J. Pharm. Pharmac., 1975, 27, 739–746 *Received March* 20, 1975

Mechanism of induction of hepatic microsomal drug metabolizing enzymes by a series of barbiturates

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The inducing effect of certain barbiturates (secobarbitone, thiopentone, pentobarbitone, allobarbitone, phenobarbitone and barbitone) on the levels of the hepatic microsomal drug-metabolizing enzymes has been studied in the rat both *in vivo* and *in vitro*. The extent of induction was related to the plasma half-lives of the barbiturates; compounds with low rates of metabolism and long half-lives were the most potent inducing agents. The latter (phenobarbitone, pentobarbitone and allobarbitone) were shown by spectral technique to interact with cytochrome P-450 suggesting that their mechanism of enzyme induction was 'substrate induction' in type. Barbiturates containing an allyl group (secobarbitone and allobarbitone) had a weaker inducing effect than expected, possibly due to their destruction of cytochrome P-450. Despite its short plasma half-life of 0·5 h thiopentone was a relatively potent inducer probably due to its metabolism to pentobarbitone, which has a much longer plasma half-life (1·3 h). Barbitone is an effective inducer of the drug-metabolizing enzymes, yet does not interact spectrally with cytochrome P-450; this is in accord with the observations that although there are increases in NADPH-cytochrome c reductase and cytochrome b_5, following administration of barbitone there is no increase in cytochrome P-450. Barbiturate pretreatment does not affect the activities of glucose-6-phosphatase, glucose-6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase.

During the last decade many hundreds of drugs and other xenobiotic chemicals have been shown to produce induction of the hepatic microsomal drug-metabolizing enzymes, with consequent effects on the rates of drug metabolism and the pharmacological activities of therapeutic agents (Conney, 1967). The chemical structure and pharmacological characteristics of these microsomal enzyme-inducing compounds are diverse and their only common property would seem to be high lipid solubility. Metabolism of these inducing agents takes place mainly in the hepatic endoplasmic reticulum and the induction of these microsomal enzymes may occur by virtue of the inducers being substrates of these enzymes (Parke, 1972), the extent of induction being dependent on the duration of binding of the inducing agents to the enzyme and hence, in general, to the rates of their metabolism. If the induction is of this 'substrate' type, rather than being hormonal in nature, it might be expected that the slower the rate of metabolism of the inducing agent, the greater would be the inductions. This has been shown to be true for certain methylenedioxy-benzene substrates, such as safrole, which remain bound to the enzyme, cytochrome P-450, for very long periods, even after metabolism, and consequently give rise to considerable induction of the hepatic microsomal enzyme system (Gray & Parke, 1973; Lake & Parke, 1972).

A series of barbiturates has now been studied to ascertain how general is the phenomenon of enzyme induction with this group of drugs, and also to examine the hypothesis that induction of the hepatic microsomal drug-metabolizing enzyme system involves 'substrate induction'.

MATERIALS AND METHODS

The barbiturates (secobarbitone, m.p. 100°, thiopentone sodium, hexobarbitone m.p. 146°, pentobarbitone sodium, allobarbitone m.p. 172°, phenobarbitone m.p. 177°, barbitone m.p. 190°), were gifts from May and Baker Ltd (Dagenham, Essex, U.K.). Solutions of the sodium salts were prepared by addition of excess M NaOH to the free barbituric acid and the solutions were subsequently adjusted with M HCl to pH 8·5. The barbiturates were found to be >99% pure by g.l.c. (Ioannides, Chakraborty & Parke, 1974) except for thiopentone which contained 2–5% pentobarbitone).

Male Wistar-albino rats, 37 days old, were dosed intraperitoneally with barbiturate (75 mg kg^{-1} in aqueous soln) daily for three days. Animals were killed by cervical fracture 24 h after the last administration; livers were excised and homogenates (25% w/v) prepared in 1·15% KCl. The liver microsomal supernatant was obtained by centrifuging the homogenate at 9000 *g* for 20 min and the microsomal suspension was prepared by centrifuging the microsomal supernatant at 105 000 *g* for 60 min and resuspending the pellet in KCl. The 105 000 *g* supernatant is referred to as the soluble fraction. For spectral studies the microsomal pellet was washed once with 1·15% (w/v) KCl and resuspended in 0·1 M tris buffer pH 7·6 (1–2 mg protein ml^{-1}).

