating the relative clearance rates of pentobarbitone in the sheep, rabbit and dog. In the cases of the dog and rabbit, receiving 40 and 30 mg/kg doses respectively, the blood concentrations at one hour were about 30 µg/ml in each case. For the sheep, with a dose of 30 mg/kg, the corresponding concentration was about 12 µg/ml. All three concentrations are greater than those found in ponies, given 10 mg/kg, whose

Table XXVII. Dosage rates of phenobarbitone and pentobarbitone used to determine clearance rates in various species.

Barbiturate	Species	Dosage Rate (mg/kg)
Pentobarbitone	Mouse	50 to I20
	Rabbit	20 to 40
	Rat	30 to 60
	Sheep	20 to 40
	Dog	25 to I00
	Man	I2
	HORSE	I0
Phenobarbitone	Mouse	I50
	Rabbit	400 mg each
	Rat	47 to 90
	Dog	5I to I00
	Man	0.75 g each
	HORSE	8

maximum blood concentration was estimated to be about I2 µg/ml at zero time. A similar comparison for phenobarbitone can be made from the results of Waddell and Butler [25] in which dogs, which had been given a dose of I25 mg/kg, had a blood concentration of about I50 µg/ml after one hour. Ponies receiving 8 mg/kg of phenobarbitone were shown to have a blood concentration of about I2 µg/ml after one hour. Similar results are obtained if dose/blood concentrations of phenobarbitone and pentobarbitone obtained by other investigators, using rabbits,[33] cows and goats [87] and man [234] are used for comparison purposes.

The low blood concentrations of the two barbiturates imposed severe limitations on the analytical technique which was originally designed for use in the forensic analysis of human blood samples where barbiturate concentrations are much higher than those encountered here. Typical blood concentrations in 42 cases of barbiturate intoxication, with various barbiturates, in man have been recorded by Wright [234] and were in the range from I0 to I77 µg/ml with a mean of about 55 µg/ml. With these concentrations an unknown blank value of from, say +I.5 to -I.5 µg/ml would have little effect on the true concentration figure, for example ± 3% at 50 ug/ml. However, with concentrations of less than I0 µg/ml, as found in horse blood, the possible maximum error increases markedly to a possible ± I5% at I0 µg/ml and ± 30% at 5 µg/ml. This error was

largely overcome in the blood clearance experiments by the ability to take a sample of blood before drug administration and so establish a value for the blank at the start of the experiment. It was then assumed that any variation from this value during the experiment would not be great. This facility would clearly not be available in forensic investigations. Limited experiments showed that the value of the blank did not move outside the limits of fresh blood blanks when the blood was allowed to stand for up to 48 hours. Thus for odd samples of blood which are to be analysed, the error due to changing blood blanks will not be greatly affected by short time standing.

As a qualitative method, the differential ultra-violet technique was also restricted in its applicability as can be seen from Figures III and IV in which the absorption of natural chromogens almost masked the typical barbiturate spectra which can be used to qualitatively identify barbiturates.[173] This degree of masking would be less marked at concentrations higher than those encountered in horse blood, but would be almost complete at lower concentrations. It must therefore follow that pentobarbitone and phenobarbitone cannot be qualitatively detected with certainty in horse blood samples using the method as described. The introduction of buffer washes of the chloroform extracts may lower the concentration of interfering chromogens. This was not tried since the presence of barbiturate was known

and no attempt was made to prove this.

That the barbiturates determined in blood were in fact pentobarbitone and phenobarbitone and not their metabolites was assumed. The justification for this assumption, with pentobarbitone, was that metabolic material was demonstrated to be absent from human blood which contained 42 µg/ml of pentobarbitone by Algeri and McBay.[42] Furthermore, the alcohol metabolites of pentobarbitone are insoluble in chloroform and are therefore unlikely to be extracted from blood by that solvent during analysis.[41] With phenobarbitone, Curry [2] has found only the parent drug in human blood, and has shown that p-hydroxyphenobarbitone was much less soluble in chloroform than phenobarbitone and is therefore presumably not so readily extracted by that solvent from blood.[3] Butler [6] found that when the metabolite was given to a dog, by intravenous injection, the greater part of it had disappeared from the plasma within one hour. Since the blood clearance rate of phenobarbitone in dogs was in the order of 20%/day it was concluded that p-hydroxyphenobarbitone would, in all probability, be removed from the blood almost as soon as it was formed. Butler [24] also showed that material determined, in human and dog plasma, as phenobarbitone when distributed between ether and glycine buffers behaved exactly as would pure phenobarbitone. This was considered as evidence of the specificity of the method which used ether to extract

buffered plasma. Since ether would extract p-hydroxypheno-barbitone under the circumstances it must be assumed that the metabolite is not present in significant amounts in human or dog plasma. There was no reason to suspect that the position in the horse was any different, so consequently it was assumed that only phenobarbitone was present in the blood.

