PHARMACOKINETICS AND METABOLISM OF BARBITURATES

TJONG DING YIH

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Promotor: Prof. Dr. J.M. van Rossum.

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CONTENTS

LIST OF ABBREVIATIONS OF THE BARBITURATES USED

Allo	: allobarbital, 5,5-diallyl barbituric acid
Apro	: aprobarbital, 5-allyl-5-isopropyl barbituric acid
Bral	: brallobarbital, 5-allyl-5-(2-bromoallyl) barbituric acid
m-Bral	: 1-N-methyl-5-allyl-5-(2-bromoallyl) barbituric acid
Dm-Bral	: 1,3-N,N'-dimethyl-5-allyl-5-(2-bromoallyl) barbituric acid
Butyl	: butobarbital, 5-ethyl-5-butyl barbituric acid
Dm-But	: 5-ethyl-5-(2,3-dimethylbutyl) barbituric acid
Cyclo	: cyclobarbital, 5-ethyl-5-(1-cyclohexen-1-yl) barbituric acid
m-Cyclo	: 1-N-methyl-5-ethyl-5-(1-cyclohexen-1-yl) barbituric acid
Dm-Cyclo	: 1,3-N,N'-dimethyl-5-ethyl-5-(1-cyclohexen-1-yl) barbituric acid
Cyclop	: Cyclopal^{R}, 5-allyl-5-(2-cyclopenten-1-yl) barbituric acid
m-Cyclop	: 1-N-methyl-5-allyl-5-(2-cyclopenten-1-yl) barbituric acid
Eunarc	: Eunarcon^{R}, 1-N-methyl-5-(2-bromoallyl-5-isopropyl) barbituric acid
Hepta	: heptabarbital, 5-ethyl-5-(1-cyclohepten-1-yl) barbituric acid
m-Hepta	: 1-N-methyl-5-ethyl-5-(1-cyclohepten-1-yl) barbituric acid
Dm-Hepta	: 1,3-N,N'-dimethyl-5-ethyl-5-(1-cyclohepten-1-yl) barbituric acid
Heptyl	: 5-ethyl-5-heptyl barbituric acid
m-Heptyl	: 1-N-methyl-5-ethyl-5-heptyl barbituric acid
Hexo	: hexobarbital, 1-N-methyl-5-methyl-5-(1-cyclohexen-1-yl) barbituric acid
m-Hexo	: 1,3-N-dimethyl-5-methyl-5-(1-cyclohexen-1-yl) barbituric acid
Hexyl	: Ortal^{R}, 5-ethyl-5-hexyl barbituric acid
m-Hexyl	: 1-N-methyl-5-ethyl-5-hexyl barbituric acid
Dm-Hexyl	: 1,3-N,N'-dimethyl,5-ethyl-5-hexyl barbituric acid
Hydroxy-Hexo	: 1-N-methyl-5-(1-cyclohexen-1-yl-3-hydroxy) barbituric acid
Keto-Hexo	: 1-N-methyl-5-(1-cyclohexen-1-yl-3-keto) barbituric acid
Neal	: nealbarbital, 5-allyl-5-neopentyl barbituric acid
Noct	: Noctal^{R}, 5-(2-bromoallyl)-5-isopropyl barbituric acid

Nonyl : 5-ethyl-5-nonyl barbituric acid

m-Nonyl : 1-N-methyl-5-ethyl-5-nonyl barbituric acid

Nor-Hexo : 5-methyl-5-(1-cyclohexen-1-yl) barbituric acid

Octyl : 5-ethyl-5-octyl barbituric acid

m-Octyl : 1-N-methyl-5-ethyl-5-octyl barbituric acid

Pentyl : 5-ethyl-5-pentyl barbituric acid

i-Pentyl : amobarbital, 5-ethyl-5-(3-methylbutyl) barbituric acid

s-Pentyl : pentobarbital, 5-ethyl-5-(1-methylbutyl) barbituric acid

Pern : Pernocton^{R}, 5-(2-bromoallyl)-5-(1-methylpropyl) barbituric acid

m-Pern : 1-N-methyl-5-(2-bromoallyl)-5-(1-methylpropyl) barbituric acid

m-Pheno : Prominal^{R}, 1-N-methyl-5-ethyl-5-phenyl barbituric acid

Propryl : 5-ethyl-5-propyl barbituric acid

Rect : Rectidon^{R}, 5-(2-bromoallyl)-5-(1-methylbutyl) barbituric acid

Rep : Reposal^{R}, 5-ethyl-5-(bicyclo-3,2,1-oct-2-en-2-yl) barbituric acid

m-Rep : 1-N-methyl-5-ethyl-5-(bicyclo-3,2,1-oct-2-en-2-yl) barbituric acid

Seco : secobarbital, 5-allyl-5-(1-methylbutyl) barbituric acid

Tal : Talbutal^{R}, 5-allyl-5-(1-methylbutyl) barbituric acid

Vinylbital : 5-vinyl-5-(1-methylbutyl) barbituric acid

The total body clearance is the kinetic parameter characterizing the elimination of drug from the body. Especially for lipophilic drugs the liver plays an important role in the elimination so that for many drugs the total body clearance equals the metabolic clearance.

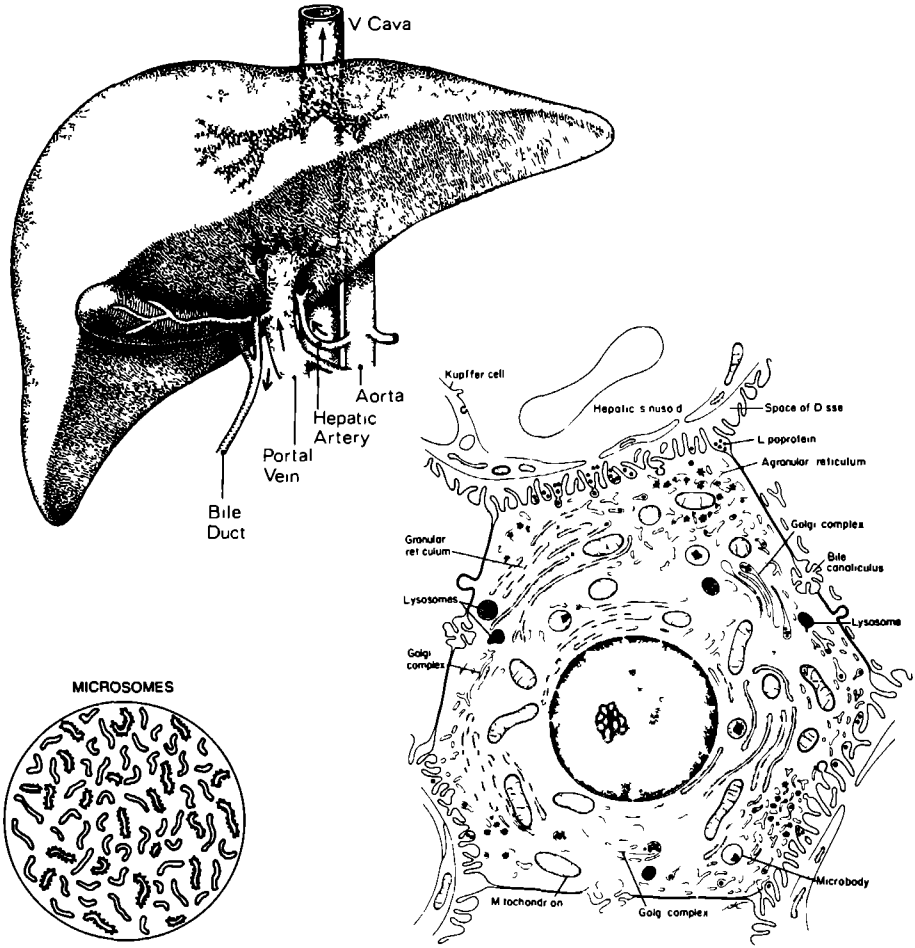
The metabolic clearance depends on the kinetics of the metabolic enzymes in the liver. This implies that there must be a relationship between the parameters governing enzymatic conversion of drugs in the liver and the metabolic clearance or total body clearance.

The object of this study is to explore the relationship between total body clearance *in vivo* and enzymatic processes *in vitro*. For this purpose the kinetics of series of barbiturates were studied in the intact rat and preparations of rat liver. The *in vitro* methods range from studies on the intact perfused rat liver via isolated liver cells (hepatocytes) to liver microsomes and the enzyme cytochrome P 450. See figure.

These studies allow us to gain information on the relevance of blood flow through the liver, the rate of penetration of drugs within the liver cells, access to the microsomes and finally binding to and biotransformation by the liver enzymes with respect to the rate of metabolic degradation.

The barbiturates were chosen because the compounds are strongly related but nevertheless they show diverse pharmacological properties. The discovery of the hypnotic properties of 5,5-diethyl barbituric acid (barbital) by Fischer and Von Mehring in 1903 led to the synthesis of numerous barbituric acid derivatives. Numerous barbiturates and barbiturate combinations have been introduced into practice as hypnotics, sedatives and anaesthetics (Bobranski, 1972). The consumption of barbiturates amounts still to enormous proportions so that many cases of barbiturate poisoning and fatalities have been reported.

In early studies with rats and mice it has been found that the "hypnotic effect", often expressed as minimum anaesthetic dose (M.A.D.), could be changed markedly by varying the substituents. See review by Doran (1959), which covers the period before 1956. The hypnotic activity in rats is different from the hypnotic potency in man. It is, however,



Survey of the metabolic systems studied in this thesis. Liver, hepatocyte and schematic representation of the microsomes

not a simple parameter but the result of complex processes, as the relationship between blood concentration and sensitivity condition, which vary with species (Stolman, 1960) and sex (Stevenson, 1974) and drug properties such as intrinsic activity, distribution through the body and especially the rate of elimination. Recent work, therefore, has been directed to biotransformation of barbiturates (Maynert, 1954; Tsukamoto, 1958). The relationship between pharmacological effect, kinetic behaviour and drug metabolism has, however, not yet been studied.

The barbiturates are eliminated mainly by metabolic conversion and the rate of elimination varies tremendously among the individual compounds. In addition homologous series are available or within our synthetic reach, so that also the relationship between chemical structure and rate of elimination could be studied.

New methods in blood sampling in rats, preparation of hepatocytes and sensitive analytical techniques have been developed to ensure the analysis of blood concentration curves in individual rats and the study on a pool of isolated liver cells. As a result the relationship between pharmacological effect and the blood concentration profile could be studied and the relationship mentioned could be investigated. Such experiments cannot be done with the common techniques of using pooled data from groups of rats.

Finally, the relevance for drug treatment in humans has been discussed, especially the extrapolation of results in rats to humans.

SECTION I

PHARMACOKINETICS

CHAPTER 1

KINETICS OF DRUG DISTRIBUTION AND ELIMINATION

INTRODUCTION

Drugs introduced into the body of man or animals by enteral or parenteral routes are distributed over the entire body and reach their target tissues by means of the blood circulation. The drug should reach a sufficiently high concentration at its site of action to cause a pharmacological response. The interaction of drug molecules with the receptors is probably a fast process (Ariëns, 1964). This implies that equilibrium is reached at the receptors although the concentration of drug in the environment of the receptors may change because of absorption and elimination processes going on.

The time course of the concentration of drug near the receptors and hence the profile of the drug concentration curve in the blood governs the intensity of the pharmacological response as a function of time. In the intact animal or in man the situation may be more complex since different types of receptors may be activated by the same drug and the various effects may mutually influence each other.

The blood concentration profile depends on the kinetic process, absorption, distribution and elimination, which occur simultaneously. The blood concentration curve can be characterized by a number of kinetic parameters if an appropriate model of the body is used. The body may be considered to exist of a number of compartments while there is exchange of drug between the blood and these compartments and elimination from the blood to the environment. Certain compartments show similar properties so that they may be grouped together. A two compartment model in general provides a good description of blood concentration curve. See Fig. 1.1a. Sometimes a single compartment suffices and this is also what one expects for a suspension of hepatocytes. See Fig. 1.1b. The volumes of the compartments and the clearance constants governing drug transport are the important parameters. Following oral administration the drug is deposited in the gastro-intestinal tract, which is a compartment outside the body.

See Fig. 1.1c. The elementary equations for these three cases will briefly be described. Extensive information on this subject can be found in the literature (Dost, 1968; Van Rossum, 1971; Riggs, 1963).

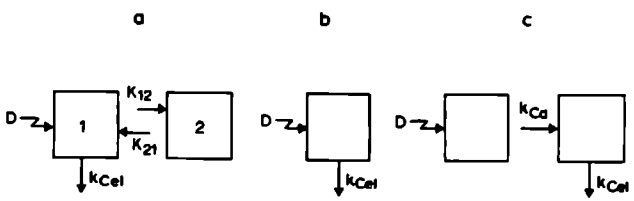


Fig. 1.1 *Block schemes of respectively the open two-compartment model, the one-compartment model and oral administration (see text). Elimination is assumed to take place from the central compartment.*

THE OPEN TWO-COMPARTMENT MODEL

Pharmacokinetic studies have shown that the elimination of many drugs, following intravenous administration, is not a simple exponential process. A graph of the logarithm of the drug concentration against the time often shows at first a steep decline, followed by a less rapid linear phase. It has been found that the two-compartment model is sufficient to describe such a curve.

In the open two-compartment model it is assumed that there is a central compartment which represents the fluids and tissues which are

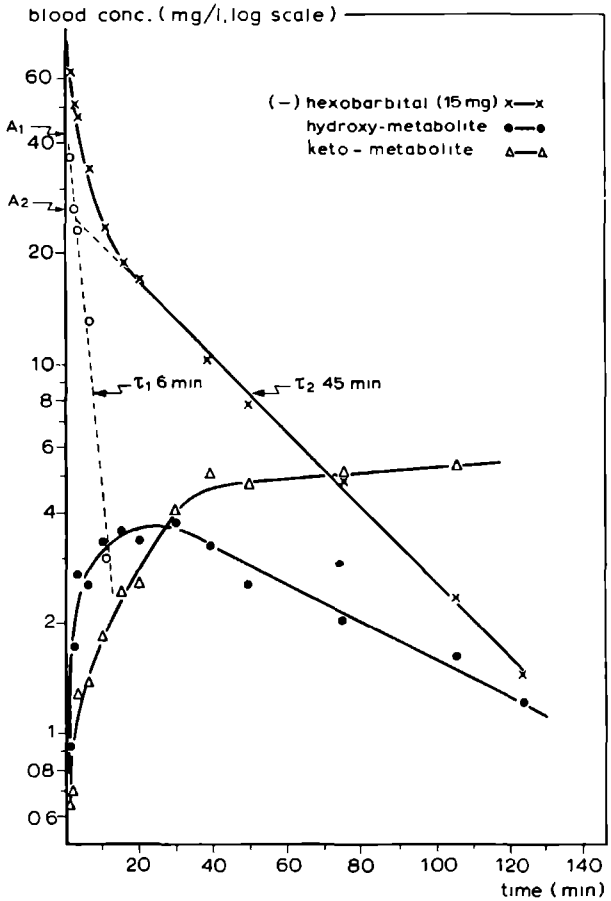


Fig. 1.2 *Blood concentration-time curve following intravenous administration in a rat. The dotted line (O---O), which represents the first term of the two-term exponential equation, can be obtained by subtracting the blood concentration values of the extrapolated elimination phase from the corresponding values on the solid line. A_1 and A_2 represent the zero-time intercepts of respectively the distribution and the elimination components of equation 1. The zero-time intercept of the biphasic curve is the sum of A_1 and A_2. It had to be noticed that metabolites appear already after 1 min, so during the distribution simultaneously metabolism occurs.*

vascularized so that they instantaneously have the same concentration of drug as the blood and a second, or peripheral, compartment which represents the fluids or tissues which are filled up at a slower rate. Elimination is assumed to take place from the central compartment because the liver and the kidney are highly vascularized. Drug transport is governed by the following differential equations:

$$V_1 \frac{dC_1}{dt} = - (k_{Ce1} + k_{C12})\ C_1 + k_{C21}\ C_2 \qquad (1)$$

$$V_2 \frac{dC_2}{dt} = - k_{C21}\ C_2 + k_{C12}\ C_1 \qquad \frac{dQ_{e1}}{dt} = - k_{Ce1}\ C_1 \qquad (2)$$

Here are V_1 and V_2 the volume of the compartments, k_{C12}, k_{C21} and k_{Ce1} clearance constants and C_1 and C_2 the concentration of drug in the central and peripheral compartment. These equations lead to the following bi-exponential equation for the concentration in the blood:

$$C_t = A_1\ e^{-t/\tau_1} + A_2\ e^{-t/\tau_2} \qquad (3)$$

where C is the blood concentration in the central compartment at any time (t) after the administration, τ_1 and τ_2 are time constants and A_1 and A_2 the zero-time intercepts of the two components of the bi-exponential curve (Fig. 1.2). The time constants are complex parameters which include all the clearance constants and volumes given in Fig. 1.1a. It is common practice to relate the biological half-life to the largest of the two time constants ($t_{\frac{1}{2}} = 0.69\ \tau_2$). From the four experimental parameters the most important pharmacokinetic parameters for a drug in the body may be calculated, using the following equation (Van Rossum, 1971):

$$k_{Ce1} = D/(A_1\tau_1 + A_2\tau_2) \qquad (4)$$

$$V_f = \frac{D\ (\tau_1^2 A_1 + \tau_2^2 A_2)}{(\tau_1 A_1 + \tau_2 A_2)} \qquad V_1 = D/(A_1 + A_2) \qquad (5)$$

where D is the dose and V_f is the apparent volume of distribution, that is the volume that would occur if under steady conditions in V_2 the same

concentration would exist as in V_1.

In our experiments with intact rats bi-exponential curves were encountered which have been analysed according to the two-compartment model.

THE OPEN ONE-COMPARTMENT MODEL

When the rate of distribution largely exceeds the rate of elimination, there is instantaneously mixing of the drug over the entire body. In this case a single compartment suffices to describe the drug profile curve. The differential equations reduce according to the model of Fig. 1.1b.

$$V_1 \frac{dC_1}{dt} = - k_{Ce1} C_1 \qquad \frac{dQ_{e1}}{dt} = k_{Ce1} C_1 \tag{6}$$

The blood levels decrease according to mono-exponential equation

$$C_t = A e^{-t/\tau_{e1}} \tag{7}$$

where A is the blood concentration at zero-time and τ_{e1} the elimination time constant. Now $V_f = V_1$. The pharmacokinetic parameters, the elimination, clearance and the volume of distribution are:

$$k_{Ce1} = D/A \tau_{e1} \qquad V = D/A \tag{8}$$

In our experiments with the isolated perfused liver, the hepatocytes and microsomes the concentration curves decrease in a mono-exponential fashion and have been analysed according to the one-compartment system.

ORAL ADMINISTRATION

In contrast with intravenous administration drugs administered orally will not reach the general circulation immediately, but first undergo several processes such as transport to the absorption site and the actual absorption. Consequently drug concentration in the blood will be initially equal to zero and increase to a maximum level as a result of drug ab-

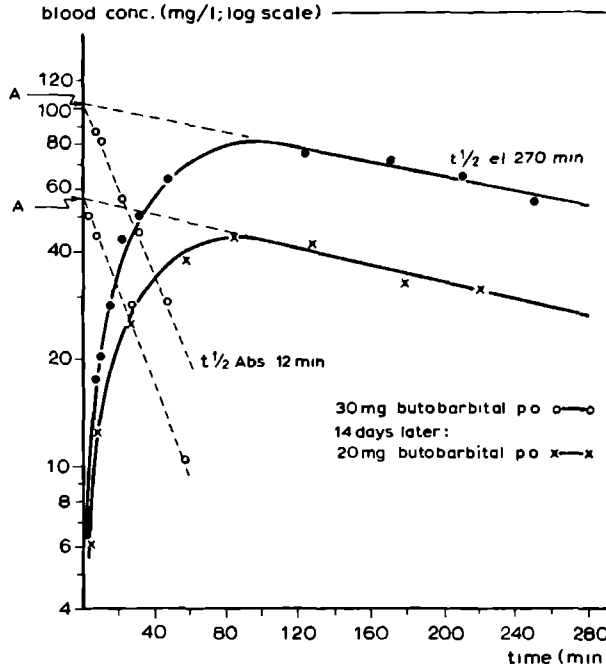


Fig. 1.3 *Blood concentration-time curves of butobarbital after oral administration of respectively 30 and 20 mg. The dotted line (o---o) represents the absorption phase and is obtained by subtraction of the blood concentration data during the absorption phase from the corresponding values on the extrapolated elimination phase. Note that in these two experiments both the absorption and the elimination time constant have the same value, indicating the reproducibility of experiment in the same animal.*

sorption. The overall rate at which the drug reaches the general circulation (absorption rate) may be determined by measuring blood levels at frequent intervals after the drug administration (Fig. 1.3). The time course of the blood concentration can be described by a three-term exponential equation. In many cases absorption is much slower than the distribution over the body so that the distribution phase is overshadowed and the body may be considered as a single compartment. The differential equations according to Fig. 1.1c are:

$$V_o \frac{dC_o}{dt} = - k_{Ca} C_o \tag{9}$$

$$V_1 \frac{dC_1}{dt} = k_{Ca} C_o - k_{Cel} C_1 \qquad \frac{dQ_{el}}{dt} = k_{Cel} C_1 \tag{10}$$

Here V_o is the volume of the zero-compartment and k_{Ca} the absorption clearance. The solution is a bi-exponential equation:

$$C_t = A (e^{-t/\tau_{el}} - e^{-t/\tau_a}) \tag{11}$$

A priori one has no notion whether the entire dose is absorbed. The value of A depends on the amount that is absorbed, the apparent volume of distribution and the time constants τ_{el} and τ_a. In formula:

$$A = \frac{F.D}{V_f} \frac{\tau_{el}}{(\tau_{el} - \tau_a)} \tag{12}$$

where F is the bioavailability or the fraction of the dose that is absorbed. In case of oral studies the clearance and the volume of distribution are overestimated if $F < 1$.

Our experiments following oral administration are analysed according to the model of Fig. 1.1c, unless a distribution phase is apparent in the curves.

FLOW-LIMITED ELIMINATION OF DRUGS

Generally the liver is responsible for the metabolic clearance of drugs. Therefore, it is evident that metabolic clearance will be limited by the hepatic blood flow. On the other hand the metabolic clearance, however, is also determined by the capacity of the enzymes and the affinity of the drugs for them. The relationship between this real metabolic clearance (k_{Cm}) and the liver blood flow (k_f), resulting in an apparent metabolic clearance ($k_{Cm\ app}$), can be given by the following equation:

$$k_{Cm\ app} = k_f\ (1 - e^{-k_{Cm}/k_f}) \tag{13}$$

When k_{Cm} is large in comparison with the liver blood flow, the apparent metabolic clearance approaches the liver blood flow. If the liver blood flow is large in comparison with the real metabolic clearance, the exponential term in eq. 13 is small, so that this term may be developed in a series of terms according to a McLaurin series (Nagashima, 1968), resulting in the following equation:

$$k_{Cm\ app} = k_{Cm}\ (1 - k_{Cm}/2k_f) \tag{14}$$

When $k_f \gg k_{Cm}$ the apparent metabolic clearance approaches the real metabolic clearance.

CAPACITY-LIMITED ELIMINATION

As mentioned previously the metabolic clearance is also determined by the capacity of the liver enzymes. Enzymatic conversion may be described by the following equation:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + \text{Products} \tag{15}$$

where E, ES and S are respectively: the enzyme, the enzyme-substrate complex and the substrate. Furthermore, per definition:

$$K_m = \frac{k_2 + k_3}{k_1} \quad \text{and} \quad K_s = \frac{k_2}{k_1} \tag{16}$$

where K_m is the Michaelis-Menten constant. Assuming that the substrate concentration in the clearance tissue equals the blood concentration, the relationship between the metabolic clearance and the metabolic enzymes may be given by:

$$k_{Cm} = \frac{Q_m}{K_m + C} \tag{17}$$

where k_{Cm}, Q_m and K_m are respectively: the metabolic clearance, the metabolic capacity of the enzymes and the Michaelis-Menten constant.
The metabolic clearance, therefore, is not a constant but is essentially dependent on the concentration. However, for low drug concentrations ($C \ll K_m$) the metabolic clearance is a constant:

$$k_{Cm} = Q_m/K_m \tag{18}$$

For most drugs the clearance is a constant in the therapeutic dosage range but it is evident that the clearance essentially will become dose-dependent at higher doses. This may be encountered in case of intoxications or suicide attempts with barbiturates.

PHARMACOKINETIC ANALYSIS

Pharmacokinetic analysis of the barbiturate blood and perfusate levels was accomplished by fitting the experimental data to equation 3 or 7 with the aid of Farmfit, a non-linear regression computer program. From the fitted A and τ values the pharmacokinetic constants were calculated (detailed information on this program is given by Breimer, 1974).

CHAPTER 2

ENZYME KINETICS ON HEPATOCYTES AND MICROSOMES

The enzyme parameters V_m and K_m (or K_s) can be calculated from experiments in which the rate of formation of a metabolite or eventually the rate of decrease of substrate is measured by using various substrate concentrations and a constant amount of enzyme. By a double-reciprocal plot denoted on the Lineweaver-Burke plot the maximum rate V_m and the Michaelis-Menten constant K_m can be found from the intercept with the ordinate and the abcis respectively. In formula:

$$\frac{1}{V} = \frac{1}{V_m} + \frac{K_m}{V_m} \cdot \frac{1}{S} \tag{19}$$

This equation is only valid in the steady state. So it is essential that the rate is linear with the time and the activity proportional to the enzyme concentration. However, the conditions in such microsomal suspension may differ considerably from the *in vivo* situation or in the hepatocytes suspension because in both these cases transport processes from blood to the endoplasmatic reticulum may have a large influence. Despite these uncertainties, it is possible to calculate the enzyme parameters *in vivo*, provided that the drug concentration is high enough ($C > K_m$, see chapter 1). In a microsomal suspension the cellular integrity is lost. It is, therefore, of advantage to calculate enzyme constants in intact cells or in isolated intact organs.

The decline in the drug concentration as function of time is given by the following equation, which holds also true for drug concentration in the order of magnitude of K_m (Van Ginneken, 1974):

$$\ln C = \ln A + \frac{A - C}{K_m} - \frac{t}{\tau_{el}} \tag{20}$$

where C and A are respectively: the concentration at any time (t) and the initial concentration extrapolated to zero-time. When the drug concentration drops to such levels that $C < K_m$ by metabolic conversion the equation reduces to the well-known linear equation. This implies that from

the straight part of the curve at low concentration τ_{el} can be calculated and from the non-linear part also K_m.

It is now possible to correlate the K_m values calculated from enzyme experiments as well as from liver microsomes, hepatocytes and intact liver preparations. Deviations may be due to transport barriers which may be different for the different barbiturates. A further correlation is possible with the binding (K_s) to cytochrome P 450, the enzyme ultimately responsible for enzymatic conversion.

It has been shown that drugs reacting with cytochrome P 450 cause a characteristic spectral change of the enzyme (Remmer, 1966), while these spectral changes are due to binding to the enzyme (Schenkman, 1967). From a plot of the spectral difference against the concentration of drug it is possible to calculate K_s.

The relationship between k_{Cel} or τ_{el} and K_m (or K_s) dictates that the smaller K_m, the smaller τ_{el}. It is then possible to gain rapidly information from *in vitro* experiments whether difficulties might be expected *in vivo*.

Since we have included in our study homologous series of barbiturates it is also possible to study the influence of gradual changes in the molecule on the enzyme constants and elimination rate constants as well as on their mutual connection.

SECTION II

MATERIALS AND METHODS

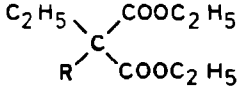
CHAPTER 3

SYNTHESIS OF BARBITURATES

Barbiturates not commercially available were synthesized by condenzing the appropriate ethyl-alkyl diethyl malonates with urea (Fischer, 1904). The malonates were prepared from diethyl malonate (Fluka A.G., Switzerland) and the alkyl bromides according to the method of Shonle (1930). Their boiling points are listed in Table 3.1.

TABLE 3.1

Boiling points of some alkyl substituted diethyl malonates, used for the synthesis of 5-ethyl-5-alkyl substituted barbiturates



R	mol.wt.	B.P.
Pentyl	202	92- 94°C/0.5 mm
Hexyl	216	114-116°C/1.0 mm
Heptyl	230	130-131°C/1.0 mm
Octyl	244	110-112°C/0.4 mm
Nonyl	258	162-164°C/1.5 mm

N-metylated barbiturates were prepared by methylation with dimethylsulphate (B.D.H. Chemicals Ltd., England) according to the method of Martin (1966). Both the mono-methyl and the dimethyl derivatives were obtained, which can be separated easily. All barbiturates were crystallized from ethanol/water (for melting points see Table 10.2). The methyl

derivatives of the 5-ethyl-5-alkyl series separated from the recrystallization solvent as an oil.

The barbiturates were checked on purity by G.C. and G.C. combined mass spectrometry (L.K.B. 9000). The purity was more than 99%. All other reagents were of analytical grade. All barbiturates were applied as their sodium salts by dissolving the free acid in 1 m-NAOH and adjusting to pH 9.0 with 1 m-HCl, except the dimethyl derivatives, which were suspended in perfusate solution (see chapter 5).

CHAPTER 4

INTACT ANIMAL EXPERIMENTS

ANIMALS

Male Wistar rats, weighing 200-300 g, were used. The animals were kept on a standard laboratory diet and fasted overnight before the experiment, but had free access to water.

ADMINISTRATION

Intravenous administration was performed by injecting the barbiturates into the dorsal penis vein. Applied in this way the drug reaches the general circulation rapidly (Nightingale, 1971). A wrongly applied injection is easily discovered by the swelling of the penis, whereas in the more commonly used tail injection control is more difficult. The mean injection time was approximately 10 sec. Oral administration was carried out with a stomach tube.

SAMPLING

At regular intervals blood samples of 0.3-0.4 ml were taken by orbita punction, using Hawksley heparinized micro-haematocrit centrifuge tubes (Cat.no. A 803). The blood was collected in tubes containing one drop of heparine. This method of sampling is very suitable for obtaining small blood samples rapidly, which is important in following the fast distribution of a drug after intravenous administration. Detailed information on this sampling method can be found in the literature (Halpern, 1951).

SLEEPING AND LATENTION TIMES

Sleeping times were determined by measuring the time between the loss and the regain of the righting reflex. Latention time was considered as the time between the injection and the loss of the righting reflex.

EVALUATION OF THE METHOD

Pilot experiments in which the time course was followed for a long period (3-4 times the apparent half-life) showed that the terminal phase was logarithmic linear. Therefore, the number of samples during this period can be restricted. It is sufficient to take 3-4 samples during the first 10 min and 5-6 during the disposition phase to characterize the blood concentration-time curve.
Since there were considerable variations in metabolic activity between the animals, series of barbiturate homologues were studied in the same animal. The barbiturates were administered to the same animal in random order with interims of two weeks. Moreover, in each series of experiment a reference compound was included (see chapter 5).

CHAPTER 5

THE ISOLATED PERFUSED RAT LIVER

PERFUSION APPARATUS

The perfusions were mainly performed according to the method of Miller et al. (1951). In Figure 5.1 a schematic drawing is shown of the recirculating perfusion system used. The essential operating principles are as follows: the perfusion medium is brought to the top of the oxygenator by means of a roller pump. A filter, obtained from a disposable blood transfusion set, prevents clots of blood from entering the circulation. The perfusate flows down as a film along the walls of the oxygenator and is collected at the bottom. From the bottom of the oxygenator the perfusate is supplied to the liver, while the excess of medium runs through the bypass to the reservoir. The oxygenator was adjusted to give a hydrostatic pressure of 20 cm H_2O. Thus a constant liver blood flow of about 10-15 ml/min.g liver was maintained. The perfusate leaving the liver by the vena cava reaches the reservoir passing a calibrated tube and is again sucked into the pump. The perfusate is oxygenated with a counter current stream of a moistened mixture of 95% O_2 and 5% CO_2, which enters the reservoir, then reaches the oxygenator via the bypass and leaves the oxygenator at the top. The whole system is placed in a thermostatically controlled cabinet (temperature 39°C).

Samples of about 0.7 ml were drawn with a 1 ml disposable syringe from the connection tube between the reservoir and the pump. The liver blood flow was determined by measuring the time needed to fill the calibrated cuvet below the liver, which was placed on a perforated sheet in the organ chamber. At regular times the pH; pO_2 and pCO_2 before and after the passage through the liver were measured.