The following enzyme determinations were carried out on: a) the microsomal supernatant: biphenyl-4-hydroxylase (Creaven, Parke & Williams, 1965), ethylmorphine *N*-demethylase (Holtzman, Gram & others, 1968) and *p*-nitrobenzoate reductase (Fouts & Brodie, 1957), modified by Gingell (1970); b) the microsomal suspension: NADPH-cytochrome c reductase (Williams & Kamin, 1962), glucose-6-phosphatase (De Duve, Pressman & others, 1955), cytochromes P-450 and b_5 (Omura & Sato, 1964), and microsomal protein (Lowry, Rosebrough & others, 1951); and c) the soluble fraction: 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase (Glock & McLean, 1953). Sleeping times were determined following a single intraperitoneal administration of hexobarbitone (100 mg kg^{-1}) as the sodium salt in water. Duration of sleeping time was taken as the time between the loss and regain of righting reflex.

Binding spectra were obtained by addition of the various barbiturates, as sodium salts in aqueous solution, to washed liver microsomes in 0·1 M tris buffer pH 7·6 to a final concentration of 4 mM (Schenkman, Remmer & Estabrook, 1967). Spectra were recorded using a Perkin-Elmer 356 dual beam spectrophotometer. The plasma half-lives of the barbiturates were determined (Ioannides, Chakraborty & Parke, unpublished) in animals of the same strain, sex and age as in the present work using a sensitive g.l.c. method (Ioannides & others, 1974).

Statistics were carried out using Student's *t*-test.

RESULTS

Table 1 shows the plasma half-lives of six barbiturates and their effect upon the hexobarbitone sleeping times in rats, the latter being a measure of the extent of enzyme induction.

Table 1. *Correlation of plasma half-lives of a number of barbiturates with their effect on the reduction of hexobarbitone sleeping time.*

Barbiturate	Chemical structure R_1	R_2	X	Plasma half-life (h)	Hexobarbitone sleeping times (min)
Control	—	—	—	—	34 ± 1
Thiopentone	$-CH(CH_3)CH_2CH_2$	$-CH_2CH_3$	S	0·5 ± 0·1	28 ± 1*
Secobarbitone	$-CH_2CH{=}CH_2$	$-CH_2CH_3$	0	0·9 ± 0·1	29 ± 1*
Pentobarbitone	$-CH(CH_3)CH_2CH_2$	$-CH_2CH_3$	0	1·3 ± 0·1	23 ± 1**
Allobarbitone	$-CH_2CH{=}CH_2$	$-CH_2CH{=}CH_2$	0	1·7 ± 0·1	22 ± 2*
Phenobarbitone	$-C_6H_5$	$-CH_2CH_3$	0	2·8 ± 0·2	17 ± 1**
Barbitone	$-CH_2CH_3$	$-CH_2CH_3$	0	7·2 ± 0·2	—

Barbiturate plasma half-lives were determined on male Wistar 40 day old rats (Ioannides and others, unpublished) by means of gas liquid chromatography (Ioannides & others, 1974). Results are expressed as mean values ± s.e.m.

* $P < 0·01$** $P < 0·001$.

Intraperitoneal administration of the various barbiturates to rats for 3 days caused varying increases in a number of liver parameters, including cytochromes P-450 and b_5, NADPH-cytochrome c reductase, and the hydroxylation of biphenyl, demethylation of ethylmorphine, and reduction of *p*-nitrobenzoic acid (Table 2). None of the other barbiturates studied were as effective an enzyme inducing agent as the classical inducer, phenobarbitone, which produced the greatest increase in cytochrome P-450

Table 2. *Effect of pretreatment of rats with barbiturates on a number of liver parameters and microsomal enzyme activities.*

Group	Liver wt (g)	100 × Liver wt/ Body wt	Biphenyl-4-hydroxylase (μmol g^{-1} liver h^{-1})	Ethylmorphine *N*-demethylation (μmol g^{-1} liver h^{-1})	*p*-Nitrobenzoate reductase (μmol g^{-1} liver h^{-1})
Control	7·0 ± 0·5	4·4 ± 0·1	1·5 ± 0·2	14 ± 1	6·5 ± 0·2
Thiopentone	8·2 ± 0·3	5·0 ± 0·1	2·8 ± 0·3	19 ± 2	5·9 ± 0·4
Secobarbitone	7·8 ± 0·2	4·7 ± 0·1	2·2 ± 0·3	15 ± 2	6·7 ± 0·3
Pentobarbitone	8·3 ± 0·1	4·8 ± 0·1	2·3 ± 0·1	23 ± 2	6·8 ± 0·2
Allobartone	8·0 ± 0·2	4·8 ± 0·1	2·4 ± 0·1	18 ± 2	7·9 ± 0·5
Phenobarbitone	9·3 ± 0·5	5·5 ± 0·1	3·4 ± 0·3	19 ± 3	7·5 ± 0·3