When considering the values of blood blanks and the recovery of the two barbiturates by the extraction procedure it was of interest to compare the results with those of Stevenson [172] who used a similar technique to determine barbiturates in human blood. He used butyl ether as the extracting medium to overcome some of the disadvantages of chloroform of which dedomposition by sodium hydroxide, to carbon monoxide whose bubbles interfere with optical density readings, is perhaps the greatest. In the present investigation, the time between extraction of the chloroform with alkali and optical density measurements was sufficiently short for bubble formation to be negligible. Stevenson used a stronger alkali solution, IN compared with 0.45N sodium hydroxide, and consequently the stronger 2.62M ethylenediamine hydrochloride buffer was required in place of the ethylamine buffer which has been described, otherwise the method was basically the same as was used in this investigation.

The values obtained for blood blanks by Stevenson for

humans and those for horses are compared in Table XXVIII.

Table XXVIII. Barbiturate blood blanks for human and horse blood.

Species	Blank (µg/ml)*	S.D.	n
Man	0.5	I.2	I6
Horse	-0.05	I.53	32

* As pentobarbitone

As can be seen from the blank variations in Table XXVIII there is little difference between the two species. Further comparison of the two methods from these sets of results was not considered justified, since small differences in the composition of the blood in horses and in man could introduce a significant unknown factor into the values of the two blank values.

A comparison may readily be made between the recoveries of barbiturate from blood by the two methods and this is done in Table XXIX. Although with each barbiturate the recoveries were similar, the recoveries using chloroform extraction were slightly lower with both barbiturates, and this may be due to the manner in which the extractions were carried out. The

Table XXIX. Comparison of the recovery of barbiturates from the blood of horses and of man.

Species	Barbiturate	Recovery (%)	S.D.	n
Man*	pentobarbitone	97	?	?
Horse**	"	95	6	9
Man*	phenobarbitone	85	?	?
Horse**	"	77	5	7

* Barbiturate concentration 20 and 5 µg/ml.

** Barbiturate concentration 7.5 to 10 µg/ml.

difference between the recoveries of phenobarbitone cannot be due to differing partition coefficients of phenobarbitone between butyl ether and water, and chloroform and water since Stevenson has shown that both have the value of 4. When the amount of each barbiturate present in the unionised form, in a dilute aqueous solution at pH 7.4 (approximate blood pH), is calculated and ionic strengths ignored, it is found that approximately 39% phenobarbitone and 80% pentobarbitone is unionised. If on extraction, with chloroform or butyl ether, some time is required for equilibrium to be reached and the ionised barbiturate to revert to the unionised form and hence be extracted it follows that if the rates of establishment of equilibrium are similar pentobarbitone will, for a short time, exist in a higher concentration in the unionised form than phenobarbitone. Should the period of extraction be less than the time required for establishment of equilibrium then the relative amount of pentobarbitone available for extraction i.e. unionised, will always exceed that of phenobarbitone. This postulation would, therefore, show why the recovery of phenobarbitone was less than that of pentobarbitone if it can be assumed that ionic equilibrium of ionised and unionised barbiturate was not established before completion of the extraction. If the extraction method of Stevenson allows this equilibrium to be nearer completion at the conclusion of the extraction than the chloroform extraction

method then more barbiturate will be unionised and able to be extracted. This is a possible explanation of why, in both cases, extraction using butyl ether gave a higher recovery of barbiturate. The validity of this arguement is supported by the results of Stevenson which showed that for 2I barbiturates the recovery fell as the polarity rose.

For qualitative determination of barbiturates the chloroform extraction method was limited by low concentrations in the horse, since the background absorption of natural chromogens masked the characteristic barbiturate ultra-violet curves. It is to be assumed that the method of Stevenson suffers similarly since insufficient detail was given on this point. However Stevenson's method was carried a stage further to enable partial identification of the barbiturate of barbiturates present in the blood to be established. This was achieved by determining the distribution of the barbiturate material between borax buffers and butyl ether.