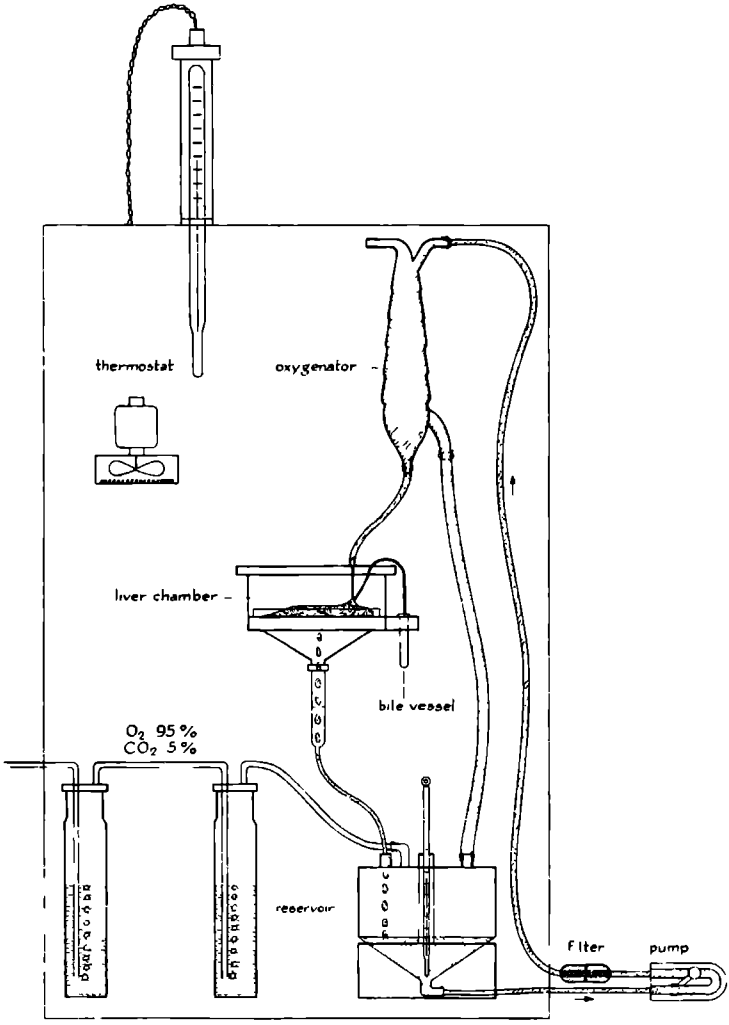


Fig. 5.1 *Schematic drawing of the apparatus used for the isolated perfused rat liver*

OPERATIVE TECHNIQUE

The liver donor rats were anaesthesized with ether, the abdomen was opened via a mid-line incision. After cutting the oesophagus between ligatures, the bile duct was located and cannulated with a polyethylene tube (Portex Ltd., England). The animal was then heparinized by injecting 1 ml heparine (1000 i.u.) into the spleen. Three loose ligatures were passed around the portal vein, which was then cannulated with a Braunula^{R} (G 16, B. Braun Melsungen A.G., West Germany, size 2 R) and the ligatures were tied, securing the cannula in place. Following these procedures the thorax was opened, the vena cava cut and the liver hanging on the diaphragm was transferred to the organ chamber. Care was taken to avoid admission of air into the cannula when connecting it with the system. About 2-4 min elapsed between the cannulation of the portal vein and the start of the perfusion. Extensive reports on liver perfusion and operation techniques have been published in the literature (Scholz, 1968).

PERFUSION MEDIUM

The volume of the perfusate was 200 ml and consisted of 80 ml freshly washed dog erythrocytes, 60 ml Hank's Eagle solution (Hanks Bss Dried, Code 5071) and 60 ml Ringer solution to which was added 6 g albumine (Sigma, no. A-4503), 400 mg glucose and 15 drops of terramycine.

PERFORMANCE AND PHYSIOLOGICAL CONTROL OF THE PERFUSION

A skilful cannulation of the liver and connection with the apparatus should result in a uniform red-brown colour of the liver surface. Bile production should start immediately. Since the medium contains phenol red, this compound will be excreted in the bile giving it a red colour.

The pO_2 before and after the liver was about 250 and 40 mm Hg respectively, the pO_2 deficit being constant for nearly 6 hours. Livers which showed a low pO_2 deficit after equilibration for half an hour were

discarded. The bile flow was ranging from 1.5-2.0 ml in 4 hours, which is in agreement with the values found by Ostahever (1960).

EVALUATION OF THE METHOD

A possible disappearance of barbiturates from the perfusate due to metabolism by the perfusate or adsorption to the tubes or walls of the system was checked. Perfusions without liver showed that these processes did not take place. Two examples with lipophilic barbiturates are shown in Figure 5.2.

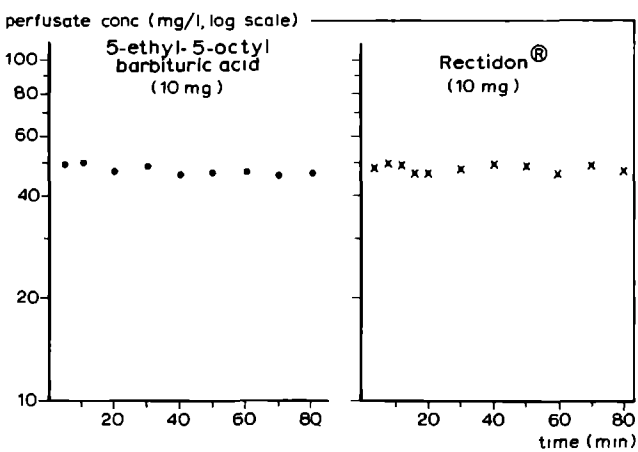


Fig. 5.2 *Control perfusion without liver of 5-ethyl-5-octyl barbituric acid (6)[a] and Rectidon[R] (12)[a]*
a. mean half-lives (min) with liver in parentheses (see chapters 11 and 12)

In contrast with the reports of Klaassen (1971) on biliary excretion of barbiturates, in our perfusion studies the amount of barbiturate excreted unchanged in the bile was always less than 0.5% of the dose administered (Fig. 5.4). Therefore, the disappearance of barbiturate from the perfusate can be attributed to metabolism by the liver.

The routine criteria for the performance of the liver as bile production, liver blood flow and oxygen consumption proved to be unsuitable to quantify the metabolic activity of the liver. The metabolic conversion of a reference compound was used as a sort of internal standard for each perfusion. Heptabarbital (5-ethyl-5-cycloheptenyl barbituric acid) was chosen as the reference compound for the following reasons:

1. Heptabarbital is eliminated mainly by metabolic conversion; no unchanged barbiturate could be detected in the urine, following administration in rats (Bernard, 1957).
2. Neither heptabarbital nor its metabolites interfere in G.C. analysis with most of the barbiturates studied.
3. It is metabolized rapidly, so there is enough time left to study another barbiturate.

With respect to the last point, it was important to investigate whether heptabarbital is eliminated flow-dependent. It was found that with flow rates higher than 15 ml/min, which is also the rate in intact rats (Liehr, 1972), heptabarbital elimination is flow-independent (Figure 5.3). In order to avoid flow-dependent elimination of the fast metabolized barbiturates, rather high flow rates were used. Comparison with perfusions at lower flow rates showed that these high flow rates did not damage the liver.

The perfusions were carried out according to the following standard scheme:

1. Equilibration for half an hour, during which time the physiological condition of the liver was checked.
2. In the following hour heptabarbital was introduced into the perfusate and 8 samples at intervals of 4 min were taken to estimate the heptabarbital clearance. Usually no more heptabarbital could be detected after 40 min.
3. After 60 min the barbiturate in study was introduced into the perfu-

sion fluid. At the introduction of each barbiturate the bile collecting vessel was replaced by another.

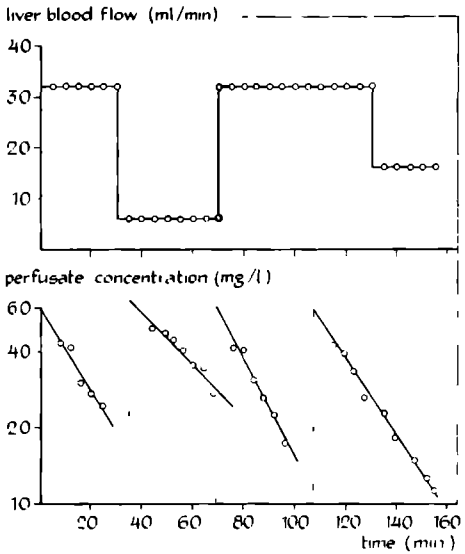


Fig. 5.3 *Influence of the liver blood flow on heptabarbital clearance. Lowering the flow to 7 ml/min affects the clearance. An decrease, however, from 32 to 15 ml/min has no effect on the clearance.*

An example of the usefulness of the standardization is given in Figure 5.4. Administration of heptabarbital and the barbiturate in study according to a cross over design did not change the heptabarbital half--life and clearance ratio.

A drawback of the method is that a possible interference with metabolites of a previously administered barbiturate cannot be excluded. However, barbiturates have been reported to be converted in hydroxy- and keto-metabolites, which are much less lipophilic than their parent compounds (Bush, 1973) and will have, therefore, a lower affinity to the

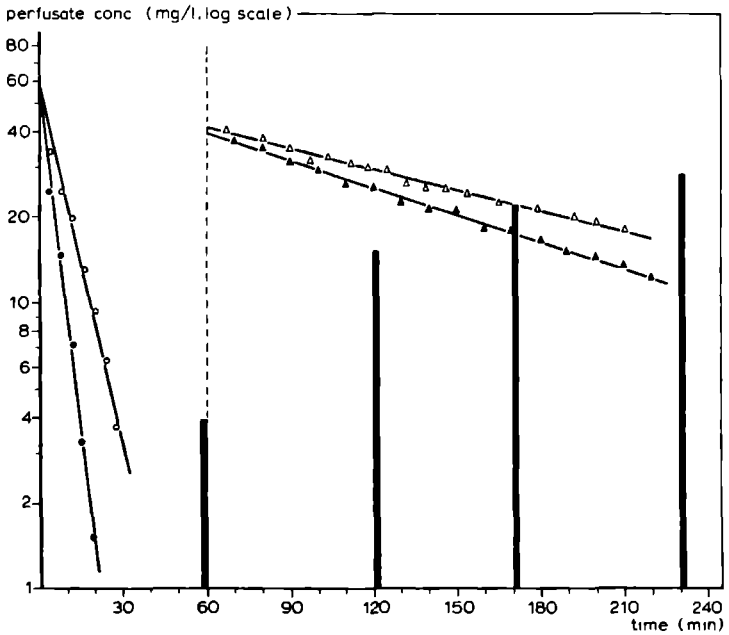


Fig. 5.4 *Comparison of the time courses of heptabarbital and Talbutal^R (5-allyl-5-(1-methyl propyl) barbituric acid) in two different perfusions*

● *heptabarbital (8 mg)* ▲ *Talbutal^R* *perfusion 10-9-74*

○ *heptabarbital (8 mg)* △ *Talbutal^R* *perfusion 12-9-74*

vertical bars: cumulative biliary excretion of Talbutal^R (µg)

metabolic enzymes. Figure 5.5 shows that a high dose of hydroxy-hexobarbital only slightly affects the time course of the parent compound.

In our studies, determination of clearance values have been performed using whole blood (perfusate) concentrations. The clearance of a drug by the liver cannot exceed the liver blood flow. Using plasma clearances, one has to reckon with a possible difference with the whole blood (perfusate) clearance, due to differences in binding to erythrocytes or plasma (perfusate) proteines. Such differences in clearance

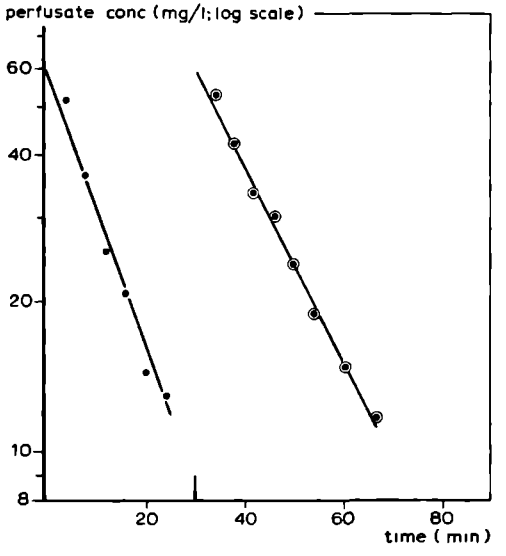


Fig. 5.5 *Influence of 3-hydroxy-hexobarbital on clearance of hexobarbital by the isolated perfused rat liver*
● *12 mg hexobarbital (control)*
⊙ *12 mg hexobarbital at time 30 min*
20 mg hydroxy-metabolite at time 25 min

have been reported for nortriptyline and desmethylimipramine (Rowland, 1972). Barbiturate binding to albumine (Goldbaum, 1954) and to erythrocytes (Brodie, 1953) seems to increase with higher lipophilicity. Possibly, the binding and debinding of barbiturates to these blood components is a rate limiting step in the metabolism of barbiturates. Differences in the rate of metabolism have been reported for hexobarbital enantiomers, whereas the proteine binding showed no significant differences (Breimer, 1974). It seems, therefore, unlikely that binding of barbiturates to blood components is a rate limiting factor in barbiturate metabolism.

CHAPTER 6

9000 G LIVER SUPERNATANT

In this study, liver homogenates represent the most simplified enzyme system with metabolizing activity. In preliminary studies on hydroxylation of barbiturates, no difference could be found between the 9000 g rat liver supernatant and the microsomal fraction, so that for convenience the first enzyme preparation has been chosen.

PREPARATION OF THE LIVER SUPERNATANT

Livers of male Wistar rats were excised immediately after decapitation and washed with ice-cold Tris buffer pH 7.4 (0.05 M) containing KCl (0.15 M). The livers were weighed, pooled and then homogenized with 4 parts (w/v) of buffer in a Potter-Elvehjem homogenizer with a Teflon^{R} pestle. Then the homogenate was centrifuged for 20 min in a refrigated centrifuge (MSE, high speed 18) at 9000 g.

INCUBATION CONDITIONS

The incubation volume was 30 cc and consisted of 5 ml barbiturate solution (10 mg/100 ml Tris pH 7.4), 5 ml liver supernatant, 5 ml of a NADPH--regenerating system and 15 ml Tris pH 7.4.
The NADPH-regenerating system contained per 100 ml: 650 mg isocitric acid (Sigma, no. I-1252), 200 mg NADP (Boehringer, Mannheim), 5 ml 0.1 M $MgCl_2$, 0.5 ml 0.1 M $MnCl_2$, 1 ml isocitric dehydrogenase (Sigma, no. I-2002, type IV; capable of generating 5.5 µmol NADPH/min.mg at 37°C) and 93.5 ml Tris-HCl buffer pH 7.4.

The mixture without barbiturate was pre-incubated at 37°C for 15 min to ensure reduction of all NADP. The reaction was started by adding the barbiturate solution. At various time intervals samples of 1 ml were taken and transferred to tubes containing 7 ml ether and internal stan-

dard.

EVALUATION OF THE METHOD

The incubation method adopted from Dewaide (1968) in which aeration was performed by shaking, proved to be unsatisfactory for experiments with barbiturates. The curves were often non-linear and the rate of disappearance of substrate from the incubation mixture was low (Fig. 6.1, I).

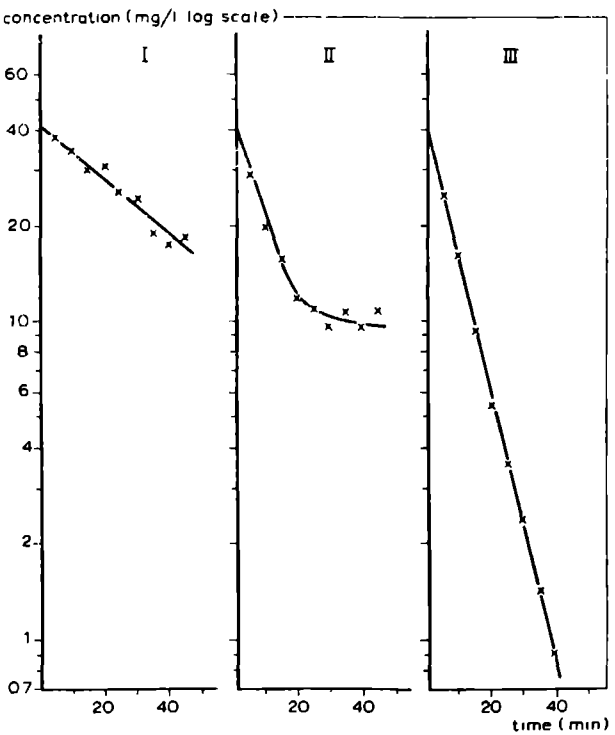


Fig. 6.1 *Evaluation of the 9000 g rat liver supernatant incubation. Rate of disappearance of heptabarbital (0.13 mmol), incubated under various conditions in a shaking waterbath at 37°C.*
I 0.11 mmol NADP; without carbogen
II 0.11 mmol NADP; with carbogen
III 0.22 mmol NADP; with carbogen

The requirement for molecular oxygen in the cytochrome P 450-mediated metabolism of drugs is well documentated (McMahon, 1969; Politzer, 1971). The addition of substrate to a microsomal suspension, containing a NADPH--regenerating system, increases the oxygen consumption (Wolf, 1974). Cumming (1970) has shown a relationship between the metabolism of hexobarbital and the oxygen tension, which in the perfused liver and in the intact rat became rate limiting at oxygen tensions below 45 mm Hg. Therefore, we have improved the oxygenation and varied the amount of NADP.

In subsequent experiments it was found that bubbling a mixture of O_2 and CO_2 (95:5) through the incubation medium resulted in a initial increase in disappearance rate of heptabarbital, followed by a decrease, probably caused by oxidation of NADPH. Doubling the amount of NADP in the regenerating system increased the linearity of the curve and lowered the half-life (Fig. 6.1). Higher amounts of NADP had no effect on the half-life.

It was found that an adequate oxygenation could be performed by stirring the incubation mixture, so that bubbling of gas through the medium, often resulting in the formation of foam, could be omitted (Fig. 6.2). The experiments were carried out in the apparatus shown in Figure 6.3. As internal control for the activity of the enzyme preparation the elimination of heptabarbital was taken (see chapter 5).

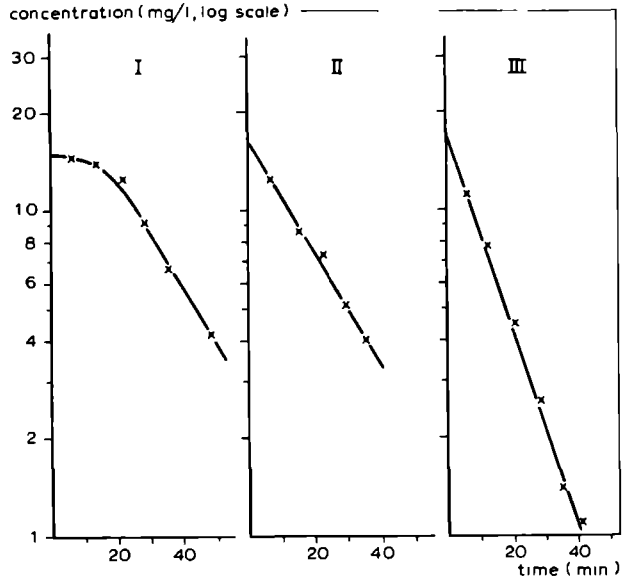


Fig. 6.2 *Evaluation of the 9000 g rat liver supernatant incubation. Rate of disappearance of heptabarbital (0.06 mMol), incubated with 0.22 mMol NADP under various conditions.*
I aeration by shaking
II with carbogen and stirring
III only stirring

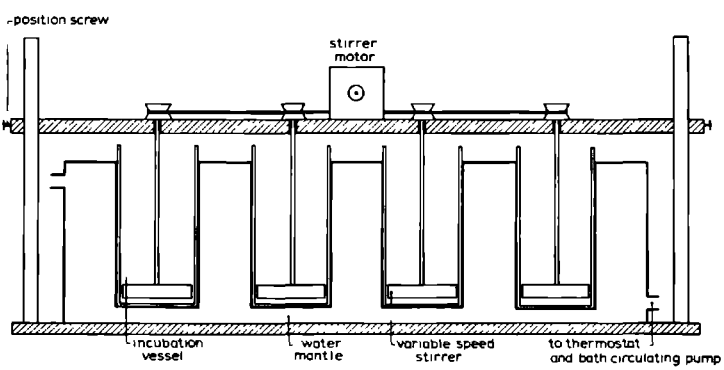


Fig. 6.3 *Schematic drawing of the apparatus used for the 9000 g rat liver supernatant and the hepatocyte experiments*

CHAPTER 7

ISOLATED RAT HEPATOCYTES

INTRODUCTION

Metabolic studies with suspensions of isolated rat hepatocytes represent a new metabolic model system, in which the intracellular structures remain intact. Mechanical separation or treatment of the liver with EDTA have already been described (Branster, 1957; Kaltenbach, 1952). These methods, however, proved to be ineffective as they produced a poor yield of cells which, moreover, often revealed extensive cellular damage (Berry, 1962).

The discovery of enzymatic methods to isolate intact rat parenchymal cells successfully (Berry, 1969; Howard, 1968) initiated the development of a metabolic tool, which has found an ever increasing employment. In this study we have isolated rat liver parenchymal cells according to the methods described by Seglen (1973) and Bojar (1975), with slight modifications.

ISOLATION PROCEDURE

Male Wistar rats weighing 200-300 g were used and fasted overnight before the experiment. After heparinizing the animal by injecting 1 ml of heparine (5000 i.u.) into the spleen, the liver was first perfused in situ through the portal vein for 5 min at a rate of about 50 ml/min, with a warm (37°C), O_2 saturated, Ca^{++}-free buffer (buffer I)[a] in order to wash the blood from the liver, while free outflow was obtained by cutting the lower vena cava. Cannulation of the portal vein was done with a Braunula[R]. While still being perfused, the liver was cut loose from the body and placed in an organ chamber as was used for the liver perfusions and the perfusion was continued for 10 min. Following this, the perfusion was switched over to a warm (37°C), O_2 saturated, collagenase buffer (buffer II)[a]. After recirculating perfusion for 15 min,

the liver was transferred to a Petri dish and gently dispersed in buffer III[a]. The suspension was then filtered through nylon mesh of 250 μ pore size and the volume adjusted to 75 ml with suspension buffer. The cells were shaken for half an hour at 37°C (damaged cells agregate) and filtered through nylon mesh of 100 μ poor size. Further removal of cell debris was accomplished by differentiate centrifuging four times for 5 min at 300 rpm. Between the centrifugings, the cells were resuspended in 75 ml of ice-cold buffer IV[a]. The final pellet was resuspended in buffer III[a] in the required cell concentration.

Liver cell concentrations were quantitated by counting aliquots of the suspension in a Bürker-Türk counting chamber to which 0.6% Trypan Blue was added. Cell yields ranged from 300-500 x 10^6 intact cells per liver, about 10% of which was stained with Trypan Blue.

EVALUATION OF THE METHOD

Incubation in glass stoppered tubes in a rotary shaker at 37°C (Notten, 1975) proved to be unpractical, because of the rapid elimination of the barbiturates and the amount of samples to be taken. Therefore, the experiments were carried out in the apparatus described for the 9000 g experiments (Fig. 6.3). After pre-incubation for 15 min, to 25 ml cell suspension 5 ml barbiturate solution (10 mg/100 ml buffer III) was added. The final cell concentration ranged from 2-3 x 10^6/ml.

Starting the incubation in this way, it was found that the reproducibility was low and the concentration-time curves often showed a deviation from linearity. It was found that again the oxygen supply was the critical parameter in these experiments. Bubbling the incubation mixture with carbogen, increased the linearity of the curves considerably but often led to formation of foam. At least, the oxygen capacity

a
Note: Composition of buffers I-IV, see Seglen (1973).

was increased by the addition of freshly washed dog erythrocytes. This proved to be quite satisfactory (Fig. 7.1). Control experiments showed that in incubations with erythrocytes, but without hepatocytes, there was no drug metabolizing activity.

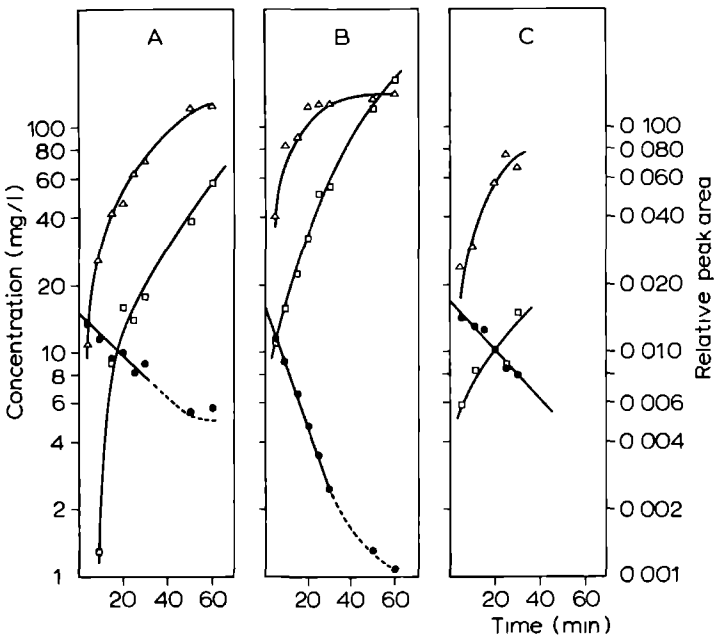


Fig. 7.1 *Influence of incubation conditions on the elimination of 1-N-methylcyclobarbital (16.5 mg/l) by isolated rat hepatocytes*
A. in presence of carbogen
B. in presence of dog erythrocytes
C. only stirring
● *1-N-methylcyclobarbital*
△ *hydroxy-metabolite*
□ *keto-metabolite*

The rate of elimination of the barbiturates from the incubation vessels appeared to be proportional to the hepatocyte concentration (Fig. 7.2). In order to obtain measurable half-lives, 2-3 x 10^6 cells/ml was chosen as the final hepatocyte concentration.

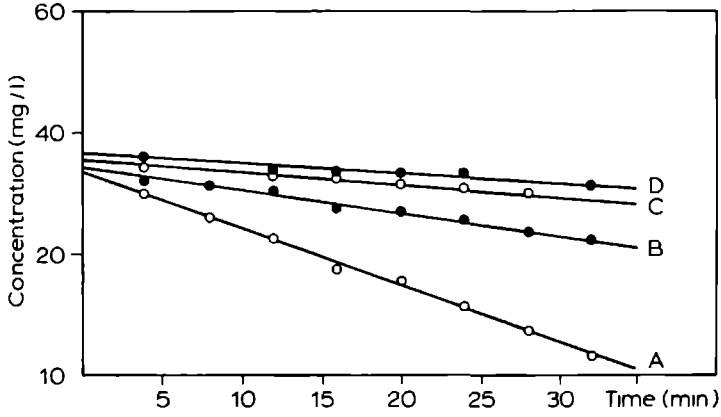


Fig. 7.2 *Influence of cell concentration on heptabarbital elimination*

Cell conc.x10^6/ml	*k_{Cel} (ml/min)*
A. 20	*A. 0.099*
B. 10	*B. 0.041*
C. 5	*C. 0.021*
D. 2.5	*D. 0.013*

Finally, the following incubation composition was chosen:
5 ml barbiturate solution (10 mg/100 ml buffer III), 10 ml erythrocyte Eagle solution (1:1 v/v) and 15 ml hepatocyte suspension.

With respect to the high affinity of the more lipophilic barbiturates to hepatocytes (see chapter 11), non-linear kinetics due to saturation could not be excluded. The concentration of barbiturate in the hepatocytes can be affected considerably by the addition of another binding possibility, e.g. albumin to the medium. Therefore, in the case of saturation, addition of albumin would result in a decrease of half-life. Figure 7.3 shows the influence of increasing albumin concentrations on

the half-life of heptabarbital. From these results it may be concluded that the elimination of barbiturate is not dose-dependent under the conditions described.

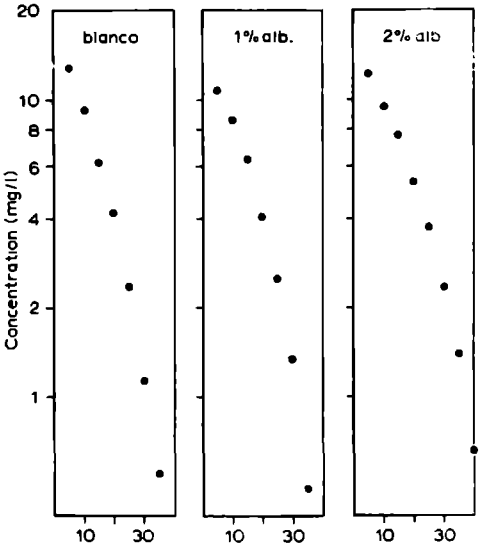


Fig. 7.3 *Influence of albumin concentration on elimination of heptabarbital (0.067 mMol) by isolated rat hepatocytes*

CHAPTER 8

PARTITION COEFFICIENTS

As already mentioned in chapter 1 the effect of a drug depends on its concentration in the biophase and its affinity to the receptor(s). This implies that transport processes of a drug to that biophase, which involves passage through hydrophilic and lipophilic barriers, is an important factor in determining the biological response. The transport to and the binding at the active site will depend, among other things, on the hydrophobic character of the drug, which can be measured in a partition system. Octanol/water partition coefficients of a number of barbiturates have been reported already in the literature (Hansch, 1967). Therefore, we have also used this system for comparison purposes. In addition another reference partition system was used, namely isolated hepatocytes/water in view of the metabolism of barbiturates, which mainly takes place in the liver.

OCTANOL/WATER PARTITION COEFFICIENTS

5 ml octanol solution containing 20 mg barbiturate per 100 ml was shaken vigorously for half an hour with an equal volume of phosphate buffer (Sorensen, 1/15 Mol). Both phases were saturated previously with each other. After equilibration and centrifuging the two layers were separated. The partition coefficient was calculated from the decrease of concentration in the octanol layer. Because of the large differences in the partition of the various barbiturates, the measurements has to be performed at different pH's. From these apparent partition coefficients (APC) the true partition coefficient (TPC) could be calculated using the following formula: $TPC = APC\ (1 + 10^{pH-pK_a})$.

To 2 ml of a cell suspension, containing 4 x 10^6 cells, was added 60 µg barbiturate. After stirring gently for 10 min, during which equilibration was reached, the suspension was centrifuged. Under these conditions no metabolism by the hepatocytes took place. From the ratio cells/water the APC at pH 7.4 was calculated.

CHAPTER 9

SPECTRAL DISSOCIATION CONSTANTS (K_s)

K_s values were determined with the help of difference spectra according to the method of Jansson (1972).

PREPARATION OF MICROSOMES

Livers of male Wistar rats were excised immediately after decapitation, weighed, transferred to 9 vol. ice-cold 0.25 M sucrose and homogenized in a Potter-Elvehjem homogenizer. Nuclei and cell debris were sedimentated by centrifuging at 600 g for 5 min; the supernatant was centrifuged at 18000 g for 15 min; the microsomal fraction was obtained by centrifuging the 18000 g supernatant for 1 h at 105000 g. The microsomal pellet was resuspended in 0.15 M KCl and centrifuged at 105000 g for 30 min, to remove haemoglobin and finally resuspended in 4 vol. 0.15 M KCl - 0.05 M Tris-HCl (pH 7.4) to a concentration of about 1 mg microsomal protein per ml. The barbiturates were dissolved in KCl-Tris-HCl buffer to concentrations ranging from 0.02-2 mMol. Two cuvettes were filled with 1 ml microsomal suspension and 2 ml barbiturate solution was added to the suspension, while to the reference cuvet an equal volume of buffer was added. With varying substrate concentrations, spectra were recorded from 500-350 nm on a Carry 118 C double beam spectrophotometer with a scattered transmission accessory. The extent of spectral change was determined as the difference in absorption at 420 and 390 nm. K_s values were obtained from reciprocal plots with the help of a linear regression computer program.

CHAPTER 10
ANALYSIS

Barbiturate concentrations were determined by means of gas chromatography according to Jain (1967).

EXTRACTION PROCEDURE

Blood and artificial fluid samples were extracted with 5 ml ether-acetone (1:1 v/v) on a whirlmixer. The extract was evaporated to dryness, the residue was redissolved in ethanol and analysed by G.C. Tissue samples were first homogenized in acetone, saturated with tartaric acid according to a modified method described by Kisser (1968). The acetone layer was evaporated to dryness and the residue redissolved in hot water. After heating for 15 min in a waterbath at 80°C, the solution was filtered and the filtrate extracted twice with ether. The ether layer was evaporated to dryness and the residue dissolved in ethanol. In order to correct variations in extraction a known amount of another barbiturate was added to the sample and used as internal standard. The concentration was calculated with the aid of a factor, derived from linear calibration curves. These curves were constructed by adding known amounts of barbiturate to blood or tissue and plotting the peak area ratio barbiturate/standard to the concentration ratio barbiturate/standard. An example of these calibration curves is shown in Figure 10.1. The recovery was about 90 per cent. for the fluid samples and somewhat lower, about 75 per cent., for the tissue samples. Concentrations down to 100 ng/ml blood and 60 ng/g tissue could be determined.

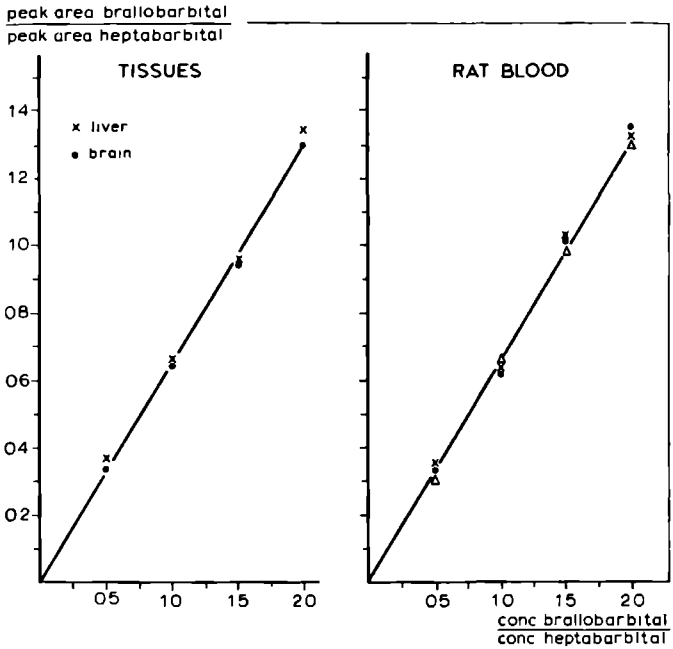


Fig. 10.1 *Calibration graphs for the determination of brallobarbital in blood and tissues, using heptabarbital as internal standard*
Blood samples: × *extraction with ether*
● *extraction with ether–acetone*
△ *extraction with tartaric acid–acetone*

GAS CHROMATOGRAPHY

Gas chromatographic analysis was done on a Hewlett-Packard gas chromatograph, type 402, equipped with a nitrogen detector. The nitrogen detector is very useful in the analysis of nitrogen containing compounds as barbiturates. The use of a nitrogen detector results in an increase not only of sensitivity but also of selectivity when compared with the normal flame ionization detector, as has been discussed extensively by Breimer (1973). The OV-17 column, which has been used, seperates clearly

barbiturate metabolites from the parent compounds (Table 10.1). Column and oven conditions were: glass column (1.8x3 mm I.D.) packed with 3% OV-17 on gas-chrom Q, 60-80 mesh; oven temperature 220-240°C, inlet and detector temperature were 260°C and 300°C respectively. Helium was used as carrier gas at a flow rate of 25-30 ml/min; hydrogen and oxygen flow rates were 30 and 200 ml/min respectively. In Table 10.2 the relative retention times are shown of the barbiturates used in this study, together with their melting points.