Group	NADPH-cytochrome c reductase (nmol g^{-1} liver min^{-1})	Cytochrome P-450 (nmol g^{-1} liver)	Cytochrome b_5 (nmol g^{-1} liver)	Microsomal protein (mg g^{-1} liver)
Control	920 ± 105	14·3 ± 1·1	7·6 ± 0·4	24 ± 1
Thiopentone	1570 ± 120	19·8 ± 1·0	9·2 ± 0·5	26 ± 2
Secobarbitone	1350 ± 110	18·7 ± 0·9	9·7 ± 0·3	29 ± 2
Pentobarbitone	1620 ± 55	24·2 ± 0·9	11·4 ± 0·4	30 ± 1
Allobarbitone	1630 ± 120	22·0 ± 1·0	11·9 ± 0·2	25 ± 2
Phenobarbitone	1630 ± 100	45·1 ± 5·5	11·9 ± 0·5	28 ± 1

Calculations were made using the following extinction coefficients: cytochrome P-450, 91 mM^{-1} cm^{-1} (Omura & Sato, 1964); cytochrome b_5, 185 mM^{-1} cm^{-1} (Omura & Sato, 1964); NADPH cytochrome c reductase, 18·5 mM^{-1} cm^{-1} (Margoliash, 1954).

Animals were pretreated with barbiturate (75 mg kg^{-1} day^{-1}, intraperitoneally, for three days). Results are the mean values of 4 animals ± s.e.m.

and biphenyl-4-hydroxylase activity and the greatest decrease in the hexobarbitone sleeping time. Administration of the barbiturates at lower dosage (50 mg kg^{-1} day^{-1} for 3 days) showed the same degree of induction (as 75 mg kg^{-1} day^{-1}) indicating that maximal induction has been achieved in all these experiments.

However, none of the barbiturates had any effect on the hepatic microsomal marker enzyme glucose-6-phosphatase, or on the soluble enzymes 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase (Table 3), from which it may be inferred that any enhanced microsomal oxygenase or reductase activity is not due merely to an increased rate of NADPH-generation.

Table 3. *The effect of barbiturates on some other hepatic enzymes.*

Group	Glucose-6-phosphate dehydrogenase (μmol NADPH g^{-1} liver min^{-1})	6-Phosphogluconate dehydrogenase (μmol NADPH g^{-1} liver min^{-1})	Glucose-6-phosphatase (μmol g^{-1} liver h^{-1})
Control	0·50 ± 0·02	1·37 ± 0·14	5·2 ± 0·6
Thiopentone	0·64 ± 0·10	1·66 ± 0·08	4·5 ± 0·3
Secobarbitone	0·47 ± 0·02	1·29 ± 0·16	5·2 ± 0·6
Pentobarbitone	0·47 ± 0·02	1·53 ± 0·08	5·8 ± 0·4
Allobarbitone	0·64 ± 0·10	1·53 ± 0·05	6·1 ± 0·4
Phenobarbitone	0·48 ± 0·10	1·51 ± 0·13	4·7 ± 0·6

Animals received daily intraperitoneal administration of barbiturate (75 mg kg^{-1}) for three days. The results are expressed as mean values ± s.e.m. from four animals.

Substrates of the hepatic microsomal drug-metabolizing enzyme system bind to cytochrome P-450, the terminal mixed-function oxidase, to produce spectral changes indicative of enzyme-substrate formation. All barbiturates used in this study, with the exception of thiopentone and allobarbitone interacted with the hepatic microsomes to form typical type I-spectra characterized by a peak at 385–390 nm and a trough at 418–427 nm as shown for pentobarbitone (see Fig. 1A). Thiopentone gave rise only to a trough followed by increased absorption without a peak, due probably to interaction of the sulphur atom with cytochrome P-450 (see Fig. 1B). Allobarbitone gave an

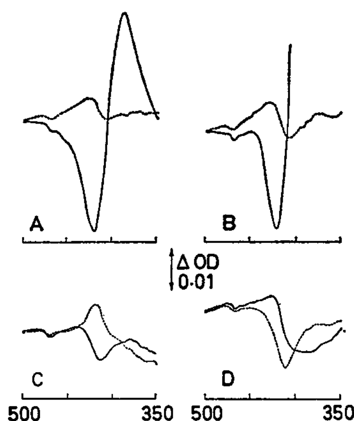

FIG. 1. Binding of barbiturates to hepatic microsomal cytochrome P-450. A. Pentobarbitone. B. Thiopentone. C. Allobarbitone. D. Barbitone. Abscissa: nm.

anomalous spectrum showing only slight absorption, which may be attributed to its destruction of cytochrome P-450 (Levin, Sernatinger & others, 1972). Furthermore, barbituric acid and barbitone also did not give the typical binding spectrum.