When a comparison is made between the results obtained for the clearance rate of pentobarbitone from horse blood and those published for other species it is found that only sheep have a similar clearance rate. The clearance rate for sheep was 49%/hour [6] compared with 46%/hour for the horse. The sheep was much less susceptible to pentobarbitone and could tolerate doses of up to 40 mg/kg i.e. four times greater than the horse. Man

who has a similar dose toleration to the horse had a very low clearance rate of about 4%/hour.[31,46] If the estimated concentration of pentobarbitone in blood at zero time is used to calculate the total quantity of the barbiturate in the blood a value of about I0% of the initial dose is found, assuming a blood volume of about I0%.[235] This would indicate no localisation of pentobarbitone in the blood, and, since the clearance curve was logarithmic from I5 minutes after administration onwards, it follows that the distribution of the barbiturate in the horse was complete within I5 minutes of intravenous administration. these conclusions agree with the results of other workers, as has been dealt with under the review section on pentobarbitone metabolism, who have shown that there is no localisation of pentobarbitone in any single organ or tissue in rats, rabbits, dogs and sheep.

Comparison of phenobarbitone clearance rates in various species with that obtained for the horse is not as straightforward as with pentobarbitone. With other species higher blood concentrations can be obtained than with the horse and, as the clearance rates are slower they can be measured for many hours and the value of the rates determined when the phenobarbitone is at equilibrium with the various organs and tissues. The horse with its low dosage rate tolerance of about 8 mg/kg did not give high blood concentrations of phenobarbitone and because

of its apparent rapid initial clearance rate this could not be followed accurately for any great length of time. It was assumed that the non-logarithmic nature of the phenobarbitone blood clearance was due to the unknown rate of absorption of the barbiturate through the wall of the gut following the oral administration.

In view of the conflicting reports in the literature, as dealt with under the review section on barbiturate protein binding, caution must be taken in comparing the results obtained for the binding of the two barbiturates to horse plasma with those published for other species. Perhaps the only conclusion able to be safely drawn is that pentobarbitone is protein bound in horse plasma to an extent approximately three times greater than phenobarbitone.

That the saliva clearance of the two barbiturates was found to parallel the corresponding blood clearance rates was considered of importance. It was of interest to compare the relative concentrations of the barbiturates in blood and saliva. In the horse there is an equality of pH between blood and saliva [231] which would lead one to suspect that the relative concentrations of barbiturate free to cross the blood-saliva barrier would be the same in both media. From the results given it would appear that the non-plasma protein bound barbiturate exists in equilibrium with the saliva-barbiturate. The same

conclusion was reached by Rasmussen [87] in his work on the parotid salivary secretion of barbiturates in cows and goats. However, in the case of cows and goats the alkaline nature of the saliva (pH about 8.3) would be expected to assist transfer of the unionised barbiturate in the blood to the saliva, where it would ionise and be unable to return to the blood across the epithelium of the saliva duct. This was, in fact, observed with the concentration of the barbiturate in the saliva being greater than the corresponding concentration in the blood, but with the ratio of the saliva-barbiturate concentrations to the blood-barbiturate concentrations remaining constant. Rasmussen considered that lipid solubility and pK_a influenced the final concentration of barbiturate in the saliva, and his results showed that barbiturates with high lipid solubilities and low degrees of dissociation reached the theoretically predicted concentrations in the saliva readily.

Since alkaline and neutral (relative to blood) saliva results in barbiturate concentrations higher or equal to blood-barbiturate concentrations respectively, it would be of interest to observe if acid saliva would have a lower barbiturate concentration than the corresponding blood. This latter postulation would be expected as the position is merely the reverse of Rasmussen's experiment with the saliva acid relative to blood instead of blood acid relative to saliva. Unfortunately

no such report appears to have been made yet in the literature. However, reference [236] has been made to the parotid salivary secretion of sulphonamides in man (saliva pH 5.5 to 7.8 [237]). Like barbiturates, the sulphonamides a weak acids so that a direct comparison of their salivary secretion would appear not invalid. Killman and Thaysen [236] found that sulphonamides were secreted in human saliva in lower concentrations than in the blood. This finding would provide support for the assumption that barbiturates, following suit, would also exist in greater concentration in the blood than in the saliva.