TABLE 10.1

Retention time ratio metabolite to parent compound of a number of barbiturates

Compound	Metabolite I	Metabolite II	Temp. (°C)
Pentyl	2.91		240
Hexyl	2.59		240
Heptyl	2.58		240
Octyl	2.56		240
Nonyl	2.68		240
Dm-Hexyl	2.49		210
Seco	2.22		230
Rect	0.74	2.15	240
m-Bral	0.76		230
m-Cyclop	2.43		230
Hexo	2.21	2.66	240
m-Cyclo	2.11	2.67	240
m-Hepta	2.26	2.56	230
m-Rep	2.15	2.71	240
Dm-Cyclo	2.05	2.45	230
m-Hexo	1.78	2.21	230

TABLE 10.2

Relative retention times[1] and melting points[2] of a number of barbituric acid derivatives

Drugs	Rel. ret. time 230° C	Rel. ret. time 250° C	m.p. 0° C
Propyl	18.9	21.4	139-142
Butyl	22.6	25.7	124-126
Pentyl	30.6	34.3	130-132
i-Pentyl	24.2	27.1	155-157
s-Pentyl	29.0	30.0	127-129
Dm-but	32.3	32.9	151-154
Hexyl	39.5	42.9	124-126
Heptyl	51.6	54.3	117-119
Octyl	72.6	75.7	114-117
Nonyl	95.2	97.1	104-107
m-Hexyl	28.2	28.6	
m-Heptyl	35.5	37.1	
m-Octyl	48.4	48.6	
m-Nonyl	64.5	65.7	
Dm-Hexyl	20.2	22.9	
Allo	22.6	24.3	167-170
Apro	21.8	25.7	140-141
Tal	25.0	27.1	224-225
Neal	27.4	30.0	154-155
Seco	33.9	35.7	94- 96
Bral	61.3	65.7	164-168
m-Bral	35.5	38.6	97- 99
Dm-Bral	24.2	28.6	36- 38
Noct	61.3	65.7	178-180
Eunarc	37.1	42.8	108-110

1) The retention time of heptabarbital has been taken as 100

2) After recrystallization from ethanol/water (see chapter 3)

TABLE 10.2

continued

Drugs	Rel. ret. time		p.m.
	230° C	250° C	0° C
Pern	80.7	80.0	132-133
m-Pern	48.4	51.1	79- 83
Rect	92.7	94.3	163-165
Cyclop	54.8	57.1	137-139
m-Cyclop	36.3	40.0	79- 80
Nor-hexo	66.9	68.6	207-109
Hexo	46.8	50.0	145-147
m-Hexo	35.5	37.1	75- 76
Cyclo	77.4	79.4	150-153
m-Cyclo	50.8	51.8	103-105
Dm-Cyclo	36.3	40.0	62- 64
Hepta	100.0	100.0	175-176
m-Hepta	68.6	68.6	95- 97
Dm-Hepta	53.2	53.9	60- 61
m-Pheno	57.3	58.2	178-179
Rep	131.7	128.6	212-215
m-Rep	85.5	88.6	164-166
Hydroxy-hexo	109.7	102.9	180-185
Keto-hexo	138.7	131.4	160-162

SECTION III

PHARMACOKINETICS OF BARBITURATES

CHAPTER 11

5-ETHYL-5-ALKYL SUBSTITUTED BARBITURATES IN THE RAT AND IN THE LIVER PERFUSION

INTRODUCTION

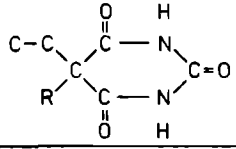
This group of barbiturates has been derived directly from 5,5-diethyl barbituric acid (barbital). The chemical structures of the barbiturates are listed in Table 11.1. Butobarbital, qua structure the first derivative of barbital used in treatment of insomnia, was developed in 1922 and is widely used as a hypnotic drug with an intermediate duration of activity (Martindale, 1972) and is marketed under the trade name Soneryl^{R}. Amobarbital (Amytal^{R}), which was introduced in 1923, has a similar minimum anaesthetic dose (M.A.D.) as butobarbital but with a shorter duration of action. The same was found for pentobarbital (Nembutal^{R}) which in addition to its use in human sleep therapy is also extensively used as veterinary anaesthetic. The hexyl derivative (Ortal^{R}) is the latest derivative of the series, which is used in clinical medicine. However, there are only a few reports on this barbiturate (Lorhan, 1944). Following intravenous administration in dogs (Gruber, 1937), Ortal^{R} was reported to produce anaesthesia lasting only one third as long as that of amobarbital. As far as known, the remaining derivatives of the series have not been employed in clinical medicine.

The major route of elimination of barbiturates is metabolism in the liver. In order to investigate the role of the liver in the elimination of the drug independent of distribution processes and other routes of elimination, the isolated perfused rat liver was selected, which can be considered as an *in vitro* system nearest to the intact animal and forming a link with the more simplified systems as liver slices, isolated hepatocytes and the 9000 g supernatant of liver homogenates.

In this chapter an attempt was made to compare the pharmacokinetics of this series of barbiturates in the intact rat and in the isolated perfused rat liver and to relate the findings to the physico-chemical properties of the drugs.

TABLE 11.1

Chemical structure, apparent (APC at pH 7.4) and true (TPC) partition coefficients of a number of barbiturates



Compound	R	pK_a [1]	log TPC octanol/water	log APC octanol/water	log APC cells/water
Propyl	-C-C-C	7.90	0.87	0.66	-0.13
Butyl[a]	-C-C-C-C	7.86	1.70	1.26	0.15
Pentyl	-C-C-C-C-C	8.00	2.23	1.78	0.43
s-Pent[b]	-C(C)-C-C-C	8.03	2.13	1.73	0.38
i-Pent[c]	-C-C-C(C)-C	7.87	2.11	1.58	0.40
Dm-but	-C-C(C)-C(C)-C	7.86	2.39	2.02	0.52
Hexyl	-C-C-C-C-C-C	8.00	3.08	2.46	0.80
Heptyl	-C-C-C-C-C-C-C	8.00	3.64	2.91	1.35
Octyl	-C-C-C-C-C-C-C-C	8.00	3.85	3.08	1.97
Nonyl	-C-C-C-C-C-C-C-C-C	8.00	4.13	3.30	2.29

1) From Doornbos (1969); Krahl (1940) and Sitsen (1973)

Generic name: a. butobarbital

b. pentobarbital

c. amobarbital

Intact rat experiments

Intravenous administration of the barbiturates resulted in biphasic blood concentration-time curves, shown in Figure 11.1. The first rapid fall in blood concentration lasted 5-10 min and is mainly due to distribution in the tissues. After the distribution phase there is a second phase which represents overall elimination. In Table 11.2 the half-lives of this elimination phase are presented as well as the ratio to heptabarbital half-life. Large differences in the half-lives of barbiturates in rats are reported in the literature. For the s-pentyl derivative the values range from 120 min (Pelkonen, 1973) to about 50 min (Alvarez, 1973; Siemens, 1973), while for the i-pentyl derivative values have been determined in the magnitude of 60 min (Pelkonen, 1973) and 150 min (Misra, 1974). Possibly interstrain differences are responsible for these variations. The values found by Pelkonen support our findings that the half-life of the i-pentyl derivative is shorter than that of the s-pentyl homologue. Table 11.2 indicates that the half-lives decrease with increasing chain length. The apparent volumes of distribution of the homologous series do not differ significantly. The volume of distribution of heptabarbital, which was used as reference compound in each animal, was significantly smaller. Since the apparent volumes of distribution of the homologous series are very similar, the difference in half-life among the members of the series can be attributed to differences in clearance. The clearance constants of elimination are presented in Table 11.3. A gradual increase in clearance is seen from the propyl to the heptyl derivative.

The isolated perfused rat liver

In Figure 11.2 some examples of blood concentration-time curves in this system are presented. The curves are clearly monophasic. This is in contrast with the type of curves observed after *in vivo* intravenous administration. A measurable distribution phase could not be distinguished.

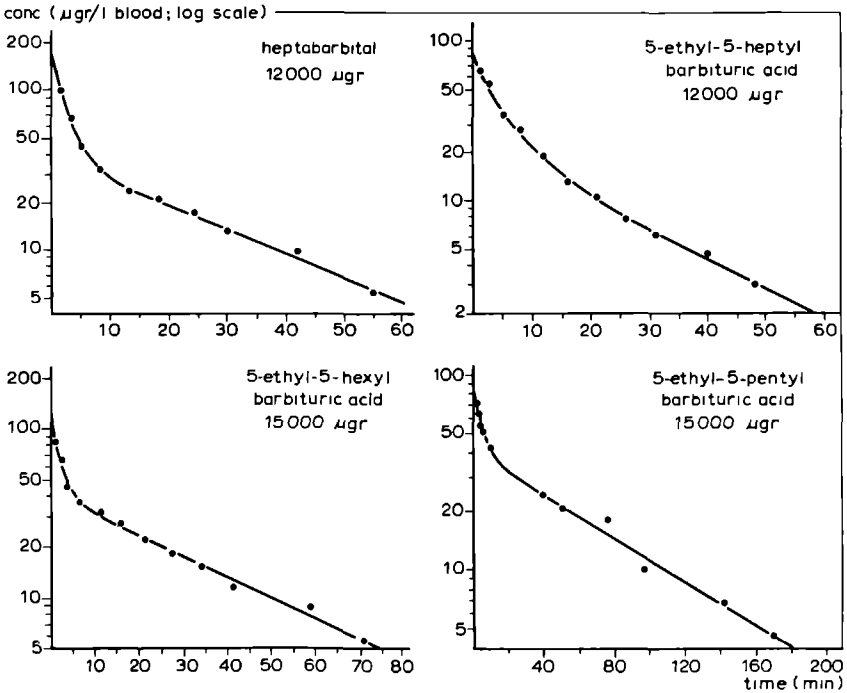


Fig. 11.1 *Blood concentration-time curves of some 5-ethyl-5-alkyl substituted barbiturates following intravenous administration in the same rat. The half-life or clearance of heptabarbital was used as parameter for the drug metabolic activity of the animal*

TABLE 11.2

Half-lives and apparent volumes of distribution of some barbiturates in the intact rat and the isolated perfused rat liver system

Compound	Half-life (min)		Half-life ratio[1]		Apparent volume of distribution (ml)	
	intact rat	perfusion	intact rat	perfusion	intact rat	perfusion
Propyl	574±83 (2)	1249±151 (2)[b]	50.1±3.6	302.5±72.8[b]	226±36	221±13
Butyl	210±37 (6)	149± 37 (5)[b]	12.8±3.0	17.0± 7.4[a]	338±66	255±26
Pentyl	48±10 (6)	48± 8 (5)[a]	3.2±0.7	4.1± 0.7[a]	305±33	234±11
s-Pent	53± 9 (4)	32± 10 (4)[b]	4.4±1.9	5.8± 0.7[a]	286±75	228±10
i-Pent	39± 9 (4)	18± 4 (4)[b]	3.2±0.5	3.6± 0.4[a]	239±13	238±21
Dm-but	32± 4 (4)	28± 5 (4)[a]	2.6±0.5	2.7± 0.7[a]	377±97	220±12
Hexyl	19± 4 (5)	13± 1 (5)[b]	1.5±0.5	1.5± 0.4[a]	250±34	241±26
Heptyl	15± 2 (5)	13± 8 (6)[a]	0.9±0.2	1.0± 0.1[a]	246±46	246±31
Octyl		6± 2 (4)		1.2± 0.2		275±21
Nonyl		7± 3 (4)		0.8± 0.1		258±14
Hepta	14± 3 (14)	13± 6 (43)[a]			162±30	255±21[c]

1) ratio half-life (derivative/heptabarbital), measured in the same rat or the same perfusion

2) mean ± S.D.; number of experiments in parentheses

a. not significantly different $P > 0.05$ *(Student's t-test)*

b. significantly different $0.001 < P < 0.05$

c. significantly different $P < 0.001$

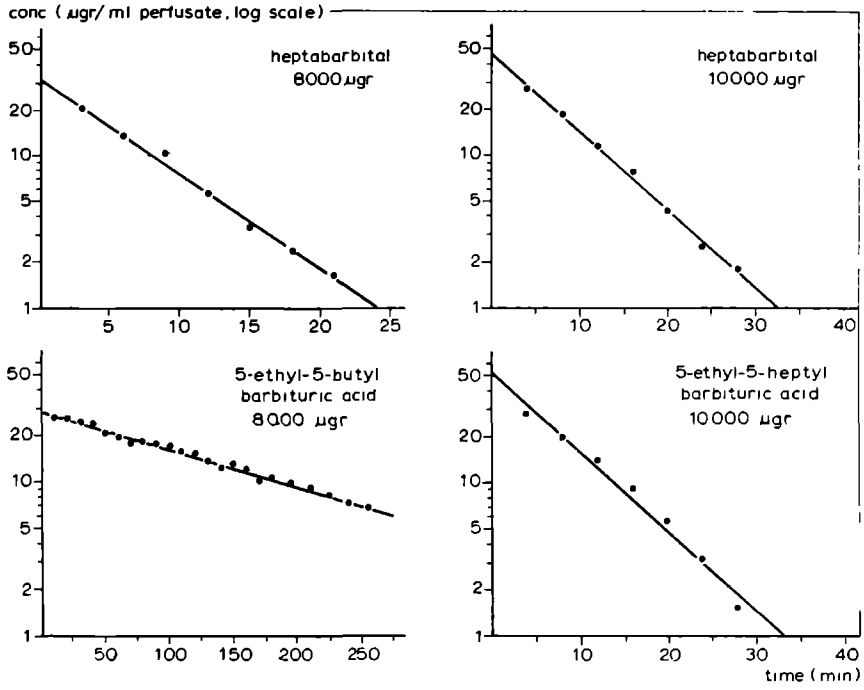


Fig. 11.2 *Blood concentration-time curves of some ethyl-alkyl substituted barbiturates in the isolated perfused rat liver system*
Right: 5-ethyl-5-heptyl barbituric acid
Left : 5-ethyl-5-butyl barbituric acid
The half-life or clearance of heptabarbital was used as parameter for the drug metabolic activity of the liver

TABLE 11.3

Clearances and clearance ratios of some barbiturates in the intact rat and the isolated perfused rat liver system

Compound	Clearance (ml/min)		Clearance ratio[1]		Clearance ratio[2]	
	intact rat[3]	perfusion	intact rat	perfusion	intact rat	perfusion
Propyl	0.34±0.09 (2)	0.14± 0.03 (2)[a]	0.026±0.001	0.004±0.001[c]	0.022±0.001	0.005±0.001[c]
Butyl	1.02±0.26 (6)	1.15± 0.39 (5)[a]	0.123±0.024	0.068±0.005[b]	0.105±0.020	0.084±0.003[a]
Pentyl	4.71±0.99 (6)	3.90± 1.04 (5)[a]	0.488±0.078	0.289±0.056[c]	0.418±0.067	0.356±0.069[a]
s-Pent	3.97±0.53 (4)	5.92± 0.93 (4)[b]	0.276±0.027	0.161±0.023[c]	0.237±0.023	0.196±0.024[a]
i-Pent	4.14±0.78 (4)	10.12± 2.15 (4)[b]	0.413±0.031	0.310±0.062[b]	0.354±0.026	0.384±0.078[a]
Dm-but	8.02±0.90 (4)	6.60± 2.22 (4)[a]	0.530±0.035	0.425±0.017[b]	0.454±0.030	0.525±0.021[b]
Hexyl	10.09±1.94 (5)	12.30± 2.16 (5)[a]	1.167±0.147	0.809±0.065[c]		
Heptyl	13.17±2.55 (5)	17.62± 9.18 (6)[a]	1.586±0.259	1.025±0.122[b]	1.360±0.222	1.266±0.151[a]
Octyl		28.01±14.57 (4)		1.110±0.272		1.371±0.336
Nonyl		29.89±12.25 (4)		1.611±0.225		1.989±0.276
Hepta	10.89±3.21 (14)	19.92± 8.94 (43)[c]				

1) heptabarbital clerance ratio *2) hexyl clearance ratio* *3) mean ± S.D.; number of experiments in parentheses*

a. not significantly different $P > 0.05$

b. significantly different $0.001 < P < 0.05$

c. significantly different $P < 0.001$

The half-lives of the perfusion studies are presented in Table 11.2. Some of these values are shorter than those found *in vivo*, while for others the difference is not significant. These differences may be caused by variation in the metabolic activity of the livers used. The half-life ratio, which is independent of such variations, indicates that with the exception of the propyl derivative the values found *in vivo* and in the perfusion are very close. There are no large differences in the volume of distribution between the members of the series. In contrast with the intact rat experiments the volume of distribution of heptabarbital and the alkyl derivatives do not differ significantly. The clearance constants and the clearance ratio are presented in Table 11.3. The heptabarbital clearance ratio found in the perfusions is lower than that found in the intact rat. For some unknown reasons the A_1 value found for heptabarbital in the intact rat is much higher than that found for the alkyl derivatives resulting in a smaller volume of distribution, as mentioned earlier. This means that the clearance constant found for heptabarbital *in vivo* is rather low (eq. 4) and the resulting heptabarbital clearance ratio is therefore high. The discrepancy between the clearance ratio *in vivo* and *in vitro* is not present when another barbiturate of the series, p.e. the hexyl derivative, is used as reference compound (Table 11.3).

Partition coefficients

The octanol/water and the hepatocytes/water partition coefficients are presented in Table 11.1. The observed values for the octanol/water TPC are in good agreement with those calculated by Hansch (1967). When the $APC_{(7.4)}$ values of the two partition systems are compared, a good correlation was found (r 0.959, s 0.255). In both partition systems there is an increase in the values for derivatives possessing more carbon atoms in the side chain. A derivative with a straight substituent seems to be more lipophilic than the one with a branched side chain having the same number of carbon atoms. According to Hansch (1973), a parabolic relationship has to be expected between the rate of elimination and the partition coefficient. Figure 11.3 presents a fitted curve showing the relationship between the heptabarbital clearance ratio and the octanol/water partition

coefficient. The correlation coefficient and standard deviation are respectively 0.977 and 0.134 (0.929 and 0.222 for the hepatocytes/water partition coefficient). When the half-life ratio is used as biological response the correlation values were for the octanol/water and hepatocytes/water system respectively r 0.968, s 0.096 and r 0.950, s 0.280.

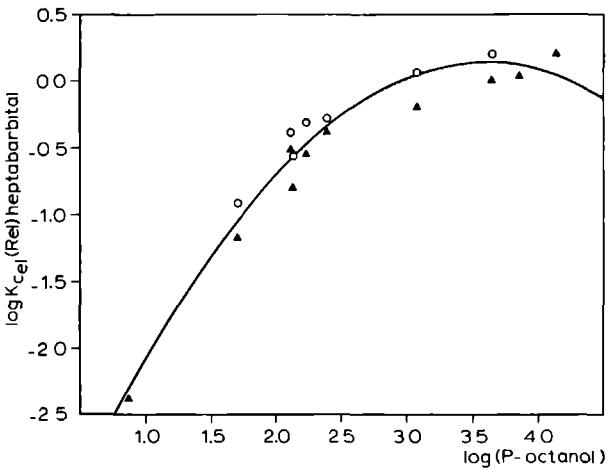


Fig. 11.3 *Relationship between log k_{Cel} (rel) heptabarbital (y) and log P-octanol (x) described by a parabolic function ($y = ax^2 + bx + c$)*
o *vivo* ▲ *perfusion*

Sleeping time and blood concentration at awakening

In order to investigate whether differences exist in central depressant potency among the members of the series, blood concentrations at awakening were measured. These concentrations were deduced from the blood concentration-time curves by measuring the sleeping time. The sleeping time and the corresponding blood concentrations at awakening are given in Table 11.4. The duration of sleeping time was dose-dependent. The blood concentrations at awakening were, however, dose-independent and rather constant for the various barbiturates. Reports from the literature con-

TABLE 11.4

Sleeping times (A,min) and blood concentrations at awakening (B,min) of a number of 5-ethyl-5-alkyl substituted barbiturates following intravenous administration (12 mg) in rats

	Butyl		Pentyl		s-Pent		i-Pent		Dm-but		Hexyl		Heptyl	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
	10	33	21	24	42	15	38[1]	21	27	14	20	14	15	12
	12	37	27	21	50	16	85[1]	22	37	20	20	16	16	15
	40	46	32	22	112[2]	13	95	17	41	18	36	15	19	15
	55[2]	31	47	21					69	19	27[2]	11	20	10
	128[2]	38	50[2]	18							28[2]	17	27[2]	11
	150[2]	34												
Mean B[3]	36 ± 5		21 ± 2		15 ± 1		20 ± 3		18 ± 3		15 ± 2		13 ± 2	

1) dose 10 mg

2) dose 15 mg

3) significantly different (F-test; $F_{(5.22)} = 37.90$)

cerning blood concentrations at awakening in rats are mainly restricted to barbital and pentobarbital. Our value for the latter is in good agreement with that reported by Kato (1967).

Starting from the butyl derivative to the heptyl derivative there is a decrease in blood concentration at awakening. The propyl derivative possesses only a weak hypnotic potency, which is in agreement with the finding of Exley (1954) in mice. The anaesthetic potency was only 41% of amobarbital.

DISCUSSION

The usefulness of the relative clearance constants for kinetic studies appears from the smaller relative errors, when compared with the absolute values. For the perfusion studies the relative error ranges from 15 to 52 per cent., while for the clearance ratio the error has been reduced to 4-24 per cent. Heptabarbital possesses various properties which make it a suitable reference compound, particulary for *in vitro* studies, as has been pointed out in chapter 5. Nevertheless, there is also a disadvantage in the use of this barbiturate as a reference as appears from the differences in the heptabarbital clearance ratio found *in vivo* and in the perfusions. However, this can be corrected easily and it plays no role when the barbituric acid derivatives are compared either in the intact animal or in the isolated rat liver perfusion system.

The majority of reports on the metabolism and elimination of barbiturates in rats are restricted to the ethyl and to the s-pentyl substituted derivatives. Our values found for the s-pentyl derivative in the intact rat are in agreement with those found by Alvarez (1973). However, his values which were found in the liver perfusion, using a perfusate volume of 100 ml, differ considerably from our values. When a comparison is made between the half-lives found by Alvarez *in vivo* and *in vitro* the latter values are too low considering the volume of distribution. Since the apparent volume of distribution in the perfusion is about twice as small as in the intact rat one would expect that the half-life in the perfusion would be half the value found *in vivo*. *In vivo* there is obviously an equi-

librium between the binding to blood components as plasma proteins and erythrocytes and the binding to other tissues as liver, brain and kidney. The apparent volume of distribution, calculated from whole blood levels, will be, therefore, in the same order of magnitude for the various barbiturates. From the studies of Goldbaum (1954) it appears that with increasing lipophilicity both the fraction bound to the blood components and the fraction bound to the other tissues increase too.

The same holds true for the apparent volume of distribution in the perfusion studies. According to the affinity towards hepatocytes (see Table 11.1) from the propyl towards the nonyl derivative, an increasing uptake by the liver has to be expected, resulting in a growing apparent volume of distribution. Our data, however, indicate that the effect of this uptake on the apparent volume of distribution is countered by binding to the perfusate components. This is supported by the studies of Jähnchen (1971) with chlorpromazine and promazine, which are reported to be bound to the erythrocytes of the perfusate up to 77 and 69 per cent. respectively.

Comparison of the clearance constant and half-life ratio measured in the intact rat and in the perfusion studies shows that, with the exception of the propyl derivative, the ratio is very similar. This indicates that for these barbiturates hepatic metabolism is the main route of elimination. This is in accord with the data in the literature dealing with urinary excretion of barbiturates. According to Buttar (1974) rats excrete only 0.6% of the administered dose of pentobarbital. Studies on the metabolism of 5-ethyl-5-alkyl substituted barbiturates in dogs showed that starting from the butyl to the hexyl derivative the barbiturates are metabolized almost completely and are excreted unchanged in the urine only to a minor extent. Taking into account the much shorter half-life of barbiturates in rats, we can assume that in rats too urinary excretion of these derivatives is negligible.

From our data we can conclude that the propyl derivative resembles the ethyl derivative, which is reported to be excreted largely in the urine of dogs (Giotti, 1951). The data presented in Table 11.1 and 11.3 show that there exists a relationship between the rate of elimination and the lipophilicity. We have fitted the data to a parabolic function after

Hansch (1973) and found a high correlation. As is the problem in most cases concerning parabolic correlations the data points cover only the left side of the parabola. As long as points are not obtained at the other side the parabolic relationship is rather speculative, in spite of the high correlation coefficients found. Attempts at fitting the data, for instance to a hyperbolic function, result in similar correlation coefficients (see Fig. 11.4).

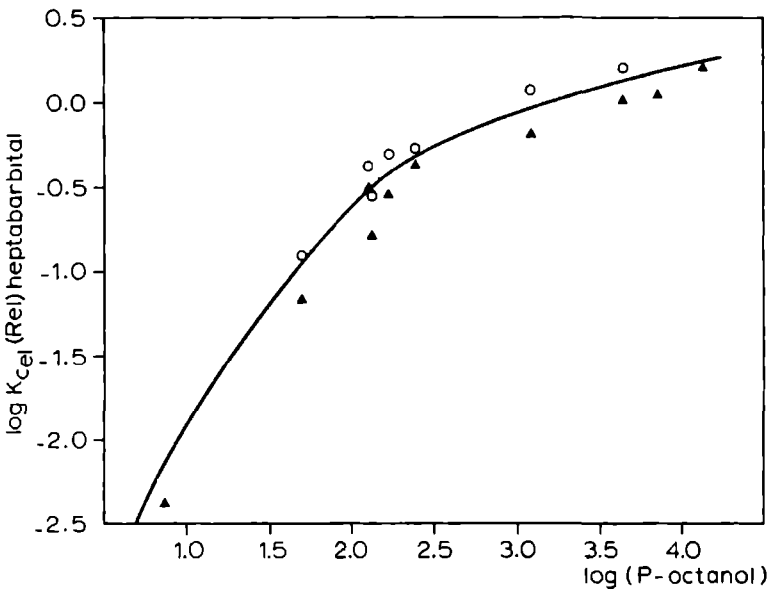


Fig. 11.4 *Relationship between log k_{Cel} (rel), heptabarbital (y) and log P-octanol (x) described by a hyperbolic function*
$(y = A.(x-B)/(x+C)$
$r = 0.979$ $s = 0.138$
o *vivo* ▲ *perfusion*

In our studies we increased lipophilicity by introducing larger substituents; but it is not unlikely that stereo-chemical rather than physico-chemical parameters cause the flattening of the curve presented in Figs. 11.1 and 11.3. It is, therefore, obviously of interest to investigate the effect of increasing the length of the substituents and the

lipophilicity on the barbiturate-microsomal enzyme interaction (see chapter 16).

Other important parameters for the biological response are the distribution in the brain and the hypnotic potency of the compounds. Blood concentration of the drug at awakening was found to correspond well with the concentration in the brain at that time (Prioux-Guyonnoneau, 1974; Smith, 1961). Autoradiographic studies on the distribution of barbiturates in the brain showed that the barbiturates do not localize in any specific part of the brain. They are rather uniformly distributed throughout the various brain areas (Domek, 1960; Lal, 1964). The differences found in blood concentration at awakening, therefore, suggest that in addition to a difference in hypnotic potency based on an unequal rate of elimination, there also exist differences in the hypnotic potency of the barbiturate itself.

The mechanism of barbiturate action is not completely known, although numerous studies have been reported in the literature concerning the mode of action of barbiturates. Many barbiturates can produce excitatory as well as depressant effects, the ratio being dependent on the chemical structure of the substituents. Neuropharmacological investigations indicate an interaction with the synaptic transmission (Richard, 1972), while studies at cellular level suggest that the activity of barbiturates may be due to an inhibition of NADPH dehydrogenase (Giuditta, 1963). Exley (1954) found differences in ganglion-depressant activity among series of barbiturates, including some ethyl-alkyl derivatives. An increasing order of activity was found with the following derivatives: propyl, s-pentyl, hexyl, butyl, isoamyl and pentyl. There is obviously a lack in correlation with the lipophilicity of the compounds. Unequal hypnotic potency has been reported for various optical antipodes of barbiturates (Breimer, 1973; Buch, 1969; Haley, 1970). This suggests that stereo-chemical rather than physico-chemical factors are responsible for the differences in the intrinsic hypnotic potency of barbiturates.

CHAPTER 12

5-ALLYL- AND 5-BROMOALLYL-5-ALKYL BARBITURATES IN THE RAT AND IN THE LIVER PERFUSION

INTRODUCTION

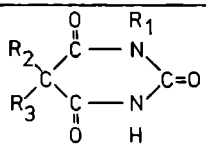
The 5-allyl and 5-bromoallyl substituted derivatives form a class of barbiturates some of which are used frequently. After the discovery of the hypnotic properties of the 5-ethyl-5-alkyl substituted barbiturates it was found that the introduction of an ethylenic group moiety enhances the hypnotic potency. This resulted in the synthesis of barbiturates with vinyl, butenyl and especially allyl groups. The allyl substituted barbiturates generally appeared to possess a higher hypnotic potency than the corresponding ethyl homologues (Nielsen, 1926). Introduction of a halogen into the allyl group, usually a bromine atom, increased the hypnotic potency (Boedecker, 1928) and led to the development of bromoallyl substituted barbiturates as Noctal^{R} (1924), Pernocton^{R} (1919) and brallobarbital (1953), a component of Vesparax^{R}. The metabolism of the 5-allyl and 5-bromoallyl derivatives is somewhat different from that of the 5-ethyl-5-alkyl series. Compared with the ethyl substituent the allyl, and especially the bromoallyl, group is more susceptible to metabolic conversion.

Mühlhauser (1974) found secodiol as a metabolite of secobarbital in dogs in addition to 3-hydroxy-secobarbital, while for Noctal^{R} and Pernocton^{R} (Boedecker, 1928), Eunarcon^{R} (Ravn-Jonsen, 1970) and brallobarbital (Keding, 1969) the conversion of the bromoallyl substituent into an acetonyl group has been reported. Although the pharmacokinetics of some members of the series have been studied intensively in man (Clifford, 1974) and in the rabbit (Somani, 1975) as far as known a systematic study of these barbiturates has not been reported.

In the course of our studies on the ethyl-alkyl substituted barbiturates we have investigated the derivatives listed in Table 12.1 and tried to find the relationship between their pharmacological behaviour and chemical structure.

TABLE 12.1

Chemical structure, apparent (APC at pH 7.4) and true (TPC) partition coefficients of a number of barbiturates



Compound	R_1	R_2	R_3	pK_a [1]	log TPC octanol/water	log APC octanol/water	log APC cells/water
Allo	H	allyl	-C-C=C	7.68	1.43	0.94	-0.02
Apro	H	allyl	-C(-C)-C	7.90	1.61	1.22	0.17
Tal	H	allyl	-C(-C)-C-C	7.91	1.76	1.35	0.22
Neal	H	allyl	-C-C(-C)(-C)-C	7.72	2.53	1.71	0.40
Seco	H	allyl	-C(-C-C-C)-C	7.90	2.59	1.97	0.54
Bral	H	bromo-allyl	-C-C=C	7.70	2.05	1.37	0.27
Noct	H	bromo-allyl	-C(-C)-C	7.70	2.21	1.47	0.28
Pern	H	bromo-allyl	-C(-C-C)-C	7.70	2.58	1.72	0.53
Rect	H	bromo-allyl	-C(-C-C-C)-C	7.70	3.13	2.09	0.70
m-Bral	CH_3	bromo-allyl	-C-C=C	7.90	2.34	1.78	0.66
Funarc	CH_3	bromo-allyl	-C(-C)-C	7.90	2.78	2.11	0.75
m-Pern	CH_3	bromo-allyl	-C(-C-C)-C	7.90	3.20	2.43	0.87

1) From Doornbos (1969) and Krahl (1940)

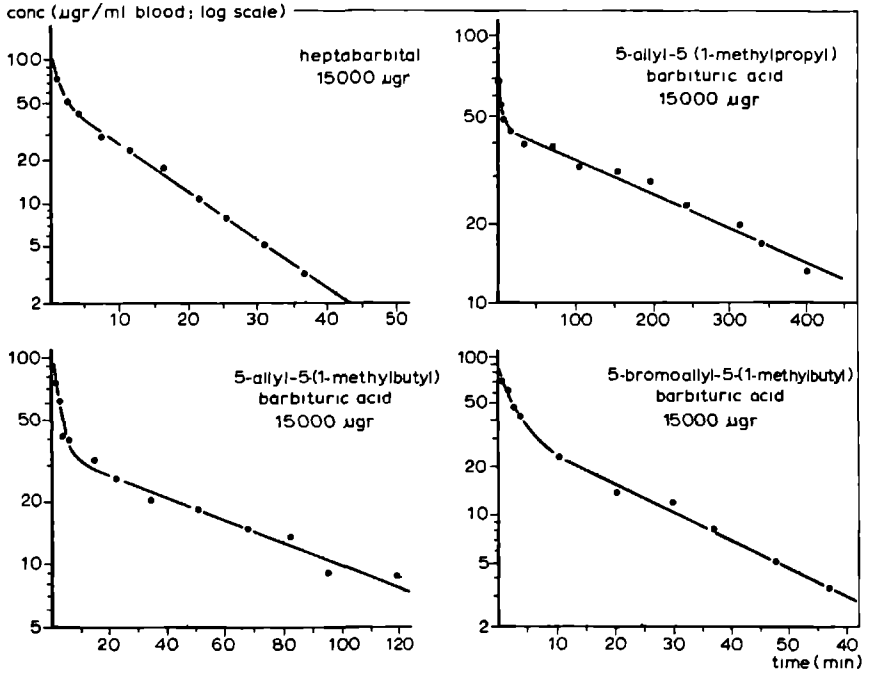


Fig. 12.1 *Blood concentration-time curves of some 5-allyl-5-alkyl and 5-bromoallyl-alkyl substituted barbiturates following intravenous administration in the same rat. The half-life or clearance of heptabarbital was used as parameter for the drug metabolic activity of the animal*

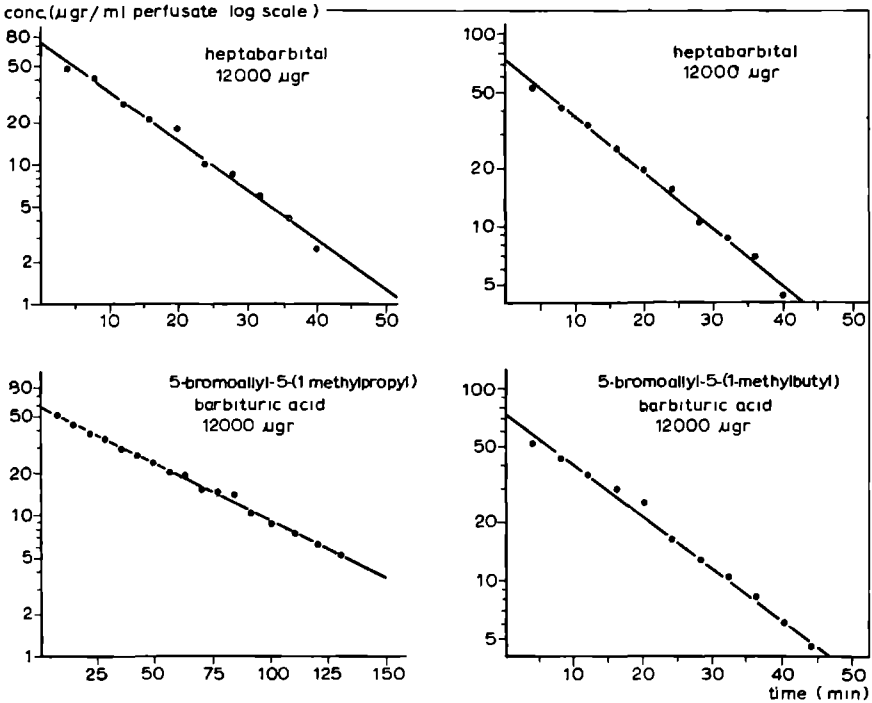


Fig. 12.2 *Blood concentration-time curves of some 5-bromoallyl-5-alkyl substituted barbiturates in the isolated perfused rat liver system*
Right: 5-bromoallyl-5-(1-methylbutyl) barbituric acid (Rectidon®)
Left : 5-bromoallyl-5-(1-methylpropryl) barbituric acid (Pernocton®)
The half-life or clearance of heptabarbital was used as parameter for the drug metabolic activity of the liver

In vivo experiments

Figure 12.1 shows the blood concentration-time curves of some derivatives following intravenous administration in the same rat. The clearance of heptabarbital was used as reference for the metabolic activity of the animal. In observing the last phase of the curves, which represent the overall elimination of the barbiturates, two distinct features can be seen. In the first place the rate at which the barbiturates disappear from the blood is higher for the derivatives with the larger substituent. Secondly, the bromoallyl substituted barbiturate has a shorter half-life than the allyl homologue. In Table 12.2 the half-lives and apparent volumes of distribution of the barbiturates studied are presented. The half-life value of 5 h in rats reported by Pelkonen (1973) for aprobarbital is in good agreement with our findings. Unfortunately there are no reports available on the half-lives of the other barbiturates.