DISCUSSION

Most barbiturates are metabolized by enzymes of the hepatic endoplasmic reticulum, and the reactions can include hydroxylation of the alkyl side-chains, *N*-demethylation, desulphuration and ring scission (Parke, 1971), reactions which would be expected to involve cytochrome P-450. All five barbiturate drugs studied were shown to interact with hepatic microsomal cytochrome P-450 producing a type I spectral change, indicative of the formation of the enzyme-substrate complex (Schenkman & others, 1967). The binding spectra obtained with thiopentone and allobarbitone are anomalous, probably because of interaction of cytochrome P-450 with the sulphur atom of thiopentone and the destructive reaction of allobarbitone on this cytochrome. However, diethylbarbituric acid (barbitone) does not readily bind to cytochrome P-450 and has previously been shown to be metabolized to only a small extent (Goldschmidt & Wehr, 1957; Ebert, Yim & Miya, 1964). This association of slow rate of metabolism with limited interaction with cytochrome P-450 in the case of barbitone may be due to its highly polar character and low lipid solubility (Mark, Burns & others, 1958; Jansson, Orrenius & others, 1972), properties also shared by barbituric acid, which similarly shows only limited spectral interaction with cytochrome P-450. For, because of the substantial phospholipid component of the endoplasmic reticulum, accessibility to the enzymes of this system is dependent on a minimum degree of lipid solubility (Lu, Kuntzman & others, 1971).

After pretreatment with the barbiturates, the observed increases in the concentration of hepatic cytochromes and drug-metabolizing enzyme activities appear to parallel the plasma half-lives of the barbiturates administered. Barbiturates that are slowly metabolized and thus exhibit long plasma half-lives, such as phenobarbitone, are the most potent inducers of the drug-metabolizing enzymes in this group of drugs. Fig. 2 shows a correlation between the plasma half-lives of the barbiturates studied and the degrees of induction of biphenyl metabolism *in vitro*, the metabolism of hexobarbitone *in vivo*, and the concentration of cytochrome P-450 (Fig. 2). These observations suggest that cytochrome P-450 may be a controlling factor in the sequence of reactions that leads to induction of the hepatic microsomal enzyme system. The low level of induction after administration of allobarbitone or secobarbitone probably results from the destruction of cytochrome P-450 (Abritti & De Matteis, 1971/1972), which is a well-known phenomenon with chemicals containing the allyl group (De Matteis, 1970; Levin & others, 1972; Levin, Jacobson & others, 1973).

However, there is but little correlation between the plasma half-life of the barbiturate on the one hand and the extent of induction of NADPH-cytochrome c reductase and of *p*-nitrobenzoate reductase on the other, showing that the *de novo* synthesis of NADPH-cytochrome c reductase does not necessarily parallel that of cytochrome P-450. It is well-known that although most drugs induce the synthesis of both cytochrome P-450 and NADPH-cytochrome c reductase, pretreatment with polycylic hydrocarbons induces only the former (cytochrome P-448) and steroids generally induce only the latter (Parke, 1975). Studies of drug metabolism in the neonatal ferret (Ioannides & Parke, 1975) show that the hepatic concentrations of NADPH-cytochrome

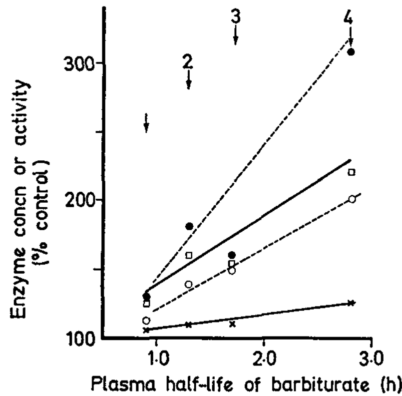

FIG. 2. Dependence of enzyme induction on rate of metabolism of barbiturate inducing agents. 1 Secobarbitone. 2 Pentobarbitone. 3 Allobarbitone. 4 Phenobarbitone. ●--● Cytochrome P-450. □–□ Biphenyl 4-hydroxylase. ○--○ Hexobarbitone metabolism *in vivo*. ×—× Liver weight; body weight ratio.

c reductase and cytochrome P-450 develop independently of each other, reaching adult levels at two weeks and at more than eight weeks respectively. It is also significant that the hepatic hypertrophy induced by the barbiturates is less related to the rate of metabolism of the inducing agent (see Fig. 2), and indeed the induction of hepatic microsomal enzymes and the accompanying hepatic hypertrophy when barbiturates are used as inducers, have been shown to be separate, independent phenomena (Seifert & Vacha, 1970).