Because of the relatively low concentrations of the barbiturates in horse saliva, a limit was imposed on the applicability of differential ultra-violet spectrophotometry to quantitative and qualitative analysis. Unlike blood samples, it has been shown that the saliva barbiturate blank value was constant despite fluctuations in the saliva flow rate, so that this factor which caused considerable interference in blood analysis did not apply to saliva samples. However, the natural ultra-violet absorbing chromogens in saliva were in sufficient concentration to cause considerable distortion of the characteristic ultra-violet absorption curves of the barbiturate material and so destroy the qualitative accuracy of the method. Although the quantitative nature of the method was high, this was only so by virtue of the availability of large samples. In

practical cases of 'dope' detection the saliva sample size available for analysis would be in the order of a few ml. This would render the method described impracticable if screening tests for several groups of drugs were required. If barbiturates alone were being tested for, the use of small volumes of alkali for the final extraction and the introduction of cuvettes of long path length would doubtless allow some use of the method to be made.

That no connection was found between saliva flow rate and saliva barbiturate concentration, and that saliva flow was not noticeably affected by the barbiturates was of interest when the results of other workers were used for comparison. Guimaris et al.,[84] have shown that provoked salivary secretion in dogs was depressed to varying degrees by different barbiturates. Of the barbiturates investigated, pentobarbitone and phenobarbitone showed the least depressive action and, if the position is the same with the horse, this may explain why no effect on salivary secretion was observed. No report of saliva flow rate inhibition was made by Rasmussen [87] in his work with barbiturates in cows and goats, although he did mention a drop in saliva-pentobarbitone concentration with an increase in saliva flow rate. this latter effect was much less marked than that reported by Burgen [8I] in a general study on the salivary secretion of non-electrolytes. The use of artificial stimulation of salivation

in the dogs used by Guimaris et al.,[84] may not be unconnected with their observation of saliva flow inhibition by barbiturates since both in the present study and in the work by Rasmussen [87] no stimulation was used and no saliva flow inhibition was reported.

The reason for the wide scattering of points, on the graphs showing the clearance rates of phenobarbitone from saliva was uncertain. It was considered not improbable that there was some relation between this scattering and the comparitively large scatter of results obtained for phenobarbitone plasma protein binding relative to the same results for pentobarbitone. The estimated variation of phenobarbitone plasma protein binding, unaccountable for as analytical error, was about ± 10% of the total amount in the blood. There may well be some unconsidered influencing factor in operation, such as the variation in the degree of plasma protein binding with barbiturate concentration as described by Campion and North.[88]

The complete lack of protein binding in saliva of pentobarbitone was in agreement with the results of Rasmussen [87] who found no evidence of protein binding in saliva with either pentobarbitone or phenobarbitone. The slight degree of protein binding with phenobarbitone in horse saliva, compared with the saliva of cows and goats,[87] maybe connected with the 'alkaline' pH of cow and goat saliva relative to the 'neutral' horse saliva,

although Waddell and Butler [25] claimed that binding to bovine serum albumin was almost independent of pH.

All the analytical results quoted, for saliva, have required that it be assumed that the barbiturate determined was in fact the barbiturate administered and not a metabolite or metabolites. The arguements against metabolic material being present in saliva are essentially those already put forward against their presence in blood, since it would not be expected that metabolic material would exist in the saliva and not in the blood.

The isolation of the 5-ethyl-5-(3-hydroxy-I-methylbutyl)-barbituric acids and of 5-ethyl-5-(I-methyl-3-carboxypropyl)-barbituric acid from the urine of horses receiving oral doses of pentobarbitone showed that the metabolism of the drug in the horse follows a similar pattern as in man, dog and rat and which has been reviewed earlier. The amount of free alcoholic metabolite excreted in the horse (33% of the administered dose) was approximately half of that excreted in the dog [44] and less than that in man (about 50% of the administered dose). Since no glucuronate was detected it was considered possible that the remainder of the pentobarbitone could be excreted as the carboxylic acid metabolite or as a sulphate or other conjugate.

Due to the difficulty involved in quantitatively determining the acid metabolite, because of the large amount of

naturally occuring interfering compounds, this was not carried out. From the intensity of the appropriate spot on paper chromatograms of urine extracts, when treated with the cobalt chloride/ammonia reagent, it was surmised that a larger amount of the acid was present than in dog urine (4.5% of the administered dose [44]). This was considered to be not impossible since *in vitro* experiments of Cooper and Brodie [59] have shown that rabbit and rat enzymes produce about equal amounts of mixed alcohols and acid.