There appears to be no structure-correlated differences among members of the allyl on the one hand and bromoallyl series on the other hand as far as the apparent volumes of distribution are concerned. This indicates that the differences in half-life of these barbiturates are based on differences in elimination clearances. In Table 12.3 the absolute and relative clearances are presented both for the allyl and bromoallyl series. With the exception of nealbarbital, there is an increase in clearance when the alkyl side chain becomes longer. This is in agreement with the results obtained in the ethyl-alkyl series.

The isolated perfused rat liver system

Apparently the time course of the barbiturates in the isolated perfused rat liver system can be described by a one-exponential equation, since there is no measurable distribution phase (Fig. 12.2). There is no considerable uptake of barbiturates by the liver, as appears from the apparent volumes of distribution, which equal almost the volume of the perfusion fluid (Table 12.2). The half-lives of the derivatives presented in Table 12.2 show for some compounds considerable differences

TABLE 12.2

Half-lives and apparent volumes of distribution of some barbiturates in the intact rat and in the isolated perfused rat liver system

Compound	Half-life (min)		Half-life ratio[1]		Apparent volume of distribution (ml)	
	intact rat	perfusion	intact rat	perfusion	intact rat	perfusion
Apro	353±27 (3)	235±78 (3)[b]	26.3±3.2	76.5±32.4[b]	291±14	242±18
Tal	240±34 (4)	113±19 (3)[b]	19.6±3.3	19.6± 3.5[a]	281±34	273±28
Neal	294±30 (3)	120±11 (3)[c]	25.1±1.9	22.1± 4.5[a]	247±27	202±13
Seco	55±16 (4)	32±13 (3)[a]	5.0±1.4	3.2± 0.4[a]	242±85	226±16
Bral	189±22 (4)	230±45 (5)[a]	14.6±1.5	28.8± 3.9[c]	249±51	263±27
Noct	163±40 (4)	128±39 (3)[a]	13.2±1.5	20.1± 2.5[b]	204±54	220±30
Pern	48±12 (3)	32±10 (3)[a]	4.0±0.8	3.7± 0.4[a]	207±36	202± 8
Rect	20± 7 (5)	12± 5 (4)[b]	1.8±0.3	1.0± 0.1[b]	279±48	192±18
M-bral	41± 6 (3)	27± 4 (3)[b]	3.7±0.9	5.4± 1.4[a]	295±14	269±50
Eunarc		18± 4 (3)		1.9± 0.2		230±12
M-pern		7± 1 (3)		1.6± 0.1		221±17
Hepta	11± 2 (24)	9± 3 (34)[b]			161±41	226±30[c]

1) ratio half-life (derivative/heptabarbital), measured in the same rat or the same perfusion

2) mean ± S.D.; number of experiments in parentheses

a. not significantly different $P > 0.05$ *c. significantly different* $P < 0.001$

b. significantly different $0.001 < P < 0.05$

TABLE 12.3

Clearances and clearance ratios of some barbiturates in the intact rat and the isolated perfused rat liver system

Compound	Clearance (ml/min)		Clearance ratio[1]		Clearance ratio[2]	
	intact rat[3]	perfusion	intact rat	perfusion	intact rat	perfusion
Apro	0.58±0.08 (3)	0.67±0.11 (3)[a]	0.048±0.002	0.015±0.005[c]	0.063±0.003	0.016±0.005[c]
Tal	0.84±0.10 (4)	1.72±0.48 (3)[b]	0.058±0.010	0.066±0.006[a]	0.075±0.013	0.068±0.007[a]
Neal	0.62±0.09 (3)	1.28±0.20 (3)[b]	0.044±0.006	0.049±0.007[a]	0.056±0.008	0.051±0.007[a]
Seco	3.14±0.45 (4)	5.76±2.72 (4)[a]	0.211±0.028	0.303±0.064[b]	0.274±0.036	0.335±0.066[a]
Bral	0.95±0.21 (4)	0.76±0.29 (5)[a]	0.116±0.006	0.037±0.005[c]	0.150±0.008	0.038±0.005[c]
Noct	1.05±0.13 (4)	1.26±0.47 (3)[a]	0.099±0.008	0.047±0.004[c]	0.128±0.011	0.048±0.004[c]
Pern	3.20±0.60 (3)	4.86±1.81 (3)[a]	0.222±0.026	0.314±0.030[b]	0.288±0.034	0.325±0.031[a]
Rect	12.29±2.14 (5)	13.12±4.45 (4)[a]	0.770±0.024	0.965±0.175		
M-bral	5.56±1.12 (3)	6.97±0.80 (3)[a]	0.603±0.051	0.270±0.060[c]	0.782±0.067	0.280±0.062[c]
Eunarc		8.11±0.75 (3)		0.524±0.042		0.544±0.066
M-pern		23.33±3+34 (3)				0.616±0.038
Hepta	12.68±3.01 (24)	22.70±9.80 (24)[c]				

1) heptabarbital clearance ratio *2) Rectidon^{R} clearance ratio* *3) mean ± S.D.; number of experiments in parentheses*

a. not significantly different $P > 0.05$

b. significantly different $0.001 < P < 0.05$

c. significantly different $P < 0.001$

with the values found *in vivo*. The differences may be based in some cases on differences in the metabolic activity between the rat and the perfused liver and then the half-life ratio found *in vivo* and *in vitro* will be similar. The half-life ratios suggest that a difference exists between the *in vivo* and *in vitro* value for aprobarbital, Noctal^{R} and brallobarbital. The clearance constants, which can be considered as metabolic clearance constants in the isolated perfused rat liver system, are shown in Table 12.3. Aprobarbital, Noctal^{R}, brallobarbital and its N-methyl derivative clearly possess a lower clearance ratio in the liver perfusion than in the intact rat.

Partition coefficients

As in the ethyl-alkyl series, the partition coefficients were determined in two partition systems, namely the commonly used system octanol/water and the isolated hepatocytes/water system. The results are presented in Table 12.1. Comparing the APC values at pH 7.4 of the two systems a good correlation was found (r 0.936, s 0.088).

There are apparently three ways to increase the lipophilicity. First there is an increase in partition coefficients resulting from the introduction of longer alkyl side chain as observed previously in the 5-ethyl-5-alkyl series. Secondly, substitution of a bromoallyl instead of an allyl group has the same effect and finally, a more lipophilic barbiturate can be obtained by the introduction of a methyl group to the nitrogen of the barbituric acid nucleus.

Relationship clearance - lipophilicity

As for the 5-ethyl-5-alkyl series we have fitted the clearance ratios to the partition coefficients using a parabolic equation. When we excluded the data obtained for aprobarbital *in vivo* and the aberrant *in vitro* values in the bromoallyl series, we found a good correlation. The correlation coefficients and the standard deviation using the octanol and the hepatocytes system were respectively r 0.964, s 0.147 and r 0.944, s 0.171.

TABLE 12.4

Sleeping times (A,min) and blood concentrations at awakening (B,min) of a number of 5-allyl-5-alkyl and 5-bromoallyl-5-alkyl substituted barbiturates following intravenous administration (12 mg) in rats

	Apro		Tal		Seco		Bral		Noct		Pern		Rect	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
	63	36	155[1]	28	100	9	15	33	50	32	59	18	25[1]	11
	74	44	27	23	95	10	62[1]	44	29	31	50	23	16	11
	75	43	27	28	51	12	58[1]	44	33	37	10	20	20	17
							6	35					30[1]	11
							47	45						
Mean B[2]	41 ± 2		26 ± 3		10 ± 2		40 ± 5		33 ± 2		20 ± 1		13 ± 3	

1) dose 15 mg

2) significantly different (F-test; $F_{(5,15)} = 28.73$)

Sleeping time and blood concentration at awakening

Differences in central depressant potency can be investigated roughly by measuring the brain drug concentration at awakening assuming a uniform drug distribution through the brain. The blood concentration at that time corresponds well with the brain drug concentration. We have, therefore, determined that blood concentration with the help of the sleeping time and the blood concentration-time curves. The results are presented in Table 12.4. It can be seen that in this series too the derivative with the 1-methyl-butyl substituent, secobarbital, has the lowest value for the concentration at awakening.

DISCUSSION

There is no clear structure-related difference in the apparent volume of distribution between the members of the 5-allyl and 5-bromoallyl series. The differences in half-life apparently reflect differences in clearances. Here too the discrepancy between the heptabarbital ratio found *in vivo* and *in vitro* is present as mentioned earlier for the 5--ethyl-5-alkyl series. The use of Rectidon^{R} as reference compound can avoid these apparent discrepancies. Observing the clearance constants found for the allyl substituted derivatives a progressive enhancement in clearance is seen for compounds with longer alkyl substituents, with the exception of nealbarbital. Metabolism of alkyl substituted barbiturates occurs mainly by hydroxylation at the penultimate carbon atom of the longest side chain (Maynert, 1965). In the neopentyl side chain this carbon atom is blocked, therefore only the allyl substituent, which is apparently less susceptible to oxidation, can be metabolized. This is confirmed by studies on nealbarbital in which as principal metabolite nealbarbital diol was found (Gilbert, 1974). A similar effect has been reported by Dixit (1969), who increased the duration of action of amobarbital in mice by two-fold by blocking the penultimate carbon atom by the introduction of a methyl group. By comparing the clearance constant ratios *in vivo* and in the perfusion it can be seen that a sig-

nificant difference exists with aprobarbital. In dog and man aprobarbital is excreted unchanged in the urine in amounts up to 24% of the dose administered (Maynert, 1949). In the intact rat the clearance constant of elimination of aprobarbital is probably representing the renal clearance constant. Renal excretion of aprobarbital is facilitated by its rather low lipophilicity. Nealbarbital, which is also metabolized slowly, but which is more lipophilic, is not excreted by the kidney Gilbert, 1974). The difference in clearance ratio found *in vivo* and in the perfusion for some 5-bromoallyl substituted derivatives cannot be due to renal excretion. Noctal^{R} and brallobarbital are reported to appear unchanged in the urine of man in negligible amounts (Keding, 1969; Maynert, 1949).

In an extensive study on the pharmacokinetics of brallobarbital it was found that the rate of elimination depends on the route of administration. Presenting this barbiturate directly to the liver resulted in an increase in the half-life (Yih, 1976). Toxic effects of Noctal^{R} in rats have been reported resulting in so-called delayed death, while for Pernocton^{R} and Rectidon^{R} a lower toxicity was reported (Holck, 1936). Obviously, the metabolic pathway of the bromoallyl group is involved in this phenomenon. With Pernocton^{R} and Rectidon^{R} hydroxylation at the alkyl substituent will become more dominant; considering their metabolism these barbiturates belong to the allyl series. The N-methylated derivatives are cleared more rapidly than their non-methylated homologues. Demethylation, therefore, would lead to accumulation of the nor-compound. In spite of reports on demethylation of N-methyl derivatives (Craig, 1970) we never found demethylation products in the intact rat and in the perfusion studies. This is in agreement with reports on the metabolism of Eunarcon^{R} (Ravn-Jonsen, 1974) and more recent studies on the metabolism of 1-N-methyl-barbital (Vore, 1975), which indicate that dealkylation is a minor route of elimination in the rat. Therefore, the higher clearance of the methylated derivatives is the result of the higher lipophilicity of these compounds. Possibly, this increase in lipophilicity results in a higher affinity of the compounds to the microsomal enzymes. For the bromoallyl derivatives on the one hand the higher lipophilicity may result in a higher clearance, while on the other hand bromination of the

allyl group will result in a substituent, which is much more susceptible to metabolism and is also faster eliminated.

The results presented in Table 12.4 show that the bromoallyl derivatives possess a higher central depressant potency than the allyl derivatives. This effect, however, is opposed by the increased metabolism of those compounds. In conclusion, it can be stated that the earlier concept of increased hypnotic action by the introduction of a bromine is questionable. Moreover, bromination enhances the toxicity of some derivatives as has already been found for Noctal^{R} and brallobarbital.

CHAPTER 13

RING-SUBSTITUTED BARBITURATES IN THE RAT AND IN THE LIVER PERFUSION

INTRODUCTION

The ring-substituted barbiturates represent a series of barbituric acid derivatives most commonly used in medical practice. Cyclopal^{R}, cyclobarbital-Ca (Phanodorm^{R}) and heptabarbital (Medomin^{R}) are employed as hypnotics, while phenobarbital (Luminal^{R}) and N-methylphenobarbital (Prominal^{R}) are used as sedatives and anti-epileptic drugs (Blum, 1932). Until recently hexobarbital (Evipan^{R}) has been employed as an anaesthetic. Moreover, hexobarbital has been used widely as a model substrate for studying the hydroxylating activity of the liver, either by measuring sleeping times or disappearance rate of hexobarbital.

The metabolic fate of these barbiturates has been investigated intensively. A series of papers dealing with the metabolism of hexobarbital in rabbits has been published by Tsukamoto (1958), followed by reports on the metabolism of cyclobarbital. Studies in rats and dogs have been carried out by Holcomb (1974) and Bush (1973) respectively.

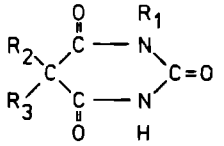
The cycloalkenyl substituted barbiturates have been reported to be hydroxylated in the cyclic substituent preferably at the 3-position and they can be converted into ketones (Nielsen, 1968). For methylphenobarbital the 4-hydroxy-phenyl derivative has been reported as principal metabolite (Mark, 1963).

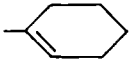
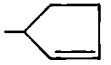
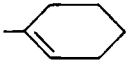
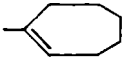
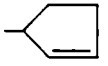
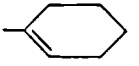
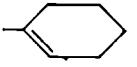
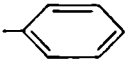
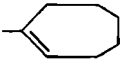
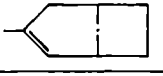
The pharmacokinetics in man of some members of the series have been studied recently (Breimer, 1974; Clifford, 1974), but a study on the structure-activity relationship (SAR) of a large number of this type of barbiturates has not yet been undertaken. For this purpose we have synthesized several N-methylated derivatives in addition to hexobarbital and methylphenobarbital.

We have studied the pharmacokinetics of some cyclic substituted barbiturates, the chemical structure of which is given in Table 13.1, in the intact rat and in the isolated perfused rat liver system. We have tried to find a relationship between their physico-chemical characteris-

TABLE 13.1

Chemical structure, apparent (APC at pH 7.4) and true (TPC) partition coefficients of a number of barbiturates



Compound	R_1	R_2	R_3	pK_a [1]	log TPC octanol/water	log APC octanol/water	log APC cells/water
Norhexo	H	-C		7.86	1.16	1.02	0.08
Cyclop	H	-C-C=C		7.80	1.64	1.51	0.22
Cyclo	H	-C-C		7.51	2.02	1.77	0.27
Hepta	H	-C-C		7.45	2.45	2.17	0.48
Rep	H	-C-C		7.52	2.78	2.53	0.57
m-Cyclop	CH_3	-C-C=C		8.26	1.95	1.89	0.41
Hexo	CH_3	-C		8.20	2.04	1.98	0.49
m-Cyclo	CH_3	-C-C		8.14	2.32	2.25	0.63
m-Pheno	CH_3	-C-C		7.70	2.03	1.86	0.48
m-Hepta	CH_3	-C-C		7.85	2.95	2.82	0.78
m-Rep	CH_3	-C-C		7.92	3.26	3.15	1.13

1) From Butler (1952); Doornbos (1969) and Kakemi (1967)

tics and their pharmacokinetic behaviour.

RESULTS

In vivo experiments

In Figure 13.1 blood concentration-time curves are shown following intravenous administration in the same rat. The curves display the same biphasic time courses as was found earlier after intravenous injection (chapters 11 and 12). The rate at which these four barbiturates disappear from the blood seems to be connected with the chemical structure

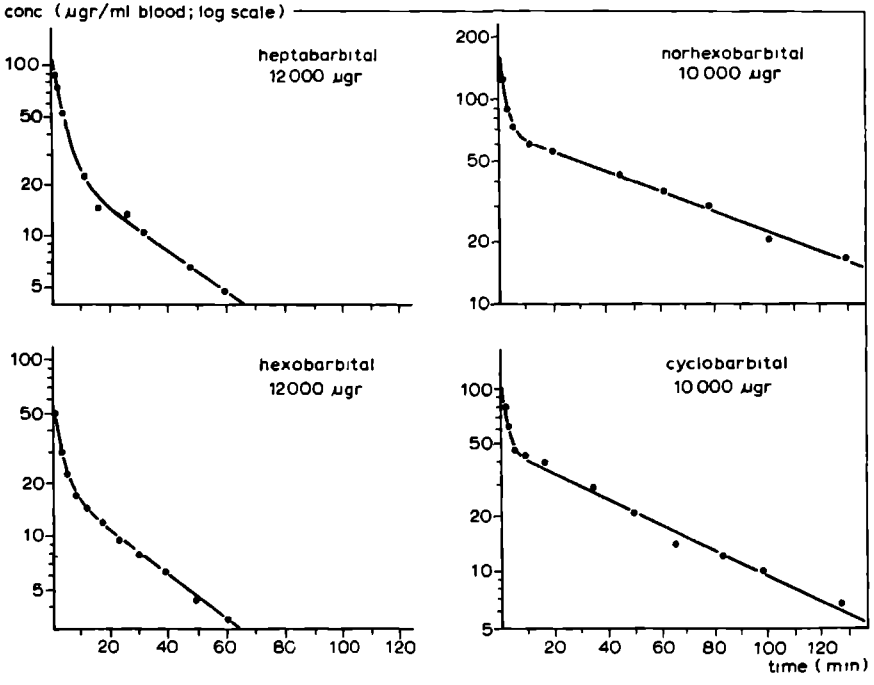


Fig. 13.1 *Blood-concentration-time curves of some cycloalkenyl substituted barbiturates following intravenous administration in the same rat. The half-life or clearance of heptabarbital was used as parameter for the drug metabolic activity of the animal*

TABLE 13.2

Half-lives and apparent volumes of distribution of some barbiturates in the intact rat and the isolated perfused rat liver system

Compound	Half-life (min)		Half-life ratio[1]		Apparent volume of distribution (ml)	
	intact rat[2]	perfusion	intact rat	perfusion	intact rat	perfusion
Norhexobarbital	78±13 (7)	108±21 (3)[a]	5.6±2.2	11.8±2.7[b]	171±222	252±28
Cyclopal[R]	75± 7 (5)	59± 5 (3)[b]	4.2±1.6	8.7±1.3[b]	313±101	230±43
Cyclobarbital	50±12 (5)	25± 4 (3)[b]	2.9±1.1	4.6±0.9[b]	291±105	226±29
Heptabarbital	17± 6 (14)	7± 2 (32)[c]			197± 55	242±19[b]
Reposal[R]		9± 1 (3)		1.4±0.2		220± 9
Methylcyclopal	18± 4 (4)	10± 1 (3)[b]	1.4±0.3	1.5±0.3[a]	263± 46	256±43
Hexobarbital	26± 3 (4)	7± 2 (4)[c]	1.3±0.5	1.1±0.2[a]	266± 31	228±21
Methylcyclobarbital	33±13 (7)	4± 1 (4)[c]	2.0±0.5	0.6±0.1[c]	895±282	249±22
Methylphenobarbital		146±16 (3)		16.6±3.2		222±15
Methylheptabarbital	10± 2 (3)	2± 1 (3)[c]	0.8±0.1	0.4±0.1[b]	187± 43	212±10
Methylreposal		3± 1 (3)		0.4±0.1		221±16

1) ratio half-life (derivative/heptabarbital), measured in the same rat or the same perfusion

2) mean ± S.D.; number of experiments in parentheses

a. not significantly different $P > 0.05$ *(Student's t-test)*

b. significantly different $0.001 < P < 0.05$

c. significantly different $P < 0.001$

TABLE 13.3

Clearances and clearance ratios of some barbiturates in the intact rat and the isolated perfused rat liver system

Compound	Clearance (ml/min)		Clearance ratio[1]	
	intact rat[2]	perfusion	intact rat	perfusion
Norhexobarbital	1.39± 0.25 (7)	1.53± 0.44 (3)[a]	0.132±0.014	0.092±0.008[b]
Cyclopal[R]	3.21± 1.66 (5)	2.81± 0.68 (3)[a]	0.277±0.073	0.134±0.010[b]
Cyclobarbital	4.36± 1.90 (5)	7.96± 0.72 (3)[b]	0.359±0.072	0.299±0.028[b]
Heptabarbital	12.21± 2.82 (14)	21.82± 5.81 (32)[c]		
Reposal[R]		17.55± 0.65 (3)		0.757±0.063
Methylcyclopal	15.01± 4.76 (4)	17.80± 4.49 (3)[a]	1.072±0.205	0.815±0.092[a]
Hexobarbital	14.53± 3.57 (4)	32.57±11.65 (4)[b]	1.331±0.316	1.252±0.054[a]
Methylcyclobarbital	22.32± 6.69 (7)	46.51±13.39 (4)[c]	2.019±0.077	1.554±0.208[c]
Methylphenobarbital		1.29± 0.24 (3)		0.076±0.009
Methylheptabarbital	19.48±12.58 (3)	57.67± 4.19 (3)[b]	1.676±0.723	2.570±0.301[a]
Methylreposal		52.61± 6.96 (3)		2.428±0.414

1) heptabarbital clearance ratio measured in the same rat or the same perfusion

2) mean ± S.D.; number of experiments in parentheses

a. not significantly different $P > 0.05$ *(Student's t-test)*

b. significantly different $0.001 < P < 0.05$

c. significantly different $P < 0.001$

and can be summarized as follows: the cycloheptenyl derivative disappears more rapidly from the blood than the cyclohexenyl substituted compound; N-methylation increases the disappearance rate; the compound with a methyl substituent at the 5-position disappears more slowly from the blood than its 5-ethyl substituted homologue.

In Table 13.2 the absolute and relative half-lives have been given together with the apparent volumes of distribution. Our value for the hexobarbital half-life is in the same range as that reported by Holcomb (1974). Unfortunately there are no reference data available for the other compounds.

The apparent volumes of distribution were subject to a considerable variation. No immediate explanation can be given for the large apparent volume of distribution, found for methylcyclobarbital.

In Table 13.3 the clearance constants of elimination and the heptabarbital clearance ratio are presented. The clearance of the N-methylated barbiturates is considerably larger than that of the corresponding parent compounds.

The isolated perfused liver

Figure 13.2 presents the time courses of some ring-substituted barbiturates in the perfusion system. As was found previously, the time courses of the barbituric acid derivatives in this system can be described apparently by a one-exponential equation. However, for the N-methylated compounds a biphasic time course has to be expected, since there exist considerable differences in clearance for the optical antipodes (Breimer, 1974).

The half-lives and apparent volumes of distribution are presented in Table 13.2. In contrast with the results found *in vivo*, the apparent volumes of distribution of the derivatives in the perfusions do not differ much and are of the same order of magnitude as the perfusion volume.

Hexobarbital is the only compound of which reference data are available in the literature. Half-lives of about 11 and 7.5 min have been reported by Holcomb (1974) and by Alvarez (1971) respectively. These authors, however, used a perfusion volume of 100 ml.

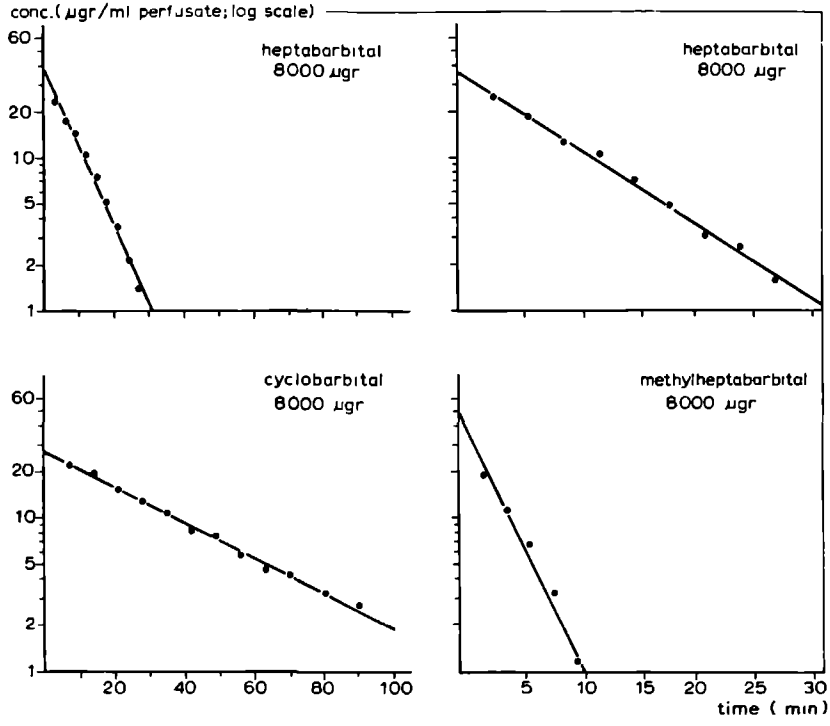


Fig. 13.2 *Blood concentration-time curves of some cycloalkenyl substituted barbiturates in the isolated perfused rat liver system*
Right: N-methylheptabarbital
Left : cyclobarbital
The half-life or clearance of heptabarbital was used as parameter for the drug metabolic activity of the liver

The clearance constants and clearance ratios shown in Table 13.3 are consistent with half-lives presented in Table 13.2 Since there are no significant differences in apparent volume of distribution between the several members of the series the differences in half-life are due to differences in clearance.

TABLE 13.4

Sleeping times (A,min) and blood concentrations at awakening (B,mg/l) of a number of cycloalkenyl substituted barbiturates following intravenous administration (12 mg) in rats

	Norhexo[1]		Cyclop		Cyclo		Hepta		m-Cyclop		Hexo		m-Cyclo	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
	88	51	22	27	2	48	4	23	3	16	3	22	5	13
	105	61	24	36	7	30	5	27	6	26	8	14	5	17
			43	20	8	38	6	21	7	29	11	19	11	11
			47	23	14	76	7	22	9	26	16	16	11	10
			60	26	14	43	7	17					15	9
							9	19					31	12
							9	18						
							9	23						
Mean B[2]	56 ± 7		26 ± 6		39 ± 7		21 ± 3		24 ± 5		18 ± 4		12 ± 3	

1) Dose 25 mg

2) Significantly different (F-test)

Partition coefficients

Octanol/water and hepatocytes/water partition coefficients are presented in Table 13.1. There was a good correlation between the values of the two partition systems (r 0.957, s 0.087). The TPC's in both systems are lower than expected on basis of the data obtained with the alkyl substituted barbiturates. Similar results have been reported by Jansson (1973) for the partition system cornoil/water and by Kakemi (1967). The partition coefficient increases with increasing ring size both for the N-methyl derivatives and the nor-compounds. The N-methylated derivatives are more lipophilic than the corresponding parent compounds.

Sleeping times and blood concentrations at awakening

In Table 13.4 the sleeping times and the blood concentrations at awakening have been listed. The value obtained for hexobarbital is in agreement with that reported by Prioux-Guyonneau (1974). Norhexobarbital displayed a poor hypnotic activity. Dosages of 12 mg induced sleeping times of 3-4 min.

DISCUSSION

The clearance ratios, presented in Table 13.3, suggest that there is a difference in clearance between the intact rat and the perfusions for norhexobarbital, Cyclopal^{R} and cyclobarbital, although not so extreme as found previously for 5-ethyl-5-propyl barbituric acid (Table 11.3) and aprobarbital (Table 12.3). It may be that in the intact animal these derivatives are eliminated partly by other routes than hepatic metabolism. This is supported for norhexobarbital by studies in dogs, in which was shown that 13 per cent. of the dose was excreted unchanged in the urine (Bush, 1953). However, Cyclopal^{R} in dogs seemed to be excreted only up to 2.5 per cent. of the administered dose (Van der Brook, 1944), while the urinary excretion of cyclobarbital in rats has been reported to account for 1.5 per cent. of the dose administered (Goldschmidt, 1959). The clearance ratios of methylcyclopal and hexobarbital found *in*

vivo and in the perfusion show a better agreement. Hexobarbital has been reported to be eliminated mainly by metabolism and is excreted in the urine of rats to a negligible extent (Holcomb, 1974).

The clearance values found for methylcyclobarbital in the intact rat are rather high and are the result of the low blood levels found after the distribution phase. First the difference in the rate of elimination between the methylcyclobarbital enantiomers was thought as origin of these low blood levels, the last part of the curve mainly representing the elimination of the slow antipode. However, intravenous administration of (-) methylcyclobarbital in rats resulted also in low blood levels. At the moment no ready explanation can be given for the extremely high apparent volume of distribution of this barbiturate. Therefore, when discussing the relationship chemical structure-clearance, we mainly restrict ourselves to the data of the perfusion studies.

Analysing the effect of substituting larger cyclic substituents to the barbiturates, one has to keep in mind that besides an enhancement of the lipophilicity also the sensitivity of the cyclic substituent may be different. This is evident when we compare methylcyclobarbital and methylphenobarbital. The size of the cyclic substituent may also play a role.

Both in the N-methyl series and non-methylated series the derivatives with a (bicyclo-3,2,1-oct-2-en-yl) substituent disappear slower from the blood than the cycloheptenyl substituted compounds. This is consistent with the values reported for the half-lives of heptabarbital and Reposal^{R} in man being 7.7 and 9.5 h respectively (Breimer, 1974; Kessing, 1963).

The data, found for the cyclohexenyl substituted derivatives, indicate that for these compounds the lipophilicity is the most important parameter in determining the rate of metabolism, since for these four barbiturates hydroxylation at the 3-position of the ring-substituent has been reported to be the major pathway of metabolism in the rat. In our studies with N-methylated barbiturates we could never detect the demethylation products, which should accumulate, since they are metabolized at a much slower rate. This is confirmed for hexobarbital by the studies of Holcomb (1974). Methylphenobarbital, however, has been reported to be

demethylated in rats in amounts up to 50 per cent. of the administered dose (Butler, 1952; Craig, 1970). Our results suggest that the rapid clearance of the N-methyl series can be contributed to the higher lipophilicity of these barbiturates and not to the existence of an additional metabolic pathway such as demethylation.