In a separate experiment (Table 4) it was shown that barbitone, which has an even longer half-life than phenobarbitone, is almost as effective as an enzyme-inducing agent, even though it is largely excreted unchanged and the overall extent of metabolism is $<5\%$ dose (Goldschmidt & Wehr, 1957; Ebert, & others, 1964). However, although it increases biphenyl hydroxylase, ethylmorphine *N*-demethylase, NADPH-cytochrome c reductase and cytochrome b_5, to extents similar to those observed after phenobarbitone, there is little or no increase in cytochrome P-450. This is in accord with the low degree of observed spectral interaction of barbitone with cytochrome P-450, and its inductive effect on the microsomal enzyme activities would seem to result from the increased activity of the NADPH-cytochrome c reductase or the even greater increase in cytochrome b_5. It is perhaps not insignificant that cytochrome b_5 can potentiate the *N*-demethylation of ethylmorphine, and other oxygenations, by providing the second electron for the reduction of the cytochrome P-450—substrate complex (Correia & Mannering, 1973), and, in the presence of FMN and NADH or NADPH, can support the metabolism of aniline to *p*-aminophenol even when no cytochrome P-450 is present (Symons & Juchau, 1974).

Table 4. *Stimulation of microsomal drug metabolism by barbitone.*

Group	Liver wt (g)	100 × Liver wt/ Body wt	Microsomal protein (mg g^{-1} liver)	Cyt.P-450 (nmol g^{-1} liver)	Cyt. b_5 (nmol g^{-1} liver)	NADPH-cyt.c reductase (nmol g^{-1} liver min^{-1})	Biphenyl-4-hydroxylase (μmol g^{-1} liver h^{-1})	Ethylmorphine *N*-demethylase (μmol g^{-1} liver h^{-1})
Control	7·2 ± 0·2	4·7 ± 0·1	22 ± 2	23·1 ± 1·1	9·4 ± 0·6	1140 ± 55	1·5 ± 0·1	17 ± 1
Barbitone	7·5 ± 0·2	5·0 ± 0·1	24 ± 1	24·2 ± 1·1	21·0 ± 1·2	1510 ± 110	2·6 ± 0·1	23 ± 1
Phenobarbitone	8·5 ± 0·3	5·6 ± 0·1	26 ± 1	31·9 ± 2·9	25·7 ± 2·9	1780 ± 45	3·0 ± 0·1	24 ± 1

Animals received daily intraperitoneal administration of barbiturate (75 mg kg^{-1}) for three days.
Figures given are mean values ± s.e.m.

Thiopentone, although rapidly metabolized, is nevertheless a relatively good inducer of the drug-metabolizing enzymes, probably because metabolism proceeds via desulphuration to give the oxygen analogue pentobarbitone (Bush, Mazel & Chambers, 1961; Mark, Brand & others, 1965; Sharma & Stowe, 1970) which is itself an effective microsomal enzyme inducing agent, and has a longer plasma half-life than thiopentone.

Pretreatment with the barbiturates has been shown not to affect the microsomal marker enzyme, glucose-6-phosphatase, or the soluble NADPH-generating enzymes, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Thus barbiturates appear to induce selectively only those enzymes directly involved in their own metabolism. Other workers have found that treatment with phenobarbitone has no effect on (Feuer & Granda, 1970), or even reduces the 'available' glucose-6-phosphatase, but produces an overall increase in the latent enzyme, which is made manifest by treatment of the microsomal preparation with deoxycholate or other detergents (Pandhi & Baum, 1970). This anomaly has been attributed to accompanying changes in conformation of the endoplasmic reticulum, but latent enzymic activity was not determined in the present study.

In a recent publication (Valerino, Vesell & others, 1974) in which the effects of a number of barbiturates on hepatic microsomal enzymes was studied (aniline hydroxylase, ethylmorphine *N*-demethylase) an association was noticed between the plasma half-lives of phenobarbitone (10·1 h), pentobarbitone (2·3 h) and hexobarbitone (0·6 h) and the degree of enzyme stimulation. This is