Little work appears to have been reported on the rate of urinary excretion of pentobarbitone metabolites. Those reports which have appeared have been based on tracer analysis [39,44,66,76] which would suggest that the difficulty in determining the metabolites chemically has proved a stumbling block. A further difficulty is doubtless in obtaining a suitable standard sample of metabolites. Due to the greater solubility of the alcohols in water than in ether and other solvents commonly used for extraction conditions must be modified for complete extraction. Such modifications, as using more suitable extracting solvents, with or without ammonium sulphate as a 'salting-out' agent, resulted in the extraction of interfering naturally occurring compounds which destroyed completely the applicability of ultra-violet spectrophotometry in the present study. Subsequent removal of such compounds was not readily carried out and if so

done removed the quantitative nature of the method. The complexometric method of Lubran overcame the difficulties incurred in the ultra-violet spectrophotometric determination and provided a rapid and convenient method of determining the alcohols when used in the form described in this work.

Determination of the acid metabolite in urine by the modified method of Lubran was not successful. Ommision of the sodium bicarbonate (which removed the acid) used in drying the ether allowed material in normal urine to reach the final reaction stage. This caused sufficient variation in the normal urine blank to completely invalidate quantitative analysis. The difficulties involved in purifying the extracted acid sufficiently for ultra-violet determinations have been reported.[42,60] It was found that the isolated acid did react positively when added to the final stage of the complexometric determination.

Insufficient data exist in the literature to allow a comparison of the urinary excretion rates of phenobarbitone in the horse with those for other species. The only conclusion that may be drawn from the results of the present study is that the total amount of phenobarbitone excreted by the horse was of similar magnitude to that in man,[5,26,34] dog [35] and rat.[8] The greater part of the phenobarbitone excreted appeared during the first 72 hours.

Of interest was the effect of vigorous exercise on the urinary excretion of phenobarbitone by the horse. As experiments along such lines were impracticable only speculation was possible. Beckett et al.,[238] in an investigation on the detection of drugs in urine, which may have been used to modify performance in sport by man, remarked on the fact that vigorous exercise resulted in the production of an acid urine. It has also been claimed [25,28] that a decrease in human urinary pH results in a decrease in the excretion of phenobarbitone. If the position in man is duplicated in the horse it would follow that a horse which had been vigorously exercised, as in a race, would excrete less phenobarbitone than the results of this investigation would suggest.

The isolation of only p-hydroxyphenobarbitone as a urinary metabolite of phenobarbitone was in accordance with the results of other investigators in dog,[1,6] man [2,3,4,5,6] and rat.[7,8] Perhaps of greater significance was the conjugation of the metabolite with glucuronic acid. In this instance the horse resembles the dog. Both man and rat excrete the metabolite unconjugated or only partly conjugated with glucuronic acid. That the metabolite was conjugated presented considerable difficulties in its determination. Although β-glucuronidase provided an easy means of liberating the metabolite it was both expensive and time consuming. Acid hydrolysis of the conjugate

was rapid but resulted in the decomposition of much material in the urine so that many stages of purification were then required with a resultant loss in quantitative accuracy. For these reasons no attempt was made to quantitatively determine the p-hydroxyphenobarbitone.

Of considerable importance was the difficulty encountered in detecting phenobarbitone in urine. Most analytical methods in use are for applications involving humam urine and have not been tested on horse urine. Differences in diet between man an horse will also doubtless affect the normal urinary constituents. These factors appear therefore to limit any possibility of detecting phenobarbitone in horse urine during routine screening for drugs in general. However the modified complexometric method of Lubran, which has been described, offers a rapid means of screening for phenobarbitone and also for barbiturates in general. Only a limited number of drugs appear to react positively to the test as originally described by Lubran. If this method is used to quantitatively determine phenobarbitone care will have to be taken with respect to the rapid fading of the colour which was experienced in this work and was not remarked on in the original paper by Lubran.

ACKNOWLEDGMENTS

This research was carried out under the supervision of Dr. F. Alexander of the Department of Veterinary Pharmacology to whom I am indebted for continued help and guidance and for allowing use to be made of his ponies in the various experiments which have been described.

Thanks are also due to Mr. M.S. Moss of the Equine Research Station at Newmarket for his introduction to some of the analytical techniques involved in the analysis of biological samples and for generously sparing time to record various infra-red spectra.

I wish to thank Mr. I.S. Warwick for technical assistance and for the construction of apparatus used in some of the experiments.

Other individuals to whom grateful thanks are due include :-

Mr. B.S. Finkle for supplying unpublished information on the analysis of blood for barbiturate.

Mr. B. Mitchell for assistance in the pentobarbitone blood clearance experiments.

Mr. Y.C. Dickert for the generous supply of a sample of 5-ethyl-5-(3-hydroxy-I-methylbutyl)barbituric acid.

Members of the Department of Statistics for their advice on interpreting phenobarbitone blood and saliva clearance rate results.

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