As in the two other series of barbituric acid derivatives we have studied the structure-clearance relationship. Figure 13.3 shows a graph of the heptabarbital clearance ratios against the partition coefficients. It seems as if the N-methyl and the nor-compounds behave as members of two different homologue series, wherein the clearance values of Reposal[R] and its N-methyl homologue lie on the descending part of the parabola. The majority of the N-methylated derivatives possess a higher central depressant potency than the corresponding nor-compounds (Table 13.4). However, this is opposed by the higher metabolic clearance. The net effect being a short hypnosis. This makes them to potential suitable

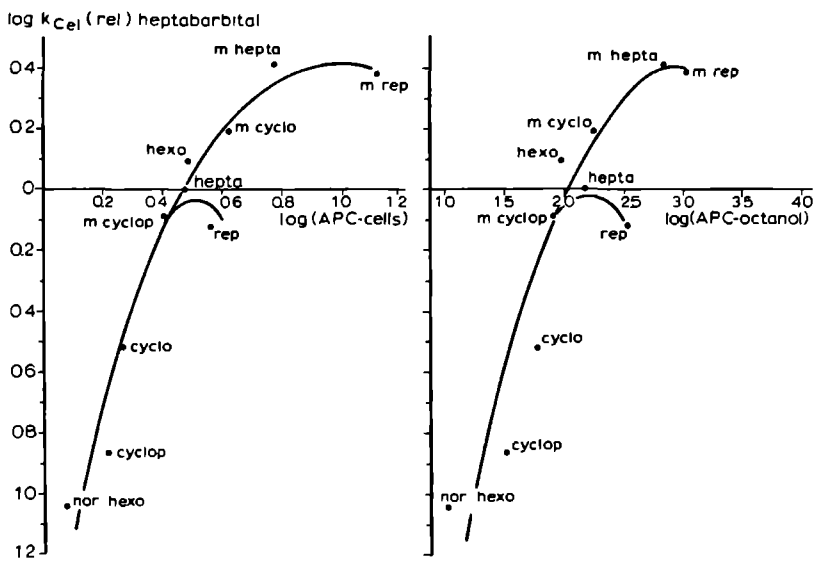


Fig. 13.3 *Graph of the logarithm of the heptabarbital clearance ratio, measured in the isolated perfused rat liver system*

drugs in hypnotic therapy in man, since the risk of residual effects is small, as has been shown for hexobarbital (Breimer, 1974).

CHAPTER 14

PHARMACOKINETICS OF BARBITURATES IN THE ISOLATED RAT HEPATOCYTE SUSPENSION

INTRODUCTION

With the development of new techniques for the enzymatic isolation of hepatocytes, it is now possible to obtain large quantities of morphological intact and biochemically functional cells. It has been shown that in hepatocyte suspensions the rate of oxygen consumption and of gluconeogenesis are similar to those observed in the intact organ (Krebs, 1975; Quistorff, 1973; Wagle, 1975).

Isolated hepatocytes have been used successfully in metabolic studies with alcohols (Grunnet, 1973; Moldeus, 1974), ethylmorphine (Erickson, 1976) and the first pharmacokinetic study in isolated hepatocytes using diphenylhydantoin as test substance has been published recently (Inaba, 1975).

In the course of our study on the relationship between the pharmacokinetics of barbiturates in intact rats, liver perfusions and enzyme preparations the isolated intact hepatocytes seem to be an ideal intermediate test system, since a number of drugs may be studied simultaneously under identical conditions using the same pool of hepatocytes. We, therefore, studied the pharmacokinetics of some homologous series of barbiturates and compared the results with those of the liver perfusions.

METHODS

Rat liver parenchymal cells were isolated according to the methods described by Seglen (1973) and Bojar (1975), with slight modifications. Liver cell concentrations were quantitated by counting aliquots of the suspension in a Bürker-Türk counting chamber to which 0.6% Trypan Blue was added. Cell yields ranged from 300 to 500 x 10^6 cells per liver, about 10% of which was stained with Trypan Blue.

Incubations were performed in the system presented in Fig. 6.2. which is a modification of the apparatus used by Jeejeebhoy (1975). The composition of the incubation medium was the following: 5 ml barbiturate solution (10 mg/100 ml buffer III), 10 ml erythrocyte-Eagle solution (1:1 v/v) and 15 ml hepatocyte suspension.

The final hepatocyte concentration was 2-3 x 10^6 cells/ml. The hepatocyte-erythrocyte solution was equilibrated for 20 min before the barbiturate solution was added. At various time intervals samples of 0.5 ml were taken and transferred to tubes containing 7 ml ether and internal standard.

RESULTS

Concentration-time curves were made from a series of barbiturates using the same pool of hepatocytes and by taking samples of the hepatocyte suspension at various periods after administration of a drug (see Fig. 14.1). A number of 5-ethyl-5-alkyl barbiturates were studied in the suspension of hepatocytes from the same liver, while heptabarbital was used as a reference drug. Apparently, the elimination in this metabolic system is a first order process in accordance with equation 7 for the open one-compartment model. In this homologous series the rate of elimination increases with increasing chain length of the alkyl substituent (see Fig. 14.1). The sequence in rate of elimination is the same as found in the intact rat and perfusion experiments, the heptyl derivative being eliminated most rapidly in all cases.

Secobarbital, Pernocton^R and Rectidon^R were the only members of the allyl and bromo-allyl series which were metabolized in the hepatocyte suspensions. Noctal^R, brallobarbital and methylbrallobarbital were not eliminated or at a very slow rate. As in the intact liver experiments the bromo-allyl derivative is eliminated more rapidly than its allyl homologue.

With the exception of heptabarbital the non-methylated derivatives of the ring-substituted barbiturates show also a low disappearance rate. N-methylation increases the rate of metabolism for all drugs studied. These N-methylated derivatives are metabolized very rapidly as is shown

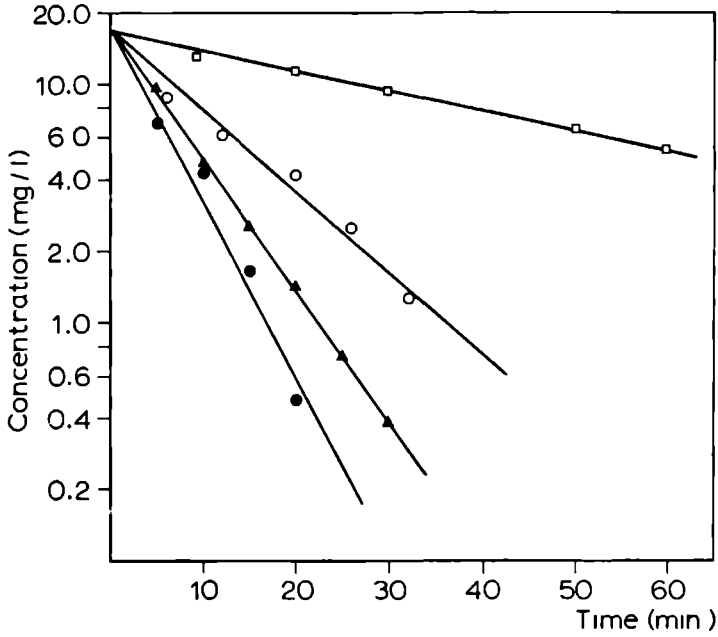


Fig. 14.1 *Elimination by isolated hepatocytes (2 x 10^6/ml)*
▲ *heptabarbital* ● *heptyl derivative*
○ *hexyl derivative* □ *pentyl derivative*
Concentration at zero-time 15 mg/l

in Fig. 14.2. They are metabolized in the same fashion as their parent compounds and not or hardly not by demethylation. Methylheptabarbital is eliminated most rapidly, while methylreposal is metabolized a little slower. This is in agreement with the results of the liver perfusion.

The half-lives and clearance constants of the derivatives studies are presented in Table 14.1, together with the values relative to heptabarbital. The importance of the standardization becomes apparent, when we compare the errors in the absolute and relative values.

For most derivatives the half-life ratios and the clearance ratios, measured in the hepatocyte and perfusion experiments, are in the same order of magnitude. An exception is methylheptabarbital. Apparently, the

turnover rate of this barbiturate in the hepatocyte suspension is higher than that in the isolated perfused rat liver system.

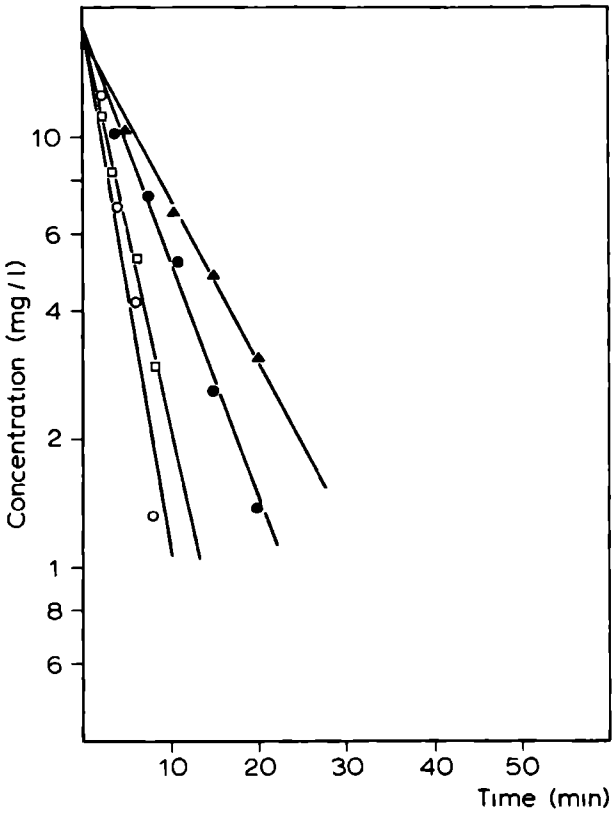


Fig. 14.2 *Elimination by isolated hepatocytes (2.5 x 10^6/ml)*
▲ *heptabarbital* ● *1-N-methyl cyclobarbital*
○ *1-N-methyl heptabarbital*
□ *1-N-methyl reposal*
Concentration at zero-time 15 mg/l

TABLE 14.1

Half-lives and clearances of some barbiturates in the hepatocyte suspension

Compound	Half-life (min)	Half-life ratio	Clearance (ml/min)	Clearance ratio
Butyl	147.7±71.8 (3)	19.8±5.7[a]	0.14±0.09	0.06±0.02[a]
Pentyl	50.5±31.3 (6)	4.8±0.9[a]	0.53±0.39	0.22±0.04[a]
Hexyl	22.9±24.0 (7)	1.4±0.1[a]	1.58±0.06	0.73±0.06[a]
Heptyl	11.2± 6.6 (4)	0.8±0.2[a]	2.46±2.23	1.27±0.22[a]
Seco	42.7±13.3 (3)	3.7±0.5[a]	0.52±0.11	0.28±0.03[a]
Pern	26.3±11.0 (3)	2.2±0.4[b]	0.80±0.23	0.43±0.04[b]
Rect	10.2± 4.2 (3)	0.9±0.1[a]	2.13±0.60	1.12±0.11[a]
m-Cyclo	5.9± 0.7 (4)	0.7±0.1[a]	3.52±0.34	1.52±0.15[a]
m-Hepta	2.2± 0.7 (4)	0.3±0.1[b]	10.40±2.66	4.38±0.77[c]
m-Rep	3.4± 0.6 (4)	0.4±0.1[a]	6.20±1.32	2.64±0.49[a]
Hepta	13.5±12.4 (28)		2.17±1.48	

1) ratio half-life and clearance (derivative/heptabarbital)

2) mean ± S.D.; number of experiments in parentheses

a. not significantly different from perfusion $P > 0.05$

b. significantly different from perfusion $0.001 < P < 0.05$

c. significantly different from perfusion $P < 0.001$

The clearance of barbiturates in a suspension of about 6.10^7 hepatocytes increases strongly with increasing chain length from butyl to heptyl. Between different batches of hepatocytes there is quite some variation because of variation in the amount and the quality of the liver cells. For this reason and in order to allow a comparison with the liver perfusion data, heptabarbital is used as a reference compound and clearance ratios have been given in Table 14.1. The clearance is linearly proportional with the number of hepatocytes in the cell suspension (see chapter 7). The intact rat liver contains about 120.10^7 hepatocytes (Zahlten, 1974), that is about 20 times more than in the suspensions used in our experiments. The clearance of the barbiturates in the perfused liver is only 7-10 times larger than in the cell suspension. This may indicate that the isolated hepatocytes have a higher metabolic capacity than the isolated liver. It is possible that in the intact liver the real number of cells, engaged with the conversion of the barbiturates, is much lower than calculated. This may be caused by the internal structure of the liver and the arrangement of the cells within the organ.

For compounds which are cleared very rapidly transport processes across the cell membrane may be rate limiting in the turnover of the compound. Accordingly, the clearance ratio for such compounds in the cell suspensions will be higher than that in the perfused liver experiments, since, as compared with the intact liver, in the hepatocyte suspension a larger surface is available for transport across the cell membrance. There are indications that this is the case with methylheptabarbital (see also chapter 15).

The metabolites formed in the hepatocyte suspension appeared to be identical to those found in the intact liver experiments, indicating that the metabolic pathways in both systems are the same.

Our findings indicate that hepatocytes as experimental material for metabolic studies are quite comparable with perfusion studies.

CHAPTER 15

PHARMACOKINETICS OF BARBITURATES IN A SUBCELLULAR SYSTEM OF RAT LIVER

INTRODUCTION

During the last ten years many studies on the metabolic conversion of barbiturates by rat liver homogenates have been published. Most of these studies deal with enzyme kinetics in liver microsomes (McCarthy, 1970; Sitar, 1973), others deal with the nature of the metabolites formed (Holtzman, 1975; Kupfer, 1973).

In spite of the numerous investigations on the metabolism of barbiturates in the intact rat and in liver microsomes, there are only a few comparative studies on drug metabolizing enzyme systems in liver homogenates and systems with intact cellular structure. However, a comparison is necessary in order to determine the reliability of data, obtained in subcellular systems with respect to systems with intact cells.

The kinetics of barbiturate metabolism has been studied in a subcellular system of rat liver (9000 g supernatant fraction) while a comparison is made between the kinetic data of the same substances in intact liver cells and the liver perfusions.

METHODS

Twenty per cent. (w/v) 9000 g homogenates in Tris (0.05 M) - KCl (0.15 M) buffer pH 7.4 were prepared according to the method of Kato (1967). The incubation mixture contained 5 ml of the supernatant fraction (equivalent to 1 gr of liver), 5 ml barbiturate solution (10 mg/100 ml Tris), 5 ml of a NADPH-regenerating system and 15 ml Tris pH 7.4. At various time intervals samples of 0.5 ml were taken and transferred to tubes containing 7 ml ether and internal standard (see also chapter 6).

The elimination of the barbiturates from the incubation medium containing rat liver microsomes follows first order kinetics as may be seen from the semilogarithmic concentration-time curves, which are straight lines. The curves for the 5-ethyl-5-alkyl substituted barbiturates are given in Fig. 15.1. With increasing chain length of the substituent the rate of metabolism increases as in the liver cells and in the intact liver. There is a good correlation between the sequence in disappearance rate in this subcellular system and the intact liver cell system (see chapter 14). The butyl derivative was metabolized so slowly that in the time course of the experiment no metabolism could be observed. The same holds for Talbutal^{R}, Noctal^{R}, brallobarbital and the N-methylated derivatives Eunarcon^{R} and methylbrallobarbital.

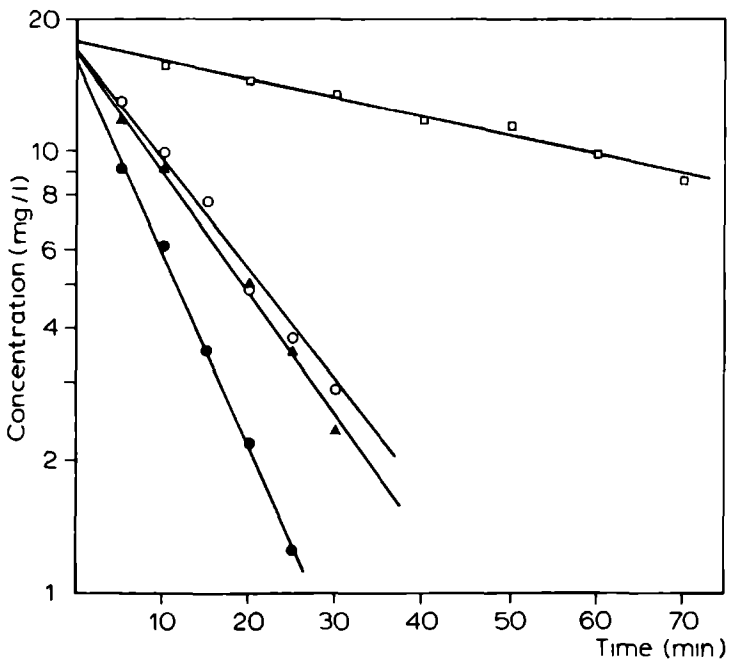


Fig. 15.1 *Elimination by 9000 g rat liver supernatant*
▲ *heptabarbital* ● *heptyl derivative*
○ *hexyl derivative* □ *pentyl derivative*

In Figure 15.2 the curves of three bromoallyl substituted derivatives are presented. The curve of Noctal^R is very flat and is therefore eliminated very slowly while Rectidon^R is eliminated very rapidly and about twice as rapidly as the reference compound, heptabarbital. This is in contrast with the results of the perfusion and cell experiments, where both drugs are eliminated at equal rate.

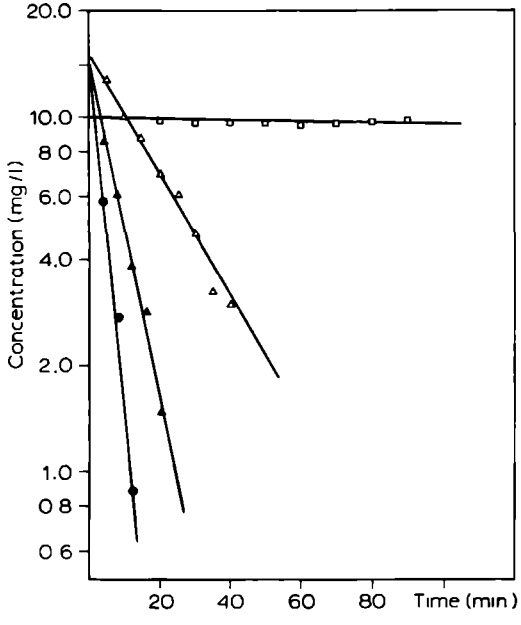


Fig. 15.2 *Elimination by 9000 g rat liver supernatant of some 5-bromoallyl-5-alkyl substituted barbiturates*
▲ *heptabarbital* ● *Rectidon^R*
△ *Pernocton^R* □ *Noctal^R*

The absolute and relative half-lives and clearance constants of the derivatives studied are presented in Table 15.1. In contrast with the results of the hepatocyte suspension, the data presented in Table 15.1 suggest that for most compounds there is a considerable difference in clearance between the liver perfusion and 9000 g supernatant experiments.

TABLE 15.1

Half-lives and clearances of some barbiturates in the 9000 g supernatant suspension

Compound	Half-life (min)	Half-life ratio	Clearance (ml/min)	Clearance ratio
Pentyl	61.7±13.3 (4)	8.7±1.9[b]	0.34±0.10	0.12±0.03[b]
Hexyl	12.5± 3.1 (4)	1.5±0.4[a]	1.68±0.44	0.70±0.12[a]
Heptyl	8.8± 5.9 (6)	0.8±0.2[b]	3.05±1.52	1.26±0.21[b]
Octyl	8.3± 4.5 (6)	0.9±0.2[b]	3.10±1.43	1.16±0.23[b]
Nonyl	10.3± 6.1 (5)	1.1±0.4[a]	2.68±1.26	1.07±0.34[b]
Seco	55.2± 5.1 (4)	6.3±1.2[b]	0.37±0.03	0.17±0.03[b]
Pern	27.2± 8.1 (5)	2.8±0.3[b]	0.77±0.27	0.36±0.04[a]
Rect	4.8± 1.4 (5)	0.5±0.1[c]	4.42±1.65	2.03±0.14[c]
m-Cyclop	32.3±12.1 (4)	2.2±0.2[b]	0.70±0.21	0.51±0.08[b]
Hexo	17.4± 4.6 (7)	1.4±0.3[a]	1.27±0.28	0.79±0.11[a]
m-Cyclo	6.0± 1.7 (8)	0.5±0.1[b]	3.93±1.21	2.31±0.40[b]
m-Hepta	1.6± 0.5 (7)	0.1±0.1[c]	14.78±3.95	8.11±1.39[c]
M-Rep	2.1± 0.7 (4)	0.2±0.1[b]	11.39±3.48	5.38±1.05[b]
Hepta	10.3± 4.4 (27)		2.26±0.82	

1) ratio half-life and clearance (derivative/heptabarbital) measured in the same experiment

2) mean ± S.D.; number of experiments in parenthesis

a. not significantly different from perfusion $P > 0.05$

b. significantly different from perfusion $0.001 < P < 0.05$

c. significantly different from perfusion $P < 0.001$

As a result these barbiturates can be separated into two groups: the derivatives of which the clearance ratio perfusion/9000 g has become higher and the derivatives of which this ratio has become lower than unity (Table 15.2).

TABLE 15.2

Heptabarbital clearance ratios of hepatocyte and 9000 g experiments versus the heptabarbital clearance ratio measured in the perfusions

Compound	$\frac{k_{Cel}\ \text{ratio cells}}{k_{Cel}\ \text{ratio perf.}}$	$\frac{k_{Cel}\ \text{ratio 9000 g}}{k_{Cel}\ \text{ratio perf.}}$
Butyl	0.82±0.26	–
Pentyl	0.75±0.19	0.42±0.11[c]
Hexyl	0.90±0.08	0.87±0.15[a]
Heptyl	1.25±0.27	0.98±0.13[a]
Octyl		1.04±0.33
Nonyl		0.66±0.35
Seco	0.93±0.22	0.54±0.16[b]
Pern	1.37±0.17	1.14±0.16[a]
Rect	1.16±0.24	2.13±0.41[c]
m-Cyclop		0.62±0.11
Hexo		0.63±0.09
m-Cyclo	0.98±0.16	1.49±0.33[b]
m-Hepta	1.72±0.36	3.13±0.68[c]
m-Rep	1.09±0.27	2.22±0.59[b]

a. not significantly different $P > 0.05$

b. significantly different $0.001 < P < 0.05$

c. significantly different $P < 0.001$

One of the most striking differences between the 9000 g rat liver supernatant and the preparations with intact liver cells is the metabolic pathway of the barbiturates. In the former the major metabolic end products are the hydroxy-metabolites, while in the latter these metabolites are oxidized further (see chapter 18). This implies that in the 9000 g experiments there is an accumulation of hydroxy-metabolites, that may inhibit the metabolism of their precursors. This product inhibition may be due to an interaction with cytochrome P 450 (Von Bahr, 1971; Stavchansky, 1974). In contradiction with this is the fact that the clearance of hexobarbital in the liver perfusion is not affected by the administration of a high dose of its hydroxy-metabolite to the perfusion medium. This metabolite penetrates into the liver cells because it is further metabolized to a keto derivative. Product inhibition may, however, occur with other members of the series.

Noctal^{R}, brallobarbital and methylbrallobarbital are not measurable metabolized in the 9000 g supernatant suspension. This supports our earlier findings that these barbiturates are eliminated at a slower rate as compared with the intact rat experiment, when presented directly to the liver enzymes (see also chapter 11 and 24). Rectidon^{R} and methylheptabarbital are very rapidly eliminated by 9000 g rat liver supernatant and intact liver cells but in the 9000 g rat liver supernatant even more rapidly than in the hepatocyte suspension when compared with the reference drug heptabarbital. This indicates that the capacity of the hepatic microsomal enzymes is higher than the supply of drug and may imply that for compounds which are rapidly eliminated transport across the liver cell membrane may become a limiting factor. Further evidence for the existence of a transport barrier in isolated hepatocytes is provided by a recent report (Von Bahr, 1974). It was shown that the hexobarbital uptake into hepatocytes occurs by a non-energy requiring process, which is dependent on the lipid solubility of the compound. Further it was shown that the rate of formation of the type I spectral change (see chapter 16) was lower for cells than for liver homogenates or microsomes.

Results from 9000 g rat liver supernatant or liver microsomes may

not be extrapolated directly to *in vivo* situation. The morphological features of the liver cells, the organization of the liver and the circulatory aspects have to be taken into consideration.

CHAPTER 16

THE DISSOCIATION CONSTANTS (K_s) OF THE BINDING OF BARBITURATES TO CYTOCHROME P 450 OF RAT LIVER AND THE MICHAELIS-MENTEN CONSTANTS (K_m) OF A NUMBER OF BARBITURATES

INTRODUCTION

For many drugs there seems to exist a relationship between the ability to produce a spectral change with liver microsomes and to undergo oxidative metabolic conversion (Schenkman, 1967). This suggests that drug binding to the microsomes is an obligatory step in the mechanism of drug oxidation. The dissociation constant, obtained by spectral difference measurement, is regarded as a measure of the affinity of the substrate for cytochrome P 450.

Although various studies have been undertaken to measure the K_s values of certain barbiturates (Degwitz, 1969; Jansson, 1972; Topham, 1970), systematic studies on large series of barbiturates are still lacking. We have, therefore, determined the K_s values of a large number of barbiturates and have correlated K_s values with the lipophilicity and the metabolic clearance of the compounds.

RESULTS

In the concentration ranges used all barbiturates showed a type I spectral change with an absorption minimum at 420 nm and a peak at 390 nm. Figure 16.1 shows the spectral change of a number of 5-ethyl-5-alkyl substituted barbiturates, added to a final non-saturating concentration of 0.22 mMol. There is an increase in spectral change from the propyl to the heptyl derivative, while a further lengthening of the alkyl side chain results again in a smaller spectral change. In Table 16.1 the dissociation constants of the 5-ethyl-5-alkyl series, based on the induced spectral change, are listed. Our values for the butyl and s-pentyl derivative are consistent with those reported by Jansson (1971). The value of

the i-pentyl derivative is in agreement with that of Topham (1970) and Sitar (1973).

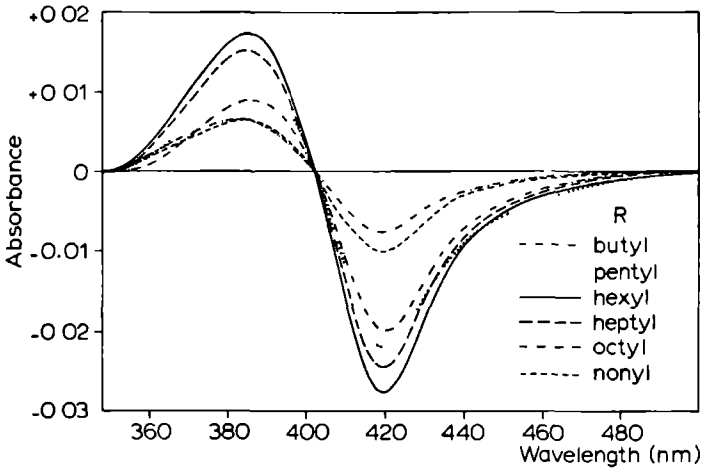


Fig. 16.1 *Type I spectral change caused by ethyl-alkyl barbiturates final concentration 0.22 mMol*

Figure 16.2 shows the reciprocal plots of induced spectral changes of a number of 5-allyl-5-alkyl substituted barbiturates. As in the previous series there is clearly a relationship with the length of the alkyl substituent. Table 16.2 presents the values of the 5-allyl-5-alkyl and 5-bromoallyl-5-alkyl series together with some N-methylated homologues. In both groups there is an increase in affinity for cytochrome P 450 with increasing alkyl side chain. The K_s values of the bromoallyl substituted barbiturates are lower than those of their allyl substituted homologues.

TABLE 16.1

K_s values of a number of 5-ethyl--5-alkyl substituted barbiturates

Compound	K_s
Propyl	0.089±0.009
Butyl	0.089±0.009
Pentyl	0.032±0.004
i-Pentyl	0.038±0.017
s-Pentyl	0.045±0.012
2,3-But	0.025±0.002
Hexyl	0.019±0.003
Heptyl	0.020±0.004
Octyl	0.024±0.002
Nonyl	0.056±0.011

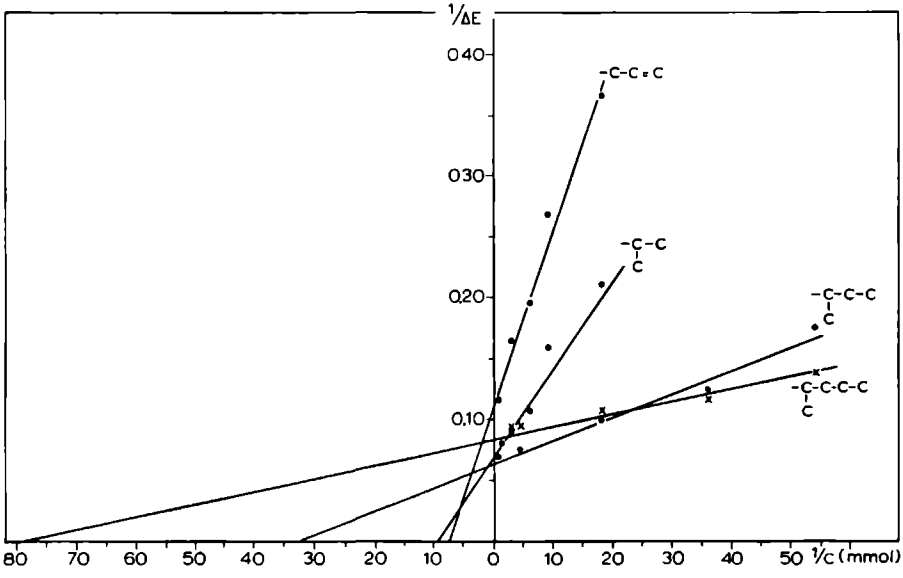


Fig. 16.2 *Reciprocal plots of changes in absorbance at 420 nm relative to 390 nm caused by consecutive additions of 5-allyl-5-alkyl substituted barbiturates to microsomal suspensions*

TABLE 16.2

K_s values of a number of 5-allyl--5-alkyl and 5-bromoallyl-5-alkyl substituted barbiturates

Compound	K_s
Allo	0.394±0.064
Apro	0.219±0.047
Tal	0.098±0.008
Neal	0.061±0.007
Seco	0.028±0.002
Brallo	0.136±0.030
Noct	0.105±0.015
Pern	0.031±0.005
Rect	0.013±0.001
m-Bral	0.103±0.013
Eunarc	0.034±0.001
m-Pern	0.012±0.001

Figure 16.3 shows the reciprocal plots of a number of N-methylated 5-ethyl-5-cycloalkenyl substituted barbiturates. There is apparently an optimum in affinity for the cycloheptenyl substituted derivative. In Table 16.3 the K_s values are listed of a number of N-methylated barbiturates with a cyclic substituent at the 5-position, together with their nor-homologues. As in the previous series the N-methylated derivatives have lower K_s values than their non-methylated homologues, they therefore bind stronger to the enzyme P 450. Topham (1970) reports a K_s value for norhexobarbital and hexobarbital of 0.08 and 0.09 mMol respectively, while Jansson (1973) reports for hexobarbital a value of 0.08 mMol. This discrepancy with our values may be caused by the fact that these authors have determined the K_s values in microsomes of phenobarbital-induced

rats. Attempts have been made to correlate the K_s values of the various barbiturates with their partition coefficients (log P).

TABLE 16.3

K_s values of a number ring-substituted barbiturates and their N-methylated homologues

Compound	K_s
Norhexo	0.099±0.003
Cyclop	0.080±0.010
Cyclo	0.041±0.003
Hepta	0.022±0.002
Rep	0.026±0.004
Hexo	0.036±0.003
m-Cyclop	0.025±0.001
m-Cyclo	0.024±0.002
m-Pheno	0.023±0.003
m-Hepta	0.015±0.002
m-Rep	0.017±0.001

In Figure 16.4 a curve is shown, which presents the relationship between log P and the affinity for cytochrome P 450, according to a parabolic function. A good correlation was found (r 0.935, s 0.105; for log P_{cells} r 0.967, s 0.121). This is consistent with the reports of Hansch (1968) on the parabolic dependence of pharmacological activity upon the lipophilic character of the compounds. In the 5-ethyl-5-alkyl series the lipophilicity was increased by increasing the chain length. Increased binding may not be caused by increased lipophilicity but by the conformation of the alkyl chain. Lipophilicity was, therefore, also increased by N-methylation while the conformation of the alkyl group

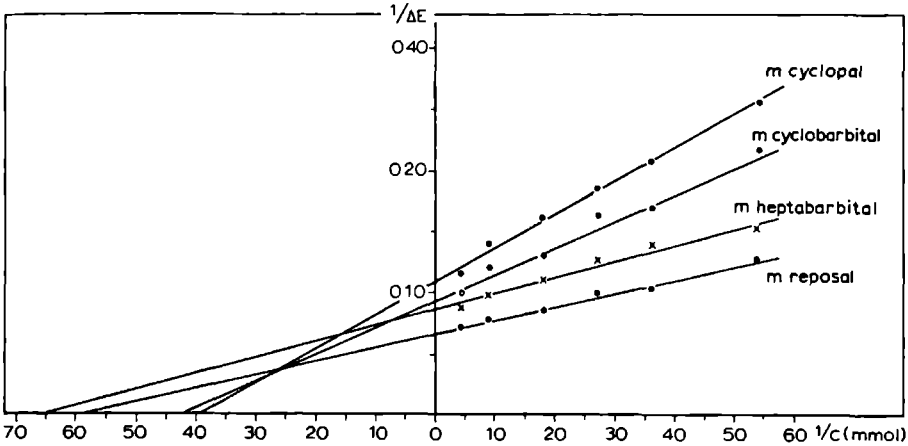


Fig. 16.3 *Reciprocal plots of changes in absorbance at 420 nm relative to 390 nm caused by consecutive additions of N-methylated ring-substituted barbiturates to microsomal suspensions*

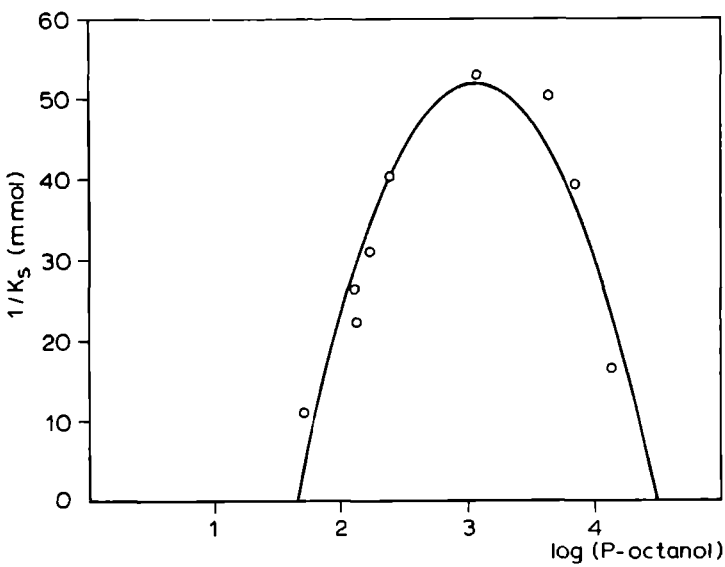


Fig. 16.4 *5-ethyl-5-alkyl series.*
Relationship between log P and the affinity to cytochrome P 450 described by a parabolic function (r 0.935, s 0.105)

remained unchanged. In Table 16.4 the K_s values of some N-methylated 5--ethyl-5-alkyl barbiturates are listed together with their octanol/water partition coefficients. The data presented in Table 16.4 indicate that the length of the substituent, rather than the lipophilicity per se is responsible for the descending part of the curve, presented in Figure 16.4.

TABLE 16.4

K_s values and partition coefficients of some N-methylated 5-ethyl-5-alkyl substituted barbiturates

Compound	K_s	log TPC oct/water
m-Hexyl	0.008±0.001	3.26
m-Hepty1	0.009±0.002	4.16
m-Octyl	0.011±0.002	4.25
m-Nonyl	0.020±0.003	4.34

As mentioned previously apparent Michaelis-Menten constants can be obtained in liver systems by administration of saturating dosages. Fig. 16.5 shows the concentration-time curve of 5-ethyl-5-octyl barbituric acid in the isolated perfused rat liver system. From this curve the Michaelis-Menten constant (K_m) and the metabolic capacity (Q_m) can be calculated with the following equations (Van Ginneken, 1974):

$$K_m = \frac{0.4343\ A}{(\log A^{\cdot} - \log A)} \qquad \text{and} \qquad Q_m = K_m k_{Cel}$$

In Table 16.5 some preliminary results are presented.

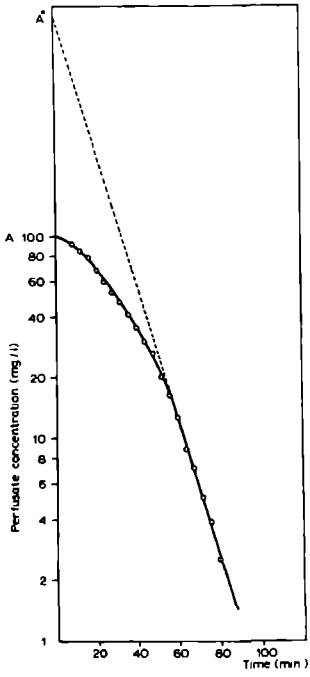


Fig. 16.5 *Saturation curve of 5-ethyl-5-octyl barbituric acid (35 mg) in the isolated perfused rat liver system*

TABLE 16.5

K_m and Q_m values of some barbiturates measured in the isolated perfused rat liver

Compound	K_m (mMol)	Q_m (uMol/min)
Hepta	0.26	2.56
	0.25	2.06
	0.29	9.26[1]
Octyl	0.24	2.41
	0.36	2.23
m-Cyclo	0.32	2.44
	0.30	2.49
Hexo	0.36	9.96[1]
	0.31	4.18[2]
Rect	0.25	3.88
	0.21	2.11

1) phenobarbital-induced

2) calculated from Stitzel (1968)

DISCUSSION

Our results suggest that there exists a relationship between the K_s values and the chemical structure of the barbiturates. The introduction of larger substituents either aliphatic or cyclic at the 5-position of the barbituric acid ring, results in a decrease in K_s. This is consistent with the data of Topham (1970). The bromoallyl substituted and N--methylated barbiturates have lower K_s values than the corresponding allyl substituted and non-methylated homologues. In the various homologous series this corresponds very well with the metabolic clearance.

Generally, compounds with a low K_s value are metabolized faster than those with higher K_s values. However, a quantitative relationship cannot be found.

Studying derivatives with no more than 6 or 7 carbon atoms in the side chain, as are used in clinical medicine, may lead to the conclusion that the K_s is only dependent on the lipophilicity of the compounds (Topham, 1970). Our results, however, indicate that with longer substituents the influence of a steric effect, opposite to the lipophilicity, becomes visible. This phenomena may be responsible for the flattening of the curve, representing the relationship between the clearance and the lipophilicity. The relationship between K_s values and metabolism is complex. Besides an affinity for the metabolizing enzymes, the rate of metabolism is dependent on the sensitivity of the substituent to metabolic attack, as appears from the K_s values and clearance ratios of nealbarbital and methylphenobarbital.

The K_s values are about a factor 10 smaller than the corresponding K_m values measured from metabolic conversion. Furthermore the metabolic clearance rate does not match the K_s or K_m values. This implies that binding to the metabolic enzymes is not the only factor responsible for the relationship between structure and pharmacokinetic properties.

SECTION IV

METABOLISM OF BARBITURATES

CHAPTER 17

PHARMACOKINETICS OF BARBITURATE METABOLITES

INTRODUCTION

The barbiturates are eliminated from the body mainly by metabolic degradation in the liver. Metabolism predominantly occurs by oxidation of the substituents at the 5-position of the barbiturate nucleus (Mark, 1969; Maynert, 1949).

Especially, the metabolism of 5-(cyclohexenyl) substituted derivatives, such as hexobarbital and cyclobarbital, has been studied intensively (Bush, 1973; Tsukamoto, 1958). Hexobarbital is generally used as model substrate for microsomal hydroxylation. In several studies the K_m value of this barbiturate has been determined, not only by following the disappearance of substrate, but also by measuring the formation of the hydroxy-metabolite (McCarthy, 1971; Sitar, 1971).

Although hydroxylation of the side chain appears to be a general route of inactivation, little is known about the fate of such hydroxy--metabolites. These metabolites lack pharmacological activity, but they may exert toxic action or in the course of their formation toxic intermediates may be formed.

In support of our studies on the pharmacokinetics of barbiturates, we have also investigated the pharmacokinetics of some of their metabolites.

RESULTS

In the isolated perfused rat liver hexobarbital is rapidly converted into a hydroxy-metabolite, which in turn is further metabolized into a corresponding keto-derivative, see Figure 17.1. The hydroxy-metabolite is more slowly eliminated than the parent compound, while the keto-product is still more resistent to elimination. The latter compound is apparently a metabolic end product that *in vivo* is cleared by the kidney. In the intact rat keto-hexobarbital is the major metabolite excreted by the

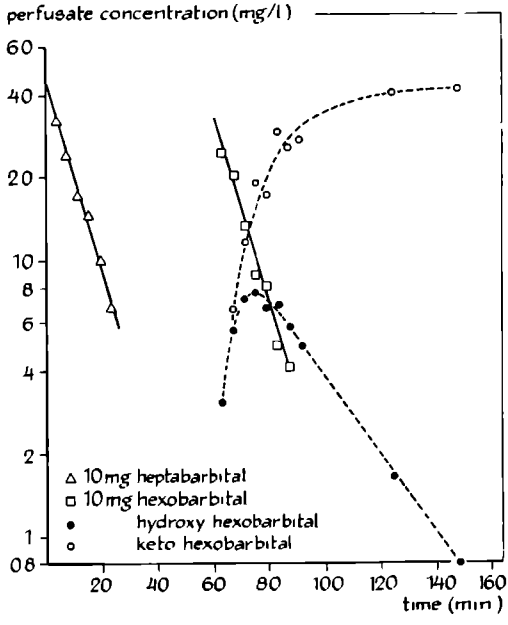


Fig. 17.1 *Time courses of hexobarbital and its metabolites in the isolated perfused rat liver system*

kidney to about 57% of the dose (Holcomb, 1974). N-demethylation does not take place in the isolated rat liver nor in the intact rat. In fact the N,N'-dimethyl and the N-methyl derivatives are metabolized according to the same pattern as the non-methylated barbiturates (see scheme in Fig. 17.2). The rate of metabolic conversion of the N,N'-dimethyl, N--methyl and non-methylated derivatives, however, is different. The N,N'--dimethyl derivatives are more rapidly metabolized than their N-methyl homologues; the latter in turn more rapidly than the non-methylated compounds.

In Figure 17.3 the time courses of N,N'-dimethylcyclobarbital and its metabolites in the perfusate are shown. As for the N-methyl derivatives the hydroxy-metabolite is converted to the ketone. This ketone,

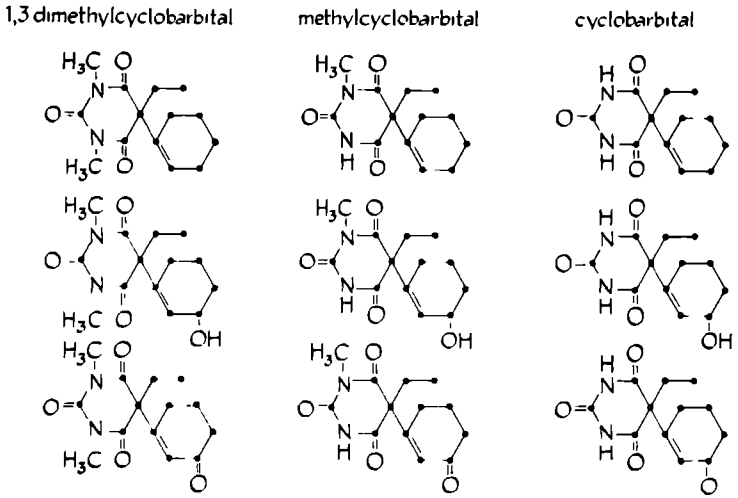


Fig. 17.2 *Scheme of metabolites of 5-cyclohexenyl substituted barbiturates*

however, disappears from the perfusate. Since in our analysis other metabolites could not be determined, the metabolic fate of this ketone is unknown. It may be converted to a malonuric acid derivative by cleavage of the barbiturate nucleus, as has been reported for hexobarbital (Bush, 1973).

In Table 17.1 the half-life ratios of the metabolites to their parent compounds are presented. The half-life ratios of the hydroxy-metabolites of the two cyclohexenyl substituted derivatives are similar.

5-Ethyl-5-alkyl substituted barbiturates have been reported to undergo side chain oxidation at the penultimate position to form an alcohol. For butobarbital, pentobarbital and amobarbital the 3-hydroxy--metabolites are reported to be the major urinary metabolites, together with the carboxy-metabolites (Maynert, 1952, 1965).

Numerous studies on the formation and the stereo-chemistry of these alcohols have been published, but only a few data are available with

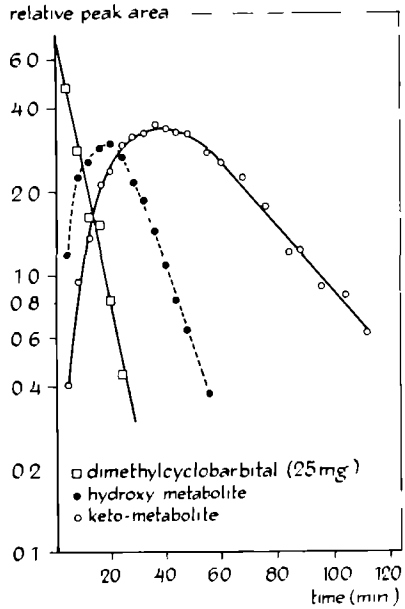


Fig. 17.3 *Time courses of 1,3-N,N'-dimethylcyclobarbital and its metabolites in the isolated perfused rat liver preparation. Since no reference compounds of the metabolites are available, the ratio of the metabolite peak areas to that of the internal standard have been plotted.*

regard to the keto-metabolites (Gilbert, 1974; Kuntzman, 1967). In our G.C. analysis of the experiments with 5-ethyl-5-alkyl substituted barbiturates only one "metabolite"-peak could be detected. However, after methylation and subsequent silylation of the samples, according to the method of Gilbert (1974), this peak resolved in our G.C. analysis into more peaks (see also chapter 19).

In Figure 17.4 the time courses of N,N'-dimethyl-5-ethyl-5-hexyl barbituric acid and its metabolites are shown. Apparently, two alcohols are formed. This is consistent with the data of Maynert (1965), who reported for 5-ethyl-5-hexyl barbituric acid the formation of two alcoholic

TABLE 17.1

Half-life ratio metabolite/parent compound of some barbiturates in the isolated perfused rat liver system

Compound	Half-life ratio	
	A	B
Hexo	2.68±0.31 (5)	
m-Cyclo	2.75±0.28 (5)	
Dm-Cyclo	1.81±0.05 (3)	4.46±0.80 (3)
m-Hepta	4.75±1.42 (3)	
m-Rep	6.71±0.91 (3)	

A. half-life ratio hydroxy-metabolite/parent compound
B. half-life ratio keto-metabolite/parent compound
Number of experiments in parentheses

metabolites. The 5-hydroxy derivative is the major alcoholic metabolite. Both alcohols are oxidized further to their keto-homologues, as appears from our G.C. and G.C. combined mass spectrometry analysis. The 4-hydroxy compound is oxidized more rapidly than the 5-hydroxy-metabolite.

The bromoallyl substituted barbiturates have been shown to be converted to the hydroxy-propyl and acetonyl metabolites (Ravn-Jonsen, 1970; Keding, 1969). For methylbrallobarbital the acetonyl-metabolite was found to be the principal metabolite. Figure 17.5 shows the time courses of this barbiturate and its metabolite in the isolated perfused rat liver system.

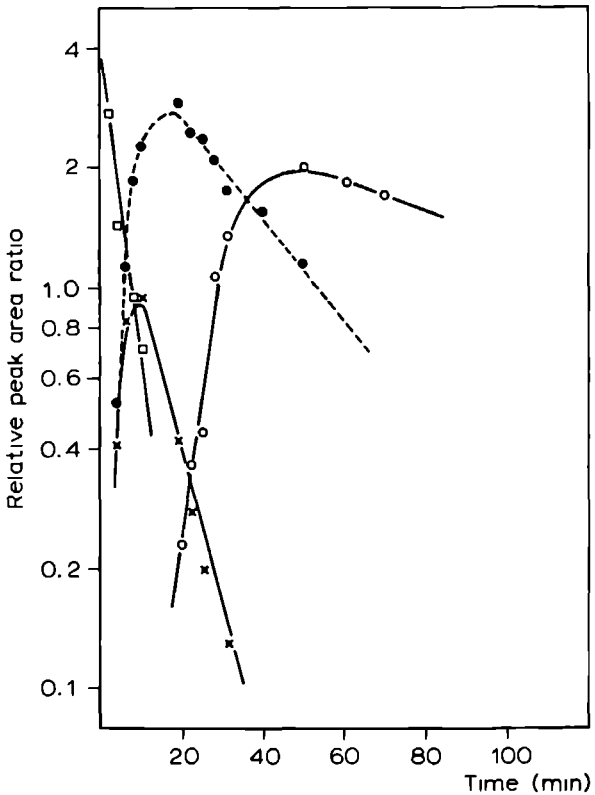


Fig. 17.4 *Metabolism of 1,3-N,N'-dimethyl-5-ethyl-5-hexyl barbituric acid by the isolated perfused rat liver*

- □ *1,3-N,N'-dimethyl-5-ethyl-5-hexyl barbituric acid*
- × *4-hydroxy-metabolite*
- ● *5-hydroxy-metabolite*
- ○ *keto-metabolite(s)*

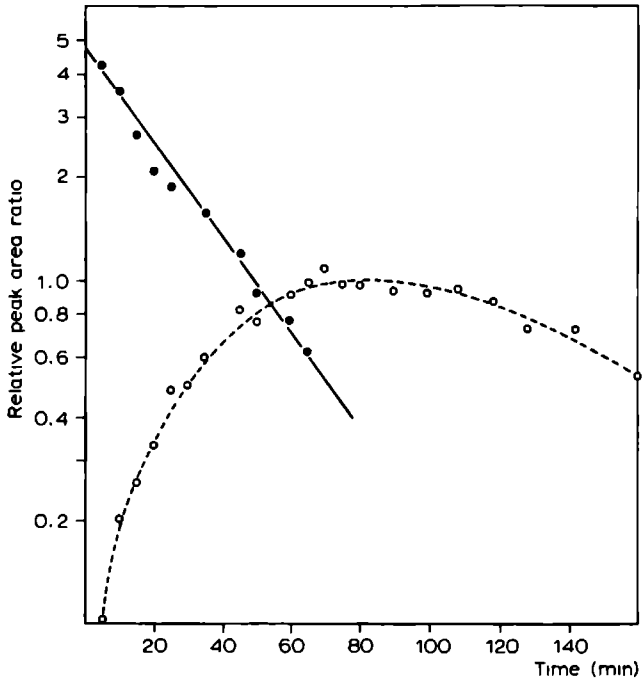


Fig. 17.5 *Metabolism of 1-N-methylbrallobarbital by the isolated perfused rat liver*
● *1-N-methylbrallobarbital*
○ *acetonyl-metabolite*

DISCUSSION

As appears from our results, the isolated perfused rat liver system is an excellent tool to study the pharmacokinetics of the metabolites of barbiturates. In this system many samples can be taken, which is necessary to follow the time courses of the metabolites accurately.

Various studies have been reported on the metabolism of hexobarbital by the isolated perfused rat liver (Bock, 1974; Holcomb, 1974; Stitzel, 1968). However, information about the pharmacokinetics of the

metabolites has not been given. This paper is the first, as far as known, which presents some information about the pharmacokinetic behaviour of barbiturate metabolites. It is shown that the metabolites formed by oxidation in the 5-substituent are more slowly eliminated than their corresponding parent compound. The keto-metabolites are more resistent to further metabolism than the hydroxy-metabolites. Knowledge of the half-lives of the metabolites is necessary with respect to a possible inhibitory action of these metabolites on the metabolism of other drugs, present in the body. Recently, it was shown *in vitro* that hydroxylated metabolites of one drug can inhibit the biotransformation of another drug (Stavchansky, 1974).

In view of the difference in lipophilicity between hexobarbital and its hydroxy-metabolite (Bush, 1973) the half-life ratio presented in Table 17.1 is rather surprising. A similar value has been reported in man for the half-life of amobarbital and its metabolite (Draffan, 1973).

Table 17.1 shows further, that for the derivatives with larger substituents, the difference between the parent compounds and their alcoholic metabolites becomes larger. Our results may indicate that either the barbiturates and their hydroxy-metabolites interact differently with the metabolizing enzyme system or with different enzymes (enzyme sites). The latter view is consistent with the observation that a soluble enzyme in the 9000 g fraction of liver homogenates is required for the formation of keto-hexobarbital (Toki, 1964) and keto-pentobarbital (Kuntzman, 1967).

CHAPTER 18

METABOLIC DIFFERENCES BETWEEN THE ISOLATED HEPATOCYTE SUSPENSION AND THE 9000 G RAT LIVER SUPERNATANT

INTRODUCTION

With the 9000 g fraction of liver homogenates one can do many parallel experiments with aliquots of material from one liver. However, one of the major drawbacks of this preparation is the destruction of the cellular integrity, which obviously has consequences for the metabolism in this preparation.

Many studies have demonstrated differences in the metabolism of barbiturates between experiments with whole animals or perfused organs and the 9000 g fraction of liver homogenates (Kuntzman, 1967; Sitar, 1973). Hydroxy-metabolites of barbiturates have been reported to be converted by enzymes, presented in the soluble fraction of the liver. Toki (1964) demonstrated the existence of several alcohol dehydrogenases for the conversion of hydroxylated barbiturates.

The development of techniques to isolate viable hepatocytes successfully made it possible to carry out parallel experiments with the preservation of the cellular structure. We have compared the metabolic performance of these two *in vitro* systems and have done some inhibition studies with ethanol in order to investigate the enzyme systems which are involved in the formation of the hydroxy- and keto-metabolites of barbiturates.

RESULTS

Figure 18.1 shows the time courses of N-methylcyclobarbital and its metabolites in a hepatocyte suspension. As in the perfusion experiments the barbiturate is converted to the hydroxy-metabolite, which in turn is oxidized rapidly to its keto-homologue. The keto-metabolite is apparently the metabolic end product.

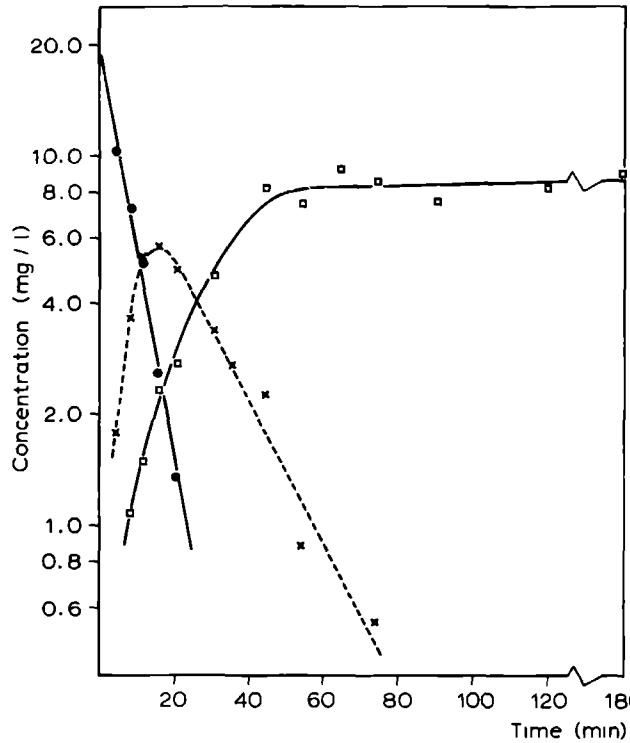


Fig. 18.1 *Metabolism of 1-N-methylcyclobarbital by isolated rat hepatocytes (3 x 10^6/ml)*
- ● *1-N-methylcyclobarbital (0.5 mg)*
- × *hydroxy-metabolite*
- □ *keto-metabolite*

Figure 18.2 presents the time course of the same barbiturate and its metabolite in the 9000 g rat liver supernatant. In this preparation the alcohol is the only metabolite, which can be demonstrated in our G.C. analysis. In contrast with the hepatocyte suspension, the alcohol is apparently not further oxidized.

Addition of ethanol to a hepatocyte suspension has no effect on the disappearance rate of the parent barbiturate and the formation of the hydroxy-metabolite, as is shown in Figure 18.3. The further oxidation of

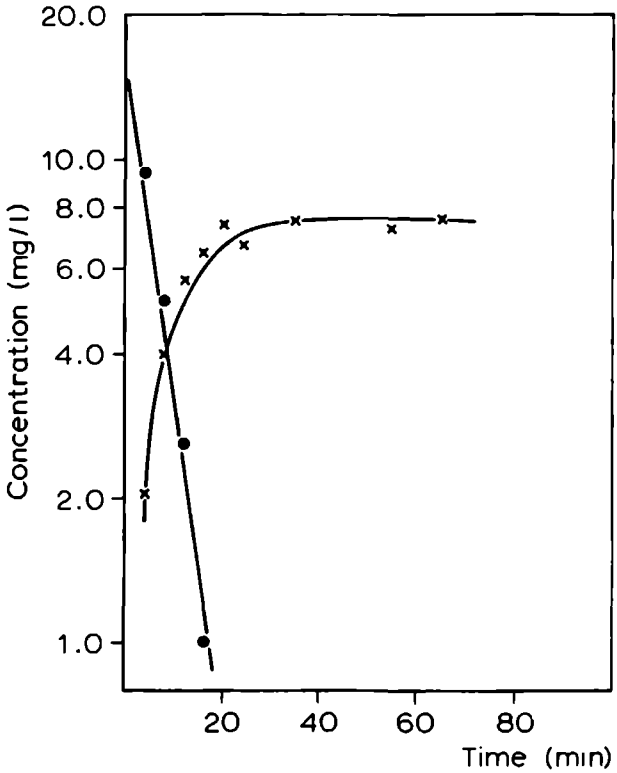


Fig. 18.2 *Metabolism of 1-N-methylcyclobarbital by 9000 g rat liver supernatant*
- ● *1-N-methylcyclobarbital (0.4 mg)*
- × *hydroxy-metabolite*

the alcohol to the keto-metabolite, however, is inhibited considerably. This results in higher levels of the hydroxy-metabolite, as compared with the control. Our results are in agreement with the data of Cinti (1973), who found that pentobarbital hydroxylation by the microsomal fraction was not affected by ethanol concentrations in the range of 2-50 mMol.

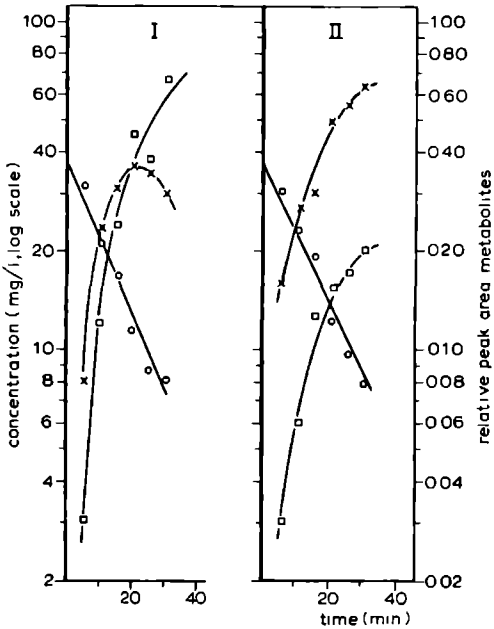


Fig. 18.3 *Effect of ethanol on the metabolism of N-methylcyclobarbital by isolated rat hepatocytes (3 x 10^6/ml)*
o *N-methylcyclobarbital (0.13 mM)*
× *hydroxy-metabolite*
□ *keto-metabolite*
I control
II 0.22 mM ethanol

DISCUSSION

Our results indicate that, contrary to the 9000 g rat liver supernatant, the isolated rat hepatocyte suspension as metabolizing system is quite comparable with the isolated perfused liver. Not only with respect to the elimination rate of the barbiturates themselves (see chapter 14), but also with respect to the formation of their metabolites.

Interesting is the selectivity of the inhibitory effect of ethanol on the metabolism of the hydroxy-metabolite. Haininen (1972) reports

that addition of ethanol to liver perfusions does not cause any redox changes in cytochrome P 450, while according to Cinti (1973) ethanol does not bind to cytochrome P 450. This may be an explanation, why the elimination of the parent compound is not inhibited.

Several authors have found a NAD dependent oxidation of alcohols to ketones by the soluble fraction in the liver. It may be that ethanol competites with the hydroxy-metabolite for the same co-factors. We have found that in damaged hepatocytes (Trypan Blue exclusion less than 50%) the elimination of the hydroxy-metabolite is much more retarded than that of the parent compound (Fig. 18.4). It may be that there is a leakage of some essential factors to the extracellular space. However, more experiments are necessary to establish the nature involved in the degradation of hydroxy-metabolites of barbiturates.

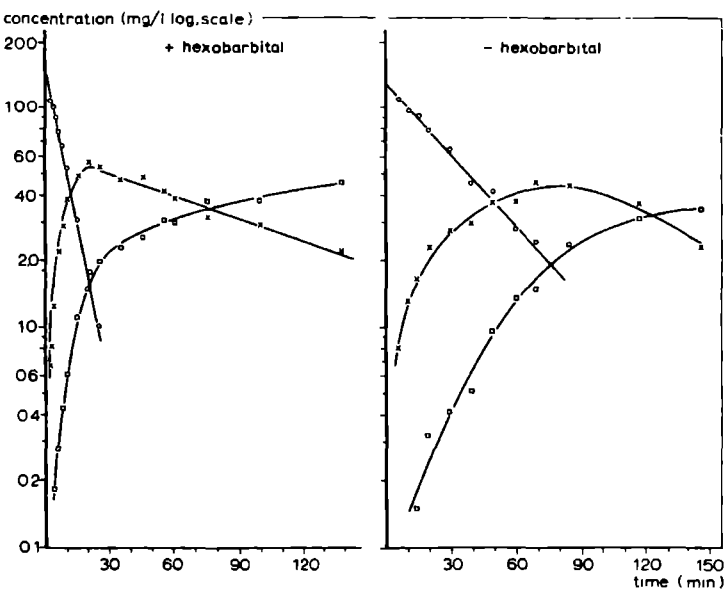


Fig. 18.4 *Influence of cellular damage on the metabolism of (+) and (-) hexobarbital*

- o *hexobarbital*
- × *hydroxy-metabolite*
- □ *keto-metabolite*

CHAPTER 19

IDENTIFICATION OF BARBITURATES AND THEIR METABOLITES

INTRODUCTION

G.C. combined mass spectrometry allows the identification of components of a mixture, which are present in low quantities by comparison with the mass spectra of known compounds. The mass spectra of barbiturates reflect the high stability of the barbiturate nucleus towards fragmentation. The 5,5-di-substituted barbiturates show preferentially radical cleavage or McLafferty rearrangement processes of the side chains at the 5-position, as appears from the numerous reports in the literature (Arnold, 1969; Coutts, 1968; Grützmacher, 1966).

The mass spectra of the 5-ethyl-5alkyl and 5-allyl-5-alkyl substituted barbiturates are characterized by the presence of abundant ions at m/e 141; 156 and m/e 167; 168 respectively, due to loss of the larger alkyl substituent. Distinction between the derivatives within each series is based on ions of low abundance and is often difficult. However, the presence of the two characteristic ions is helpful in the recognition of the "barbiturate" character of compounds as well as in synthesis of barbiturates as in metabolic studies. The 5-alkyl-5-cycloalken-1-yl substituted derivatives can be identified more easily, since they lose preferentially the short alkyl side chain (Gilbert, 1970). This results in abundant ions, the mass of which depends on the cyclic substituent. Barbiturates are reported to be metabolized mainly to hydroxy- and keto--metabolites. Identification of these metabolites can be facilitated by conversion to derivatives, p.e. hydroxyl groups may react with silylating reagents, which often results in a shift in G.C. retention time; N-methylation of the barbiturates usually gives greatly improved results with respect to the G.C. analysis.

EXPERIMENTAL

Mass spectrometric conditions

Mass spectra were obtained with a L.K.B. 9000 gas chromatograph–mass spectrometer. The apparatus was equipped with a glass column (1.8 m, 3 mm I.D.), packed with 3% OV-17 on gas-chrom Q 60-80 mesh. Helium was used as carrier gas at a flow rate of 10-20 ml/min. Temperature: oven 200-240°C; separator 260°C; ion source 170°C. Electron energy 20-70 EV, accelerating voltage 3.5 kV, trap current 60 μA, U.V. recorder paper Kodak, type 1895, spec. III.

Methylation and silylation

Methylation with diazomethane and silylation with bis-trimethylsilyltrifluor acetamide (BSTFA) was performed according to Gilbert (1974).

RESULTS

The metabolites of non-methylated and N-methylated derivatives have after conversion to the dimethylated derivatives with diazomethane the same G.C. retention times and mass spectra as the metabolites of the dimethylated barbiturates. This indicates that N,N'-dimethyl, N-methyl and non-methylated barbiturates are metabolized similarly, while they only differ in the velocity of metabolism. Therefore, the N,N'-dimethylated barbiturates are excellent tools to study the metabolism of barbiturates, since they are converted most rapidly.

Cycloalkenyl substituted barbiturates

In Figure 19.1 the gas chromatogram of a sample of an incubation with methylhexobarbital is shown. The treatment with BSTFA results in the formation of two other peaks before the original position of peak 1. In Figure 19.2 the mass spectrum and fragmentation scheme, analogous to Arnold (1969) of peak 1a is shown. Comparison with the mass spectrometric

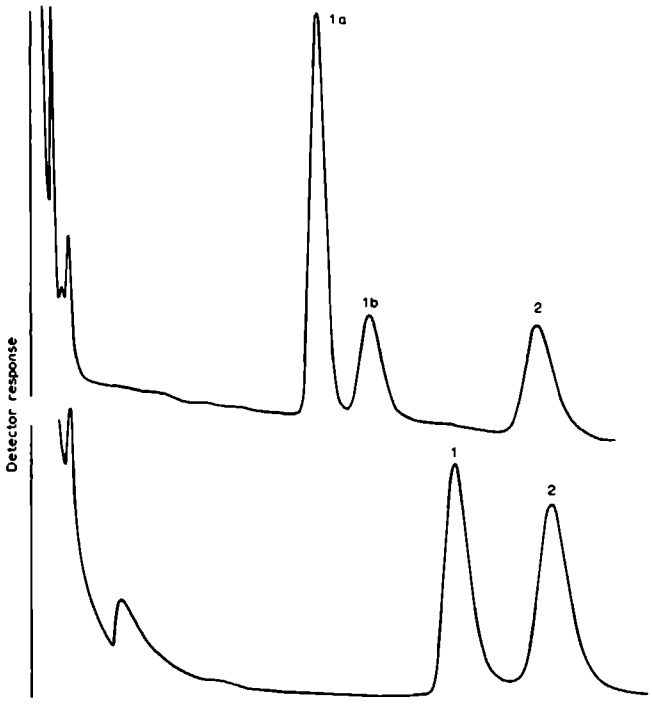


Fig. 19.1 *Gas chromatograms of a sample of a perfusion experiment with methylhexobarbital before (lower) and after (upper) silylation*

data of 3-hydroxy-hexobarbital (Gerber, 1971) and 3-hydroxy-heptabarbital (Gilbert, 1974) suggests that this compound is identical with the 3-hydroxy-metabolite.

The identity of peak 1b is still unknown. Peak 2 does not show a shift in G.C. retention time on silylation. The mass spectrum and fragmentation scheme, analogous to that presented for 3-keto-hexobarbital (Arnold, 1969) is shown in Figure 19.3. The ion at m/e 95 points to the presence of a cyclohexenone group (Arnold, 1969). The mass spectrum of peak 2 is identical with that of methylated 3-keto-hexobarbital (Breimer, 1974).

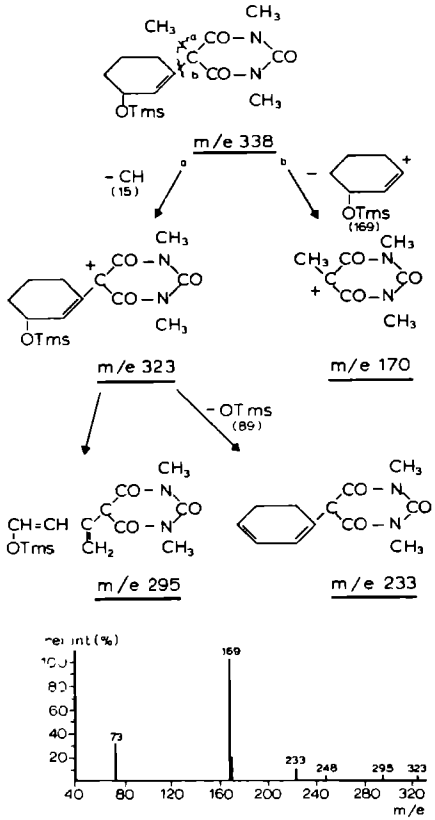


Fig. 19.2 *Fragmentation scheme of silylated hydroxy-methylhexobarbital and the mass spectrum of peak 1a*

5-Ethyl-5-alkyl substituted barbiturates

In contrast with the cycloalkenyl substituted barbiturates in our G.C. analysis of experiments with 5-ethyl-5-alkyl barbiturates and their methylated homologues there is always only one "metabolite"-peak present. In Figure 19.4 two gas chromatograms of experiments with the N,N'-dimethylated hexyl derivative are shown, after silylation. Before silylation there was only a rather broad peak at the position of peak 5. The

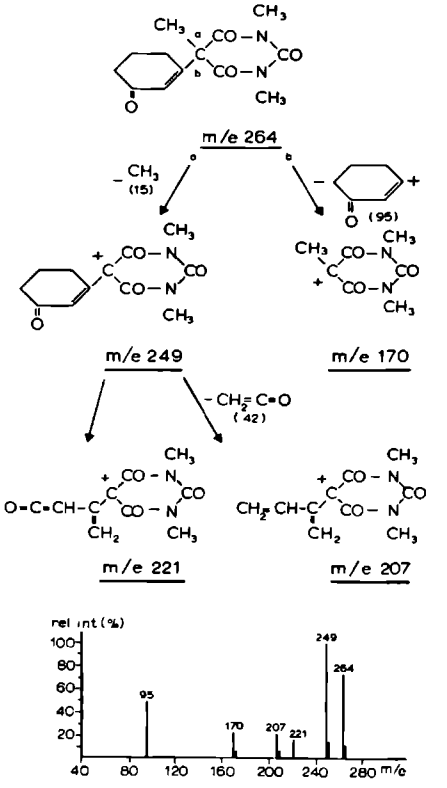


Fig. 19.3 *Fragmentation scheme of keto-methylhexobarbital and the mass spectrum of peak 2*

shift of peak 3 and peak 4 on silylation suggests the presence of hydroxyl groups in these compounds. Peak 5 is not present in the 9000 g sample.

There are indications that these peaks represent some hydroxy- and keto-metabolites (see also chapter 17, Fig. 17.4). However, the final proofs of their structures await the synthesis of the reference compounds.

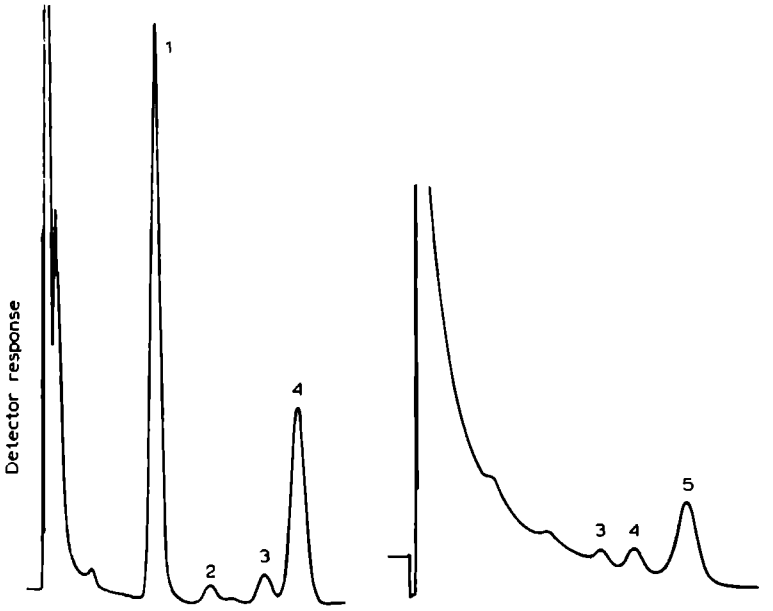


Fig. 19.4 *Gas chromatograms of silylated samples of 1,3-N,N'-dimethyl--5-ethyl-5-hexyl barbituric acid in the 9000 g rat liver supernatant incubation (left) and liver perfusion (right)*

5-Bromoallyl-5-alkyl substituted barbiturates

In our G.C. analysis of experiments with N-methylbrallobarbital a "metabolite"-peak always appears with a shorter retention time than the parent compound. Figure 19.5 shows the mass spectrum and fragmentation scheme, analogous to that described for a metabolite of Noctal^{R}, 5--bromoallyl-5-isopropyl barbituric acid (Arnold, 1969).

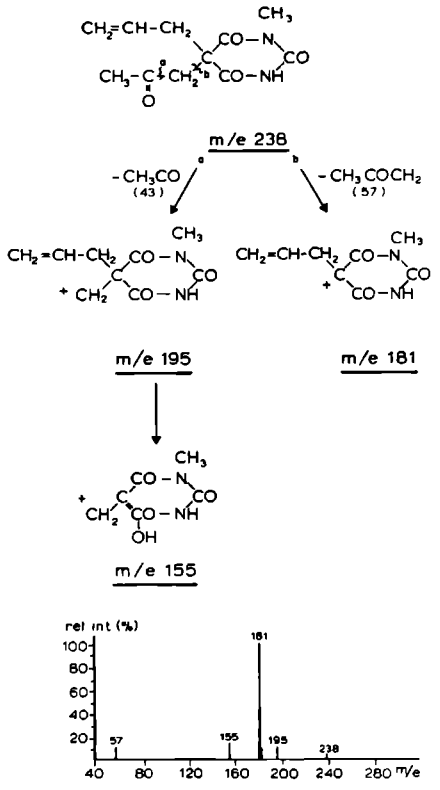


Fig. 19.5 *Fragmentation scheme of a metabolite of N-methylbrallobarbital in the isolated perfused rat liver*

DISCUSSION

The formation of 3-hydroxy- and 3-keto-methylhexobarbital as principal metabolites of methylhexobarbital is in agreement with the numerous reports on the metabolism of hexobarbital (Gerber, 1971; Holcomb, 1974). The shift and the resolution in two other peaks on silylation of peak 1 in Figure 19.1 suggest the presence of two alcoholic metabolites. This would be in agreement with the report on the metabolism of heptabarbital (Gilbert, 1974). This author describes the presence of two alcoholic

metabolites. The compound with the shortest G.C. retention time is identical with the 3-hydroxy-metabolite, while the other compound with a longer G.C. retention time is supposed to be the 7-hydroxy-homologue.

The presence of probably two hydroxy-metabolites in the experiments with the N,N'-dimethylated hexyl derivative (Fig. 20.1) is consistent with the findings of Maynert (1965).

Hydroxylation, resulting in the formation of several alcoholic metabolites, has been reported too for 1-N-butyl-5,5-diethyl barbituric acid (Vore, 1974). Moreover, it was shown that N-dealkylation of barbiturates in rats is negligible.

The mass spectrum of the metabolite of 1-N-methylbrallobarbital corresponds with that of the Noctal^{R} metabolite and this suggests that this compound is identical with the acetonyl-metabolite of methylbrallobarbital in dogs, in which as principal urinary metabolite the acetonyl compound was demonstrated (Keding, 1969). Different findings have been observed for the urinary metabolites of Eunarcon^{R} (1-N-methyl-5-bromoallyl-5-isopropyl barbituric acid) in rats. As principal metabolite was found 1-N-methyl-5-(2'-oxo-3'-hydroxypropyl)-5-isopropyl barbituric acid (Ravn-Jonsen, 1970, 1973).

It is important to elucidate the metabolic fate of the bromoallyl substituent, since bromoallyl substituted barbiturates have been reported to possess toxic properties (Holck, 1936; Yih, 1976).

SECTION V

INTRAVENOUS AND ORAL ADMINISTRATION OF BARBITURATES

CHAPTER 20

PHARMACOKINETICS OF BUTOBARBITAL AND CYCLOBARBITAL FOLLOWING INTRAVENOUS AND ORAL ADMINISTRATION

INTRODUCTION

In clinical medicine, barbiturates are generally administered orally. This implies that the drug is involved in many processes, before it reaches the general circulation, such as absorption from the stomach and the passing through the liver by the portal system. These processes may result in changes in metabolic elimination (Barber, 1974). Therefore, we have studied the pharmacokinetics of two commonly used barbiturates, butobarbital and cyclobarbital, following intravenous and oral administration.

RESULTS

In Figure 20.1 the blood concentration-time curves of butobarbital are presented following intravenous and oral administration in the same rat. The half-lives of the two curves are similar. The bioavailability of the oral administration, determined by comparison with the intravenous curve, is about 1. This could be expected considering the ratio of the hepatic clearance and the liver blood flow (Rowland, 1972).

Figure 20.2 shows the curves of cyclobarbital after intravenous and oral administration in the same rat. The absorption rate of cyclobarbital in rats is very high, so that often a part of the distribution phase becomes visible. The half-lives of the elimination phase of the two curves do not differ significantly. The bioavailability of cyclobarbital after oral administration is low, as appears from the difference in the levels of both curves.

For butobarbital we have determined the blood concentration and some tissue levels at the moment of the loss and the regain of the righting reflex. The results are presented in Table 20.1. Intravenous injection of butobarbital in rats results in the loss of the righting

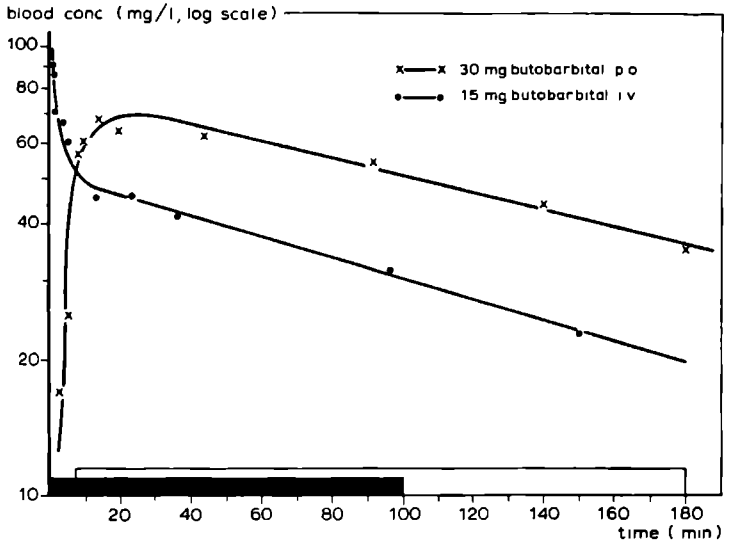


Fig. 20.1 *Blood concentration-time curves of butobarbital following intravenous and oral administration in the same rat Horizontal bars: sleeping times (min)*

reflex within 2 min. At that moment the equilibrium between the blood and the brain is clearly not reached, as is the case for the liver and the kidneys. The blood-brain ratio at the loss of the righting reflex and the values at awakening of both routes of administration become unity. In contrast with the brain, the liver and the kidneys equilibrate very rapidly with the blood and can be considered to belong to the central compartment. The differences in blood and brain concentration at the loss and the regain of the righting reflex in the oral administration are not significant. There are no indications for the development of acute tolerance, as has been reported for other barbiturates (Aston, 1965; Turnbull, 1976).

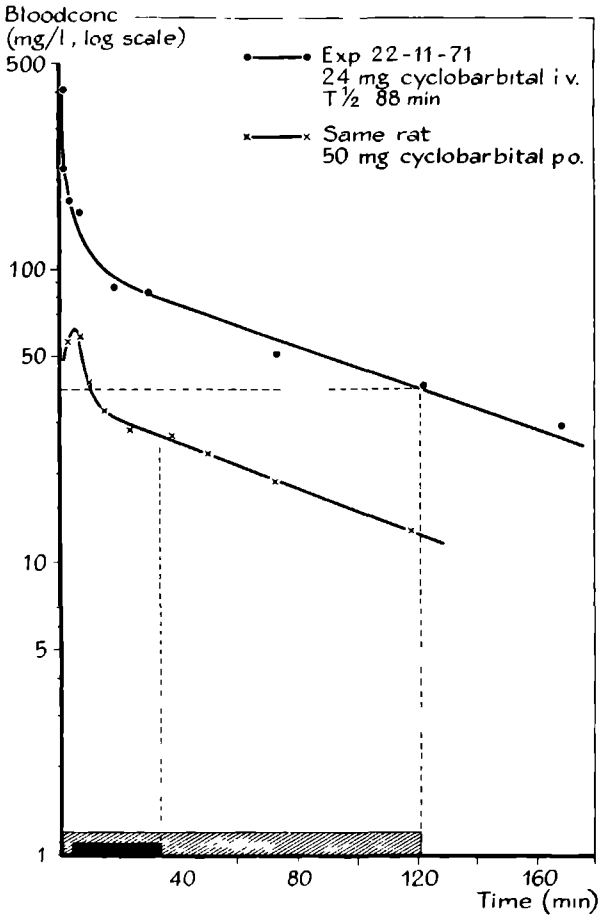


Fig. 20.2 *Blood concentration-time curves of cyclobarbital following intravenous and oral administration in the same rat Horizontal bars: sleeping times (min)*

TABLE 20.1

Butobarbital concentrations in blood (mg/l) and tissues (mg/kg) at the moment of the loss (upper) and the regain (lower) of the righting reflex

Dose (mg)		Blood	Brain[1]	Liver[1]	Kidneys[1]
15	i.v.	164	48 (0.29)	289 (1.76)	201 (1.23)
15	i.v.	230	65 (0.28)	371 (1.61)	192 (0.83)
15	i.v.	173	43 (0.25)	288 (1.66)	233 (1.75)
25	p.o.	55	54 (0.98)	117 (2.13)	98 (1.78)
25	p.o.	86	70 (0.81)	157 (1.83)	107 (1.24)
25	p.o.	55	66 (1.20)	129 (2.34)	806 (1.45)
15	i.v.	53	48 (0.91)	95 (1.70)	54 (1.02)
15	i.v.	53	59 (1.11)	103 (1.94)	75 (1.41)
15	i.v.	37	41 (1.10)	82 (2.21)	59 (1.59)
25	p.o.	57	58 (1.02)	95 (1.67)	101 (1.77)
25	p.o.	43	47 (1.09)	115 (2.67)	122 (2.84)

1) tissue/blood ratio in parentheses

DISCUSSION

Our results indicate that for the studied derivatives the half-lives are not affected by the route of administration. Similar results were obtained in man with pentobarbital (Smith, 1973) and hexobarbital (Breimer, 1974).

Cyclobarbital, administered orally, has a low bioavailability, compared with the intravenous route. The bioavailability of cyclobarbital, administered orally in man, is about 0.95, calculated after Breimer (1974). Since in rats the ratio hepatic clearance/liver blood flow is much higher, the bioavailability of cyclobarbital administered orally will be considerably lower.

The data presented in Table 20.1 suggest that equilibrium between blood/liver and blood/kidneys has been reached already after 2 min, since the ratios do not change further with time. For the blood/brain ratio the equilibrium is reached much slower.

CHAPTER 21

PECULIAR PHARMACOKINETICS OF BRALLOBARBITAL AS A SOURCE OF COMPLICATIONS IN VESPARAX® INTOXICATION

INTRODUCTION

Vesparax®, a potent barbiturate-hydroxyzine combination (50 mg brallobarbital, 150 mg secobarbital, 50 mg hydroxyzine-HCl), has been used frequently in suicide attempts (Verheist, 1970). It is difficult to treat such intoxications successfully. In the course of a comparative study of barbiturates in rats, we studied also brallobarbital. In preliminary studies was found that the hypnosis after oral administration of brallobarbital was much longer than after intravenous injection.

Since there are also clinical indications that the toxic effects of Vesparax® may be caused by brallobarbital, we have studied the pharmacokinetics of brallobarbital in detail both in the intact rat and in the isolated perfused rat liver preparation.

RESULTS

Intravenous administration

Figure 21.1 shows the time courses of a low and a high intravenous dose of brallobarbital in the same rat. The half-lives are about 3 h and independent of the dose. Intravenous injection of doses ranging from 25 to 40 mg/kg results in the loss of the righting reflex within 15 sec and produces a quiet sleep; the blood concentration at the moment of awakening is about 40 mg/l (Table 21.1). After high doses, however, the animals often die after 3-4 h, apparently by respiratory failure accompanied by convulsions.

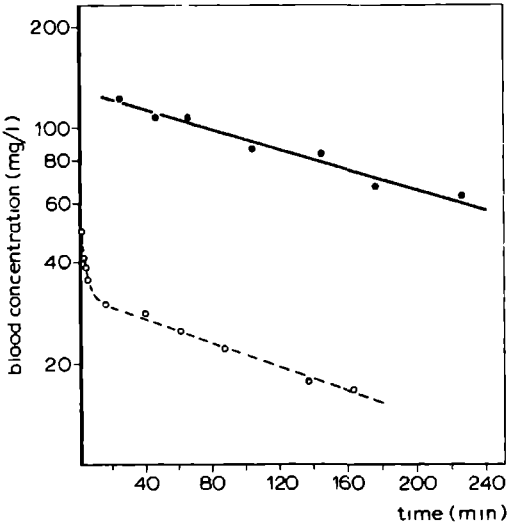


Fig. 21.1 *Time courses of brallobarbital following intravenous administration in the same rat* ●——● *92 mg/kg* ○----○ *28 mg/kg*

Oral administration

After oral administration there is a latent period of about 15-25 min. The onset of hypnosis coincides with convulsions and the sleep is much longer than after intravenous injection (Table 21.2). This corresponds with the sustained blood level, which becomes nearly constant after the absorption phase and starts to decrease only after 3-4 h.

As brallobarbital is present as its calcium salt in the commercial Vesparax^{R} tablet, this too was studied. Figure 21.2 presents the blood concentration-time curves of brallobarbital-Ca and the free acid (suspended in 10% Tween 80) in the same rat. The calcium salt and the free acid gave blood curves similar to the sodium salt administered orally (see also Table 21.2). Doses administered orally are absorbed via the

TABLE 21.1

Blood half-lives, sleeping times and blood concentrations at awakening in rats following intravenous administration of brallobarbital

Dose (mg/kg)	$t_{\frac{1}{2}}$ (min)	Sleeping time (min)	Blood concentration at awakening (mg/l)
16	180	-	-
29	184	6	35
33	183	15	33
39	190	47	45
39	195	58	44
42	191	62	44
80	176	-+	-
92	184	-*	-
Mean	185±6		40±5

+, died without awakening after 190 and 245 min respectively*

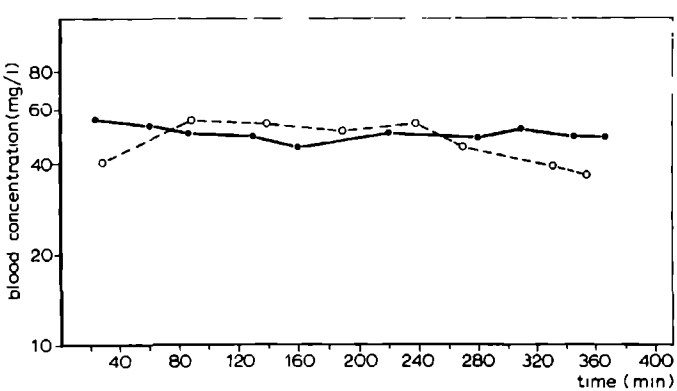


Fig. 21.2 *Time course of brallobarbital after oral administration in rats*
○ *calcium salt (95 mg/kg)*
● *free acid (95 mg/kg)*

TABLE 21.2

Blood levels in rat following administration of brallobarbital orally and via the portal vein

Dose	Blood concentration (mg/l) at time (min)					Latent period	Sleeping time	C_{aw} [1]
(mg/kg)	30	60	120	180	240	(min)	(min)	(mg/l)
Oral administration								
20	14	14	14	13	12	-	-	-
61	20	21	24	26	25	-	-	-
75	39	38	34	34	34	25	202	34
125	82	81	81	65	60	16	334	43
95	54	52	49	47	49	16	294	49
95	40	47	53	50	49	14	315	38
Portal vein administration								
57	66	62	57	59	45	-	-	-
60	69	68	63	62	59	-	-	-
45	53	54	45	43	40	-	-	-

1) blood concentration at awakening

mesentery blood system, which via the portal vein leads directly to the liver. Drugs, injected into the dorsal penis vein, however, first enter the general circulation (Nightingale, 1973). It was, therefore, interesting to follow the fate of brallobarbital administered directly to the liver with exclusion of absorption processes. This was done in two ways: first by injection of the barbiturate into the portal vein, secondly in the isolated perfused rat liver system.

Administration by the portal vein

Injection of brallobarbital into the portal vein resulted in blood concentration-time curves, which have the same time course as found after oral administration. An example is shown in Figure 21.3. Therefore, absorption can be ruled out as the origin of the retarded elimination.

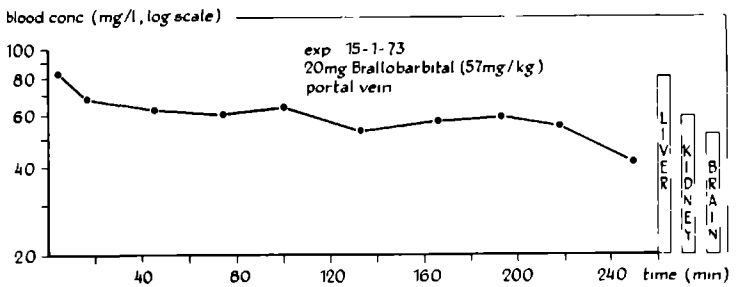


Fig. 21.3 *Blood concentration-time curve and tissue concentrations (mg/g) of brallobarbital following administration via the portal vein*

Liver perfusion

The rate of elimination of brallobarbital in the isolated perfused rat liver system is slower than in the intravenous experiments. There is a highly significant difference between the heptabarbital half-life ratio and heptabarbital clearance ratio measured in the intact rat and in the perfusion (see also chapter 12, Tables 12.2, 12.3).

Liver concentration

In order to investigate if there is a difference in the amount of brallobarbital following the different routes of administration, liver concentrations were measured. The results presented in Table 21.3 show no essential differences in the blood/liver concentration ratio following the different routes of administration. In spite of the high concentration of

TABLE 21.3

Blood and liver concentrations of brallobarbital after different routes of administration

Dose (mg/kg)	Route of administration	Time after dosage (min)	Concentration in blood (mg/l)	Concentration in liver (mg/kg)	Ratio blood liver
32	i.v.	2	52	89	0.58
41	i.v.	100	37	81	0.46
41	i.v.	120	34	68	0.50
92	i.v.	240	52	92	0.57
57	p.v.	240	50	80	0.63
45	p.v.	240	41	64	0.64
115	p.o.	400	53	112	0.47
122	p.o.	330	55	87	0.63
150	p.o.	24	114	152	0.75
165	p.o.	60	174	223	0.78

i.v., intravenous; p.v., portal vein; p.o., oral administration

brallobarbital, attained after intravenous injection, elimination is normal and this suggests that it is not brallobarbital itself which is responsible for the retarded elimination, observed after oral administration.

Combination with other compounds

The retarded elimination may be caused by inhibition of the liver enzymes. Therefore, the influence of brallobarbital given orally on the elimination of heptabarbital, Eunarcon^{R} and Rectidon^{R} was studied. In the intact animal and in the perfusions the retarded elimination appeared to be restricted to brallobarbital, whereas the other barbiturate was eliminated normally. Figure 21.4 presents a typical example with Rectidon^{R}.

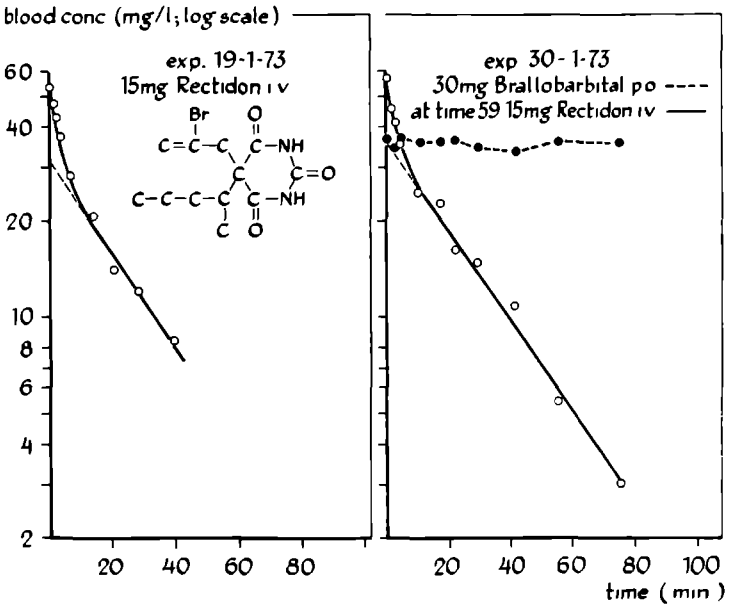


Fig. 21.4 *Influence of brallobarbital administered orally on the elimination of an intravenous dose of Rectidon*^{R}
a. Control Rectidon^{R} *(32 mg/kg) intravenously* o——o
b. Brallobarbital (18 mg/kg) orally ●----●, *plus Rectidon*^{R} *(32 mg/kg) intravenously, 50 min later*

Further we have studied the influence of some hypothetical metabolites of brallobarbital. According to Keding (1969) brallobarbital is metabolized in man according to the following scheme (Fig. 21.5). The principal urinary metabolites are compounds III and IV. Compound IV is used as ataractic drug under the name Ipronal^{R}, which is metabolized in man to non-barbiturate metabolites, such as compound X and XI (Bobranski, 1961). Oral administration of these compounds resulted in a retarded elimination of brallobarbital, when administered intravenously 20 min afterwards. The blood concentration curves are the same as found in the oral experiments (Fig. 21.6).

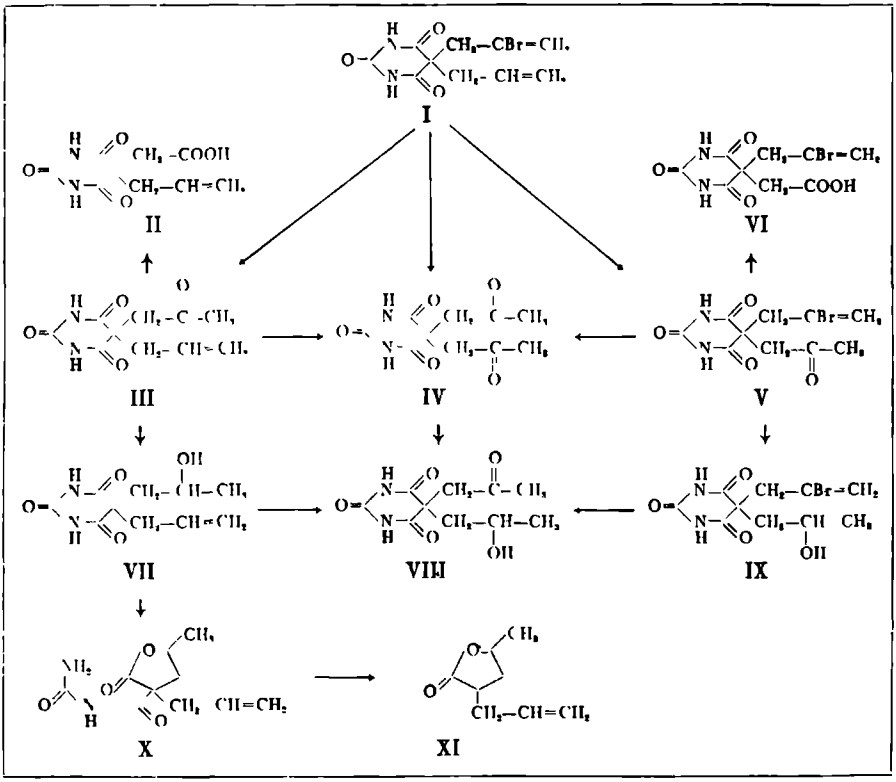


Fig. 21.5 *Hypothetical scheme of the metabolism of brallobarbital after Keding (1969)*
I 5-(2'-bromoallyl)-5-allyl barbituric acid; II 5-allyl-5-carboxymethyl barbituric acid; III 5-acetonyl-5-allyl barbituric acid; IV 5-diacetonyl barbituric acid; V 5-acetonyl-5-(2'-bromoallyl) barbituric acid; VI 5-(2'-bromoallyl)-5-carboxymethyl barbituric acid; VII 5-allyl-5-(2'-hydroxypropyl) barbituric acid; VIII 5-acetonyl-5-(2'-hydroxypropryl) barbituric acid; IX 5-(2'-bromoallyl)-5-(2'-hydroxypropyl) barbituric acid; X α-allophanyl-α-allyl-γ-valerolacton; XI α-allyl-γ-valerolacton

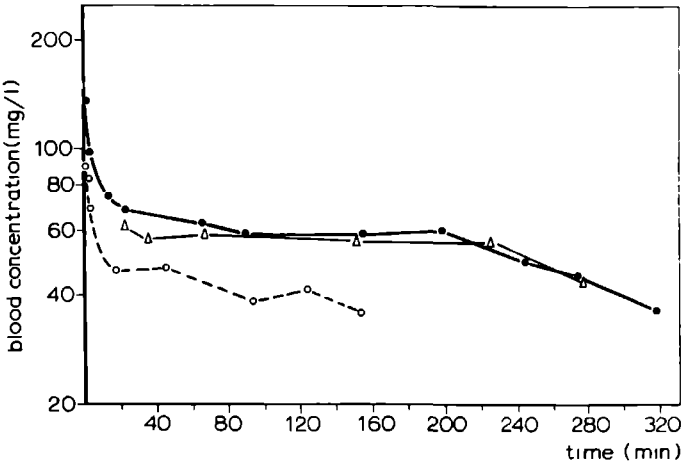


Fig. 21.6 *Influence of metabolites administered orally on the elimination of an intravenous dose of brallobarbital, given 20 min afterwards*
o----o *Ipronal*^{R} *(55 mg/kg, orally); brallobarbital (40 mg/kg)*
●——● *Valerolacton (63 mg/kg, orally); brallobarbital (40 mg/kg)*
Δ----Δ *Reference, brallobarbital (104 mg/kg) orally*

Combination with secobarbital and hydroxyzine

We have investigated the influence of the other components of Vesparax^{R} on the elimination of brallobarbital. Table 21.4 shows that both components increase the half-life of brallobarbital; secobarbital by a factor two and hydroxyzine is even more effective.

Induction with phenobarbital

Since our experiments suggested that the retardation of brallobarbital elimination after oral administration was due to one or more of its metabolites, we decided to increase the formation of these metabolites by induction of the enzymic system with phenobarbital. Rats were pretreated with phenobarbital, 20 mg orally, for ten days.

TABLE 21.4

Influence of other compounds on the blood half-life of brallobarbital

Compound	Dose (mg/kg)	$t_{\frac{1}{2}}$ brallobarbital (min)	Sleeping time (min)	Blood concentration at awakening (mg/l)
Secobarbital	53	380	204	37
	51	380	110	41
Hydroxyzine	60	500	120	41
	51	660	145	39
Ipronal^{R}	55	400	131	37
	67	370	115	39
Valerolacton	53	520	220	40
	63	615	273	45

The compound was administered orally; brallobarbital (35 mg/kg) was given intravenously 20 min afterwards

Figure 21.7 shows the blood concentration-time curves of brallobarbital in the same rat, intravenously administered, before and after phenobarbital induction. The half-life is shortened with a factor 5 to 35 min. Oral administration of brallobarbital in a phenobarbital-induced animal resulted in a blood concentration-time curve with a half-life of about 60 min (Fig. 21.8). An explanation may be that the breakdown of the metabolite(s) itself is also enhanced, the nett effect being a decrease in retardation.

In addition to our rat experiments we studied the peculair behaviour of brallobarbital in dog. Figure 21.9 shows that also in this species the difference in rat of elimination between oral and intravenous dosage is present. This appears clearly from the difference in sleeping time.

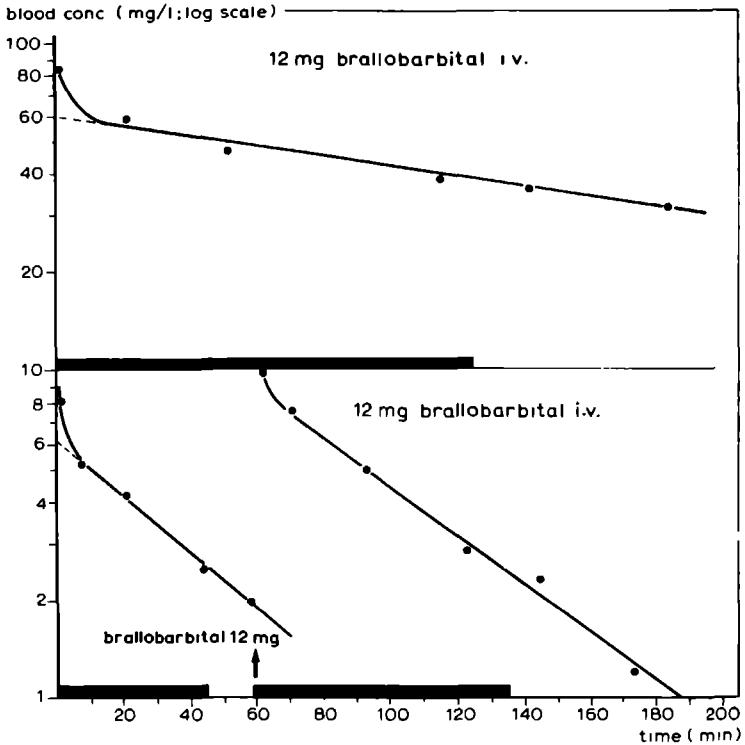


Fig. 21.7 *Blood concentration-time curves of brallobarbital following intravenous administration in the same rat before (upper) and after (lower) phenobarbital induction*
Horizontal bars: sleeping times (min)

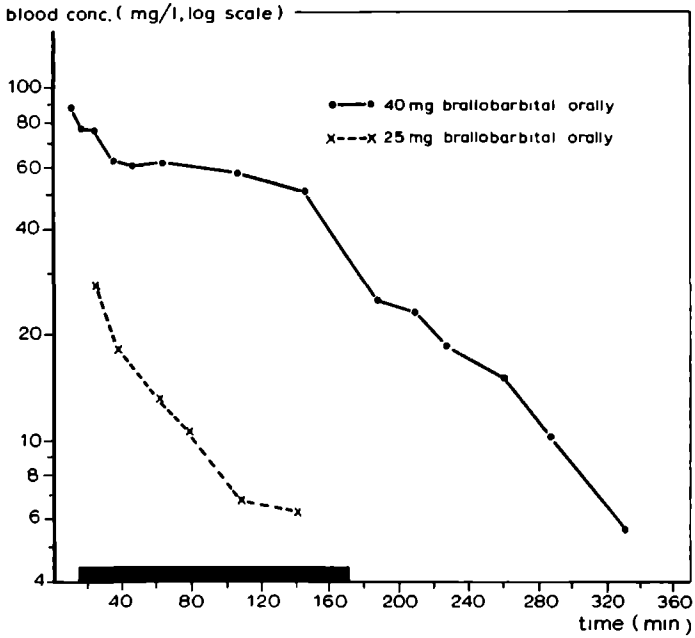


Fig. 21.8 *Blood concentration-time curves of brallobarbital following oral administration in an phenobarbital-induced rat Horizontal bar: sleeping time (min)*

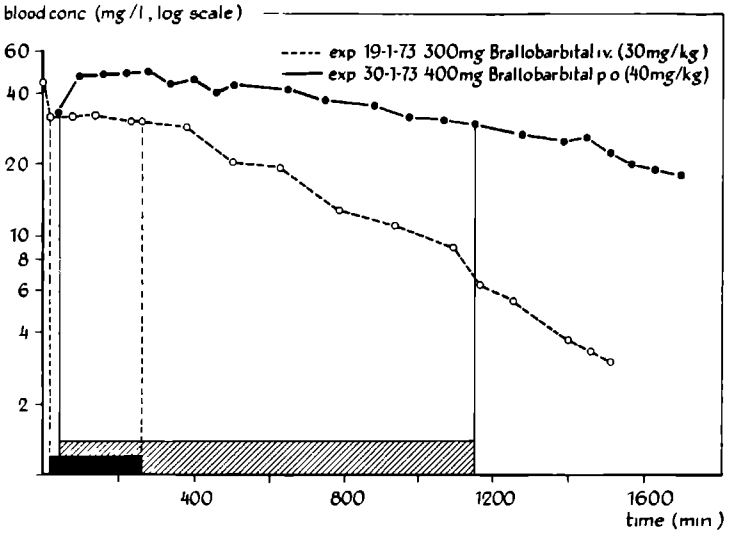


Fig. 21.9 *Blood concentration-time curves of brallobarbital following intravenous and oral administration in a Beagle dog Horizontal bars: sleeping times (min)*

DISCUSSION

Pottier (1951) has made similar findings to ourselves using mice. He found that sleeping times, following administration of brallobarbital and a series of structurally related barbiturates, were longer after oral than after intravenous dosage. However, this effect was not discussed.

The convulsions, which are so characteristic of the "hypnosis" produced by brallobarbital orally, appear about 20-30 min after dosing, suggesting that they are caused by a metabolite rather than by brallobarbital itself. The differences in sleep patterns between the two routes of administration may be caused by differences in the formation of this metabolite. Unfortunately, in our G.C. analysis no metabolites of brallobarbital could be detected. For N-methylbrallobarbital the acetonyl-meta-

bolite could be detected as well after oral as intravenous dosage. This N-methyl derivative too exhibits the difference in elimination between the two dosage forms as can be seen in Figure 21.10.

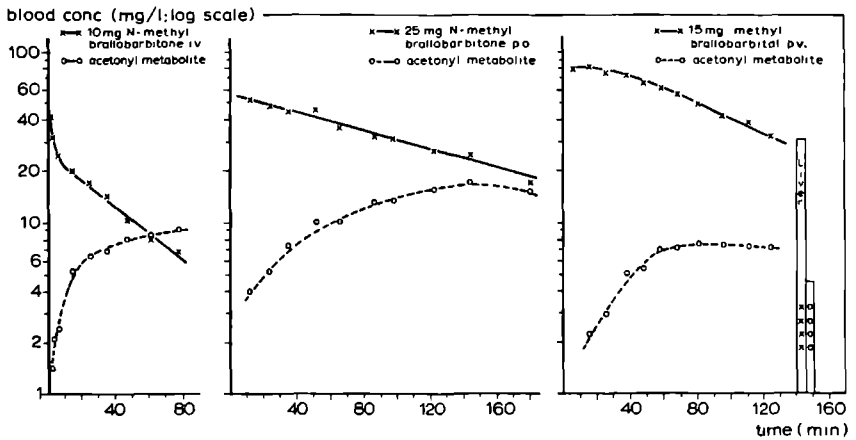


Fig. 21.10 *Time courses of N-methylbrallobarbital in the intact rat following intravenous (left), oral (middle) and portal vein (right) administration*

Unfortunately, the half-life of brallobarbital administered intravenously to phenobarbital-treated animals was not increased as we had anticipated. It may be that in induced animals the compound responsible for the retarded elimination is in turn broken down rapidly. This aspect requires investigation in detail.

5-Allyl-5-(2'-hydroxypropyl) barbituric acid (Ipronal^{R}) prolongs sleep induced by phenobarbital and hexobarbital (Hano, 1962) and some animals, receiving these barbiturates in sub-lethal doses, died. This supports our findings that the metabolism of brallobarbital is inhibited by Ipronal^{R}.

Another important finding concerning the toxicity of Vesparax^{R} is that the elimination of brallobarbital is also retarded by secobarbital and hydroxyzine. This is in agreement with the results of Hermans (1973)

and Gainotti (1967). According to the last author hydroxyzine provokes a longer duration of sleep in man and seems to be responsible for a slight somnolence upon awakening.

Although further study will be necessary to elucidate the problems of the toxicity of Vesparax^{R}, it seems most likely that they are caused by a delayed elimination of brallobarbital, when taken orally. This inhibition seems to be due to a metabolite of brallobarbital formed especially after oral administration of this barbiturate and to an inhibition by the other components of Vesparax^{R}, namely hydroxyzine and secobarbital.

On the basis of our findings we may conclude that, from a pharmacokinetic point of view the specific combination of drugs as present in Vesparax^{R} is far from optimal.

SECTION VI

SPECIES AND SEX DIFFERENCES IN ELIMINATION OF BARBITURATES

CHAPTER 22

COMPARISON OF THE PHARMACOKINETIC DATA OF BARBITURATES IN MAN AND RAT

Studies on the pharmacokinetics and metabolism of drugs in experimental animals are of interest with regard to the evaluation of both the pharmacodynamic properties and the toxicity of the compounds. However, in the extrapolation of these data to man species differences in response and metabolism must be considered. Despite the long tradition of barbiturates as hypnotics, there were until recently no reliable data on their pharmacokinetic behaviour in man.

In the present chapter we have investigated if there is some conformity between our data found in rats and the pharmacokinetic parameters known in man. For the 5-ethyl-5-alkyl substituted derivatives in both species penultimate hydroxylation and oxidation is the main route of metabolism (Freudenthal, 1973), while cyclobarbital, hexobarbital and heptabarbital are metabolized at the 3-position of the cycloalkenyl substituent (Gilbert, 1974; Mark, 1963).

In Table 22.1 some pharmacokinetic data of barbiturates in man and rats are presented. The apparent volumes of distribution in both species do not show such big differences as the half-lives. Therefore, we may conclude that the differences in half-lives are based on differences in the metabolic activity in the two species. This appears directly from the clearance values. Investigations reported in the literature indicate that the hexobarbital oxidase activity of human adult liver enzymes lies below that of rat livers, the difference being, however, only a factor two (Pelkonen, 1973). The cytochrome P 450 contents of human and rat liver are 13.6 and 31.0 nMol/g liver respectively. An explanation of the large difference between man and rat cannot yet be given in term of enzyme constants such as V_m, K_m and K_s, since a systematic study in liver microsomes (or hepatocytes) has not been done in human material.

As in the rat experiments we have correlated the clearance of the barbiturates to that of heptabarbital (Breimer, 1974). However, it should be noticed, that the data in man show a considerable intersubject variation and that in contrast with the rat experiments the heptabarbital

TABLE 22.1

Comparison of some pharmacokinetic parameters in man and rat

Compound	Half-life (h)			App. volume distribution (l/kg)		Clearance (ml/min.kg)			Heptabarbital k_{Cel} ratio		Ref
	rat	man	ratio $\frac{m}{r}$	rat	man	rat	man	ratio $\frac{m}{r}$	rat	man	
Heptabarbital	0.22	7.7	35	0.65	1.31	43.6	1.92	23	1.00	1.00	1
Butobarbital	3.50	37.5	11	1.35	0.78	4.1	0.24	17	0.13	0.12[a]	1
Pentobarbital	0.88	22.3	25	1.14	0.99	15.0	0.51	29	0.28	0.27[a]	2
Amobarbital	0.65	22.7	35	0.96	0.87	16.5	0.51	29	0.41	0.41[a]	3
Vinylbital	1.65	24.0	14	0.98	0.74	7.0	0.36	19	0.16	0.18[a]	1
Cyclobarbital	0.83	11.6	14	1.45	0.50	17.4	0.49	35	0.36	0.26[b]	1
Hexobarbital	0.43	4.41	10	1.06	1.10	58.1	3.57	16	1.33	1.92[b]	1

1) Breimer (1974) *2) Ehrnebo (1974)* *3) Balasubramian (1970)*

a. not significantly different $P > 0.20$

b. not significantly different $P > 0.05$

clearance was not measured in the same subject.

The data presented in Table 22.1 show that for the majority of the barbiturates the heptabarbital clearance ratios found in man and rat are similar. There was a strong correlation between the two species (r 0.991, s 0.054). This suggests that it is possible to predict the clearance values in man by studying relative values in rats.

CHAPTER 23

SEX DIFFERENCES IN THE ELIMINATION OF HEXOBARBITAL AND HEPTABARBITAL IN THE RAT

INTRODUCTION

Several reports have been published on sex differences in drug metabolism by rat liver. The magnitude of the sex difference varies with the substrate. Brodie (1968) reported a clear sex difference for hexobarbital, whereas for the hydroxylation of aniline no sex difference was found (Kato, 1965). The results of some preliminary experiments with the isolated perfused female rat liver are presented in this chapter.

RESULTS

Figure 23.1 shows the curves of heptabarbital and hexobarbital in the isolated perfused female rat liver.

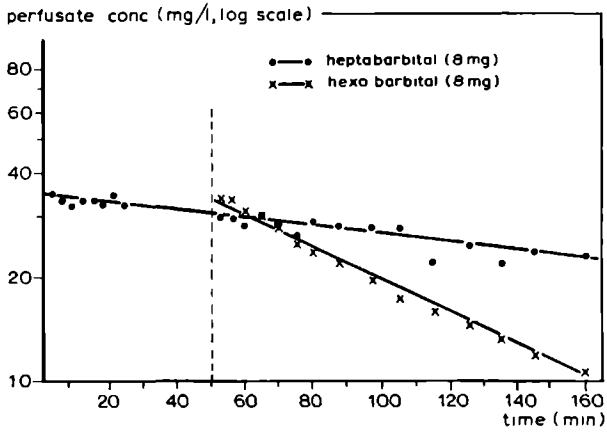


Fig. 23.1 *Concentration-time curves of heptabarbital and hexobarbital in the isolated perfused female rat liver*

The half-lives and clearances of heptabarbital and hexobarbital are 238 and 56 min and 0.64 and 2.80 ml/min respectively. The heptabarbital half-life and clearance ratio of hexobarbital are 0.24 and 4.38 respectively. The half-life of hexobarbital is 4 times smaller and the clearance 4.4 times larger than of heptabarbital. In the male rat as used throughout the previous chapters the rate of elimination of both barbiturates is 10-30 times faster. See chapter 13.

DISCUSSION

The clearance of heptabarbital in the isolated perfused female rat liver is a factor 34 lower as compared with the male rat liver perfusion; for hexobarbital this factor is approximately 11. According to Kato (1965) hexobarbital is metabolized about thrice as rapidly by the 9000 g liver supernatant of male rats as by that of female animals. The cytochrome contents of livers of male and female rats are of the same order of magnitude (Gram, 1970). The high sex differences found in the perfusions therefore cannot be attributed to different cytochrome P 450 contents.

Although many studies have been undertaken on sex differences in metabolizing activity, e.g. effect of starvation and steroids (Kato, 1965), the nature of it is still obscure.

Pharmacokinetic studies on barbiturates in man are mainly restricted to men. There is only one report on the pharmacokinetics of amobarbital in men and women (Balasubramaniam, 1970). According to this author there was no sex-dependent difference in amobarbital metabolism. Since, however, the sex difference in the metabolism of barbiturates apparently varies markedly with the barbiturate studied, it would be recommendable to investigate the rate of elimination of a barbiturate such as heptabarbital in females.

This thesis describes pharmacokinetic studies concerning the hypnotic activity, kinetic behaviour and metabolism of a large number of barbituric acid derivatives in relation to their chemical structure. The studies have been carried out *in vivo* as well as *in vitro*; the *in vivo* studies were done with rats, while the *in vitro* experiments were performed in various rat liver preparations of gradual increasing simplicity, namely: the isolated perfused rat liver, the isolated rat hepatocyte suspension and the 9000 g rat liver supernatant fraction. Further the interaction of the barbiturates with the metabolizing enzyme system was studied by measuring K_m and K_s values.
The relevance of the pharmacokinetic data obtained in rats have been discussed with respect to human sleep therapy.

The above mentioned approaches are elucidated in the general introduction.

In Section I, chapters 1 and 2, the theoretical aspects of pharmacokinetics are discussed, such as the pharmacokinetic models used and the pharmacokinetic analysis of the data and the calculation of enzymic constants from saturating curves.

Section II contains the chapters concerning materials and methods. One of the main drawbacks in structure-activity relationship (SAR) studies on barbiturates is the use of small miscellaneous sets of barbiturates. In our study we have used large homologous series of barbiturates, the synthesis of which is described in chapter 3.

In chapter 4 the methods used in the intact animal experiments, are presented. The pharmacokinetic parameters were derived from blood concentration-time curves, which were obtained from one animal. This was possible by using a special sampling technique, "the orbita punction". Moreover, in each series of experiments a reference compound was included to correct for interindividual variation in the drug metabolizing activity of the animals.

Chapter 5 deals with the isolated perfused rat liver. In the majority of the pharmacokinetic studies using this preparation, a little attention

is paid to the physiological state and the drug metabolic performance of the liver. We have measured the liver blood flow and the bile production and determined the pH, pCO_2 and pO_2 before and after the liver at regular time intervals. In addition, in each perfusion heptabarbital was used as reference compound for the drug metabolizing activity of the liver. The clearance of a derivative studied was related to the clearance of heptabarbital (heptabarbital clearance ratio).

In the chapters 6 and 7 the methods and evaluation of the experiments with isolated rat hepatocytes and the 9000 g rat liver supernatant are described. It has been shown that the oxygen supply is the most critical factor in this sort of experiments.

The last three chapters of this section describe the methods for the determination of partition coefficients, K_s values and the analysis of the samples. In chapter 10 some tables are presented, showing the relative G.C. retention times of the barbiturates used in this study together with those of the metabolites which could be detected in our assay.

Section III contains the experimental chapters. In the chapters 11, 12 and 13 a comparison is made between the elimination of the barbiturates *in vivo* and in the isolated perfused rat liver system. The rate of elimination of the barbiturates is clearly dependent on the length of the side chain at the 5-position of the barbituric acid nucleus; the bromoallyl substituted barbiturates are cleared more rapidly than their allyl homologues and N-methylation of the barbiturates results too in a higher clearance. A relationship was found between the rate of disappearance of the barbiturate and their lipophilicity, which was expressed either as octanol/water or hepatocyte/water partition coefficient. For the majority of the compounds a good correlation was found between the clearance *in vivo* and in the perfusions. An exception was formed by some bromoallyl substituted barbiturates, which displayed in the perfusion a considerably lower heptabarbital clearance ratio than in the intact animal.

Chapter 14 presents the pharmacokinetics of a number of barbiturates in the isolated rat hepatocyte suspension. It was shown that for the majority of the compounds studied, the heptabarbital clearance ratio was similar to that found in the perfusion studies. However, the absolute

clearances, corrected for the number of cells, suggest that the isolated rat hepatocyte suspension has a higher metabolic activity than the isolated perfused liver.

Chapter 15 deals with the pharmacokinetics of barbiturates in the 9000 g rat liver supernatant. The pharmacokinetic behaviour of the majority of the barbiturates studied was different from that found in the hepatocyte suspension. Some compounds are metabolized much faster than in the hepatocyte suspension, as appears from the heptabarbital clearance ratio. This phenomenon is discussed and associated with differences in transport processes between these metabolic test systems.

In chapter 16 the binding of the barbiturates to cytochrome P 450 has been studied. The bromoallyl substituted barbiturates have a higher affinity for cytochrome P 450 than their allyl homologues; N-methylated barbiturates too display a higher affinity for cytochrome P 450 than their non-methylated homologues. In all homologous series the affinity for cytochrome P 450 seems to increase with longer side chains. However, in the 5-ethyl-5-alkyl series the derivatives with an octyl and nonyl substituent clearly have a lower affinity for cytochrome P 450 than the hexyl and heptyl substituted barbiturates. Apparently the length of the side chain is a factor to reckon with.

Further some preliminary results of the determination of Michaelis--Menten constants (K_m) from curves obtained following administration of a saturating dose to liver perfusions are presented. The K_m values obtained from these curves differ from the values obtained with the conventional methods (i.e. Lineweaver-Burke plots). Phenobarbital induction apparently does not affect the K_m value; the metabolic capacity of the liver, however, is increased considerably.

In chapter 17 the pharmacokinetics of some barbiturate metabolites have been studied. The alkyl and cycloalkenyl substituted barbiturates are converted to hydroxy-metabolites which in turn may be metabolized further to their keto-homologues.

In chapter 18 the metabolic differences between intact cell and subcellular test systems are discussed. In the intact cell systems, such as the intact rat, liver perfusion and isolated hepatocyte suspension, the alcoholic barbiturate metabolites are converted further to the keto-

nes, whereas in the 9000 g rat liver supernatant the hydroxy-metabolites are not converted further. Ethanol inhibits the metabolism of the hydroxy-metabolites, whereas the metabolism of the parent compounds remains unaffected. This suggests that the metabolism of the parent barbiturates and their hydroxy-metabolites is mediated by different enzymes.

Section IV deals with the identification of the various barbiturate metabolites. Methylation and silylation techniques have been used to improve the G.C. response and the separation of the hydroxy- and keto-metabolites.

Section V deals with the pharmacokinetics of barbiturates following different routes of administration. Chapter 20 shows that the half-lives of butobarbital and cyclobarbital are independent from the route of administration. The bioavailability of cyclobarbital is much lower than that of butobarbital.

Chapter 21 deals with the pharmacokinetics of brallobarbital following intravenous, oral and portal vein administration. It was shown that after oral and portal vein administration the half-lives of brallobarbital and its N-methyl homologue were longer than after intravenous administration. Moreover, it was found that the half-life of brallobarbital was increased following administration of secobarbital and hydroxyzine. From these results it has been concluded that the combination of drugs as present in Vesparax^{R} (50 mg brallobarbital-Ca, 150 mg secobarbital-Na and 50 mg hydroxyzine-HCl) is far from optimal.

In Section VI species and sex differences in the elimination of barbiturates have been studied. In chapter 21 a comparison is made between the pharmacokinetic data obtained in rat and man. It is shown that rats metabolize the barbiturates more rapidly than man; the values, however, of the clearances relative to heptabarbital were similar in both species. This suggests that it is possible to predict the clearance values in man by studying relative values in rats.

In the last chapter of this study some preliminary results of sex differences in clearance of heptabarbital and hexobarbital are presented. Heptabarbital is eliminated a factor 30 as low in the female rat liver perfusion as compared with the male rat liver perfusion. For hexobarbital this factor is about 11.

Our studies indicate clearly that the results obtained with each *in vitro* technique cannot be extrapolated directly to the *in vivo* situation without considering the limitations of each technique. The differences in morphological features of the liver cells, the organization of the liver and the circulatory aspects have to be taken into consideration.

In dit proefschrift is farmacokinetisch onderzoek beschreven betreffende de hypnotische werking, farmacokinetiek en metabolisme van een groot aantal barbituraten in relatie tot hun chemische structuur. Het onderzoek is uitgevoerd zowel *in vivo* als *in vitro*. De *in vivo* experimenten werden gedaan met ratten; de *in vitro* proeven werden uitgevoerd in verschillende rattelever preparaten met een geleidelijk afnemende complexiteit, namelijk: de geïsoleerde doorstroomde rattelever, een suspensie van geïsoleerde rattelever parenchym cellen en de 9000 g supernatant fractie van de rattelever. Verder werd de interactie barbituraat-enzym onderzocht door het meten van de K_m en K_s waarden.
De betekenis van de farmacokinetische resultaten, gevonden in ratten, voor de slaaptherapie in de mens wordt ter discussie gesteld.

De hierbovengenoemde benaderingen worden toegelicht in de algemene inleiding.

In Sectie I, hoofdstukken 1 en 2, worden de theoretische aspecten van de farmacokinetiek behandeld, zoals het gebruik van de verschillende modellen, de farmacokinetische verwerking van de meetpunten en het bepalen van enzym constanten met behulp van verzadigingscurven.

Sectie II bevat de hoofdstukken die handelen over de gebruikte stoffen en methoden. Een van de meest beperkende factoren in reeds gepubliceerd werk over de relatie structuur-werking van barbituraten is het gebruik van een klein aantal, qua structuur totaal verschillende barbituraten. In ons onderzoek hebben we gebruik gemaakt van grote reeksen homologen. De synthese hiervan wordt behandeld in hoofdstuk 3.

Hoofdstuk 4 geeft de methoden, die gebruikt werden voor de proeven met intacte ratten. De farmacokinetische parameters werden bepaald uit curven, die verkregen werden van één dier. Dit was mogelijk door een speciale bloedafname techniek te gebruiken, "de orbita punctie". Bovendien werd in elke serie experimenten een referentie stof meegenomen ter correctie voor variatie in metabole activiteit tussen de verschillende dieren.

In hoofdstuk 5 wordt de leverperfusie behandeld. In de meeste artikelen over farmacokinetisch onderzoek, verricht met deze techniek, wordt weinig aandacht besteed aan de fysiologische toestand en activiteit van de lever. Wij hebben de bloedstroom door de lever en de galproduktie gemeten en verder op gezette tijden de pH, pCO_2 en pO_2 vóór en achter de lever bepaald. Tevens werd in elke perfusie heptabarbital meegenomen als referentie voor de metabole activiteit van de lever. De klaring van de onderzochte barbituraten werd gerelateerd aan de klaring van heptabarbital.

Hoofdstukken 6 en 7 beschrijven de methoden, gebruikt voor de experimenten met geïsoleerde levercellen en de 9000 g rattelever supernatant. In beide preparaten blijkt de zuurstof toevoer de kritische factor te zijn.

De laatste drie hoofdstukken geven de bepalingsmethoden voor de verdelingscoëfficiënten, K_s waarden en de analyse van de monsters. In hoofdstuk 10 worden de relatieve retentietijden voor de onderzochte barbituraten opgegeven, samen met die van de metabolieten die gaschromatografisch aangetoond konden worden.

Sectie III bevat de experimentele hoofdstukken. In de hoofdstukken 11, 12 en 13 wordt een vergelijking gemaakt tussen de klaring van de verschillende barbituraten in de intacte rat en in de perfusies. De snelheid, waarmee de barbituraten uit het bloed verwijderd worden, is duidelijk afhankelijk van de substituent op de 5-plaats van de barbituraat ring; de broomallyl gesubstitueerde barbituraten worden sneller gemetabolizeerd dan hun allyl homologen, terwijl de N-methyl derivaten ook sneller geklaard worden dan de niet gemethyleerde barbituraten. Voor de meeste barbituraten bestaat er een goede overeenkomst tussen de eliminatie in de rat en in de perfusie. Een uitzondering wordt hier gevormd door enkele broomallyl gesubstitueerde barbituraten, die in de perfusie een duidelijk lagere klaring, gerelateerd aan heptabarbital, hebben dan in de intacte rat.

Hoofdstuk 14 behandelt de farmacokinetiek van een aantal barbituraten in de levercel suspensie. Voor de meeste barbituraten blijkt de klaringsratio t.o.v. heptabarbital overeen te stemmen met die welke in de perfusie gevonden werd. De absolute waarden, gecorrigeerd voor het

aantal cellen, doen echter vermoeden dat de cel suspensie een hogere metabole activiteit bezit dan de geïsoleerde lever.

Hoofdstuk 15 gaat over de farmacokinetiek in de 9000 g rattelever supernatant. Voor de meeste barbituraten was er een verschil, vergeleken met de cel suspensie. Sommige barbituraten werden veel sneller omgezet. Dit fenomeen wordt besproken en in verband gebracht met verschillen die er bestaan tussen beide modellen in transport door celwanden.

In hoofdstuk 16 wordt de interactie van de barbituraten met cytochroom P 450 besproken. De broomallyl en de N-methyl barbituraten bezitten een hogere affiniteit voor cytochroom P 450 dan respectievelijk de allyl en niet gemethyleerde verbindingen. In alle series blijkt de affiniteit voor cytochroom P 450 groter te worden naarmate de zijketens langer worden. In de 5-ethyl-5-alkyl serie blijken de octyl en de nonyl derivaten echter een lagere affiniteit te bezitten dan de hexyl en de heptyl verbindingen. Blijkbaar begint voor de octyl en de nonyl derivaten de lengte van de zijketen een storende factor te worden.

Verder worden enige voorlopige resultaten getoond betreffende de bepaling van Michaelis-Menten constanten (K_m) uit curves, verkregen na toediening van een verzadigende dosering aan leverperfusie. Deze K_m waarden verschillen van die, welke op de conventionele manier (Lineweaver-Burke) verkregen worden. Inductie met phenobarbital geeft geen verandering van de K_m, wel een duidelijke verhoging van de metabole capaciteit van de lever.

In hoofdstuk 17 wordt de farmacokinetiek van enkele barbituraat metabolieten onderzocht. De alkyl en cycloalkenyl gesubstitueerde barbituraten worden omgezet in hydroxy-verbindingen, die op hun beurt verder gemetabolizeerd kunnen worden tot ketonen.

In hoofdstuk 18 worden de verschillen in metabolisme tussen de test systemen met intacte cellen en subcellulaire fracties besproken. In de systemen met intacte levercellen, zoals de intacte rat, leverperfusie en de levercel suspensie, worden de hydroxy-metabolieten omgezet in ketonen, terwijl dit in de 9000 g rattelever supernatant niet gebeurd. Verder blijkt ethanol het metabolisme van de hydroxy-metabolieten te remmen, terwijl het metabolisme van de barbituraten zelf onaangetast blijft. Dit suggereert dat de omzetting van barbituraten en hydroxy-

barbituraten verloopt via verschillende enzymsystemen.

Sectie IV behandelt de identificatie van de verschillende barbituraat metabolieten. Er is gebruik gemaakt van verschillende technieken, zoals methylering en silylering, om de gaschromatografische response en de scheiding van de metabolieten te verbeteren.

Sectie V behandelt de farmacokinetiek van barbituraten bij verschillende toedieningsvormen. In het eerste hoofdstuk van deze sectie wordt aangetoond dat de halfwaardetijd van zowel butobarbital als cyclobarbital onafhankelijk is van de wijze van toediening. De biologische beschikbaarheid van cyclobarbital is echter veel lager dan die van butobarbital.

Hoofdstuk 21 behandelt de farmacokinetiek van brallobarbital na intraveneuze en orale toediening en toediening via de poortader. Er werd gevonden dat na orale en poortader toediening de halfwaardetijden van brallobarbital en zijn N-methyl homoloog veel langer zijn dan na intraveneuze toediening. Bovendien werd aangetoond dat de halfwaardetijd van brallobarbital verlengd was na toediening van secobarbital en hydroxyzine. Hieruit wordt geconcludeerd dat de combinatie van stoffen, zoals bij Vesparax^{R} (50 mg brallobarbital-Ca, 150 mg secobarbital-Na en 50 mg hydroxyzine) verre van optimaal is.

In Sectie VI worden species en sex verschillen in de eliminatie van barbituraten besproken. In hoofdstuk 21 wordt een vergelijking gemaakt tussen de resultaten verkregen in de rat en in de mens. Ratten blijken barbituraten veel sneller om te zetten dan mensen; de klaringen, gerelateerd aan heptabarbital, blijken in beide species gelijk te zijn. Dit suggereert dat het mogelijk is klaringsconstanten in de mens te voorspellen door de relatieve klaringen in de rat te bestuderen.

In het laatste hoofdstuk van dit proefschrift worden enkele voorlopige resultaten van sex verschillen in de eliminatie van heptabarbital en hexobarbital besproken. Heptabarbital blijkt dertig maal zo langzaam gemetabolizeerd te worden door levers van vrouwelijke ratten dan van mannelijke ratten, voor hexobarbital is deze factor circa 11.

Uit ons onderzoek blijkt duidelijk dat de resultaten verkregen met de diverse *in vitro* technieken niet direct geëxtrapoleerd mogen worden naar de *in vivo* situatie, zonder zich de beperkingen van iedere techniek te realiseren. Men moet rekening houden met de verschillen in de morfo-

logie van de levercellen, de structuur van de lever en de aspecten betreffende de circulatie.

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De auteur werd geboren op 22 juli 1946 te Utrecht. Hij bezocht het Titus Brandsma Lyceum te Oss, alwaar hij in 1964 het diploma gymnasium β behaalde. Van 1964 tot 1971 studeerde hij biologie aan de Katholieke Universiteit te Nijmegen. Het doctoraalexamen werd op 6 juli 1971 afgelegd met als hoofdvak zoölogie en als bijvakken genetica en farmacologie. In augustus 1971 trad hij in dienst van de Katholieke Universiteit te Nijmegen als wetenschappelijk medewerker op het Farmacologisch Laboratorium. Hij heeft als lid van de werkgroep farmacokinetiek onder leiding van Prof.Dr. J.M. van Rossum onderzoek verricht naar de farmacokinetiek en het metabolisme van barbituraten, voornamelijk bij de rat en in verschillende *in vivo* systemen. Uit het onderzoek zijn de volgende publicaties voortgekomen:

T.D. Yih, A.W. Boxman and J.M. van Rossum, Influence of the liver blood flow on the metabolic clearance of barbiturates in the intact rat and in the isolated liver, Pharm. Wkbl. 110, 1236-1240 (1975).

T.D. Yih and J.M. van Rossum, Peculiar pharmacokinetics of brallobarbital as source of complication of Vesparax^{R} intoxication, Xenobiotica 6, 355-362 (1976).

STELLINGEN

I

Een grootheid als oxidatie snelheid, zoals gedefinieerd door Bock (1970), is concentratie-afhankelijk en kan beter vervangen worden door een grootheid als de klaring

Bock K W , Naunyn Schmiedeberg's Arch Pharmacol 283,319 (1970)

II

In studies, betreffende inhibitie van metabolisme, is het vereist, dat de te remmen stof in een dusdanige concentratie wordt toegediend, dat zijn metabolisme niet verzadigd is

Stitzel, R E , Tephly, T R and Mannering, G J Mol Pharmacol 4,15 (1968)

III

De metabole activiteit van *in vitro* preparaten wordt vaak gerelateerd aan de hoeveelheid microsomaal eiwit of aan het gehalte cytochroom P-450 Gelet op de variatie in deze waarden, is het beter om de metabole activiteit te betrekken op de omzetting van een referentie stof

Litterst, C L , Gram, T E , Mimnaugh, L G , Leber, P , Emmerling D and Freudenthal, R I Drug Metab Dispos 4,203 (1976)

IV

Steeds vaker wordt de klacht gehoord, dat het aantal geneesmiddelen op de nederlandse markt te hoog is Wanneer alle oude geneesmiddelen opnieuw getoetst zouden worden, conform de eisen, die gesteld worden aan nieuwe toe te laten spécialites, zou deze markt aanzienlijk uitgedund worden

V

In studies betreffende de relatie structuur-werking wordt vaak gebruik gemaakt van reeksen homologe stoffen Het is hierbij van belang de betrekkelijkheid van het begrip homoloog te onderkennen

Jansson, I , Orrenius S and Ernster, L Arch Biochem Biophys 151,391 (1972)

VI

Studies betreffende de invloed van catacholamines op het agressieve gedrag bij mieren kunnen beter verricht worden met soorten als Formica fusca L en Formica rufibarbis F dan met een agressieve species als Formica rufa L

Kostowski, W., Tarchalska, B and Wanchowicz, B. Pharmacol. Biochem. Beh. 3,337 (1975)

VII

Het feit, dat het combinatie preparaat Optalidon ® een populariteit geniet als doping bij wielrenners, doet twijfelen aan de werkzaamheid van het in dit preparaat aanwezige barbituraat Sandoptal ®

Lavene, D., Longchampt, J , Guillaume, M F and Kiger, J.L. Int J Clin. Pharmacol. 13,235 (1976)
Doping Centre, Dept of Pharmacology, Nijmegen.

VIII

Barbituraten hebben een tweeledige werking
De patient bezorgen zij slaap, de toxicologen slapeloosheid

IX

De toepassing van compartiment modellen, zoals bijvoorbeeld gebruikelijk is in de farmacokinetiek, is een mathematische variant van de hokjesgeest van deze tijd.

T.D. YIH **NIJMEGEN, 10 SEPTEMBER 1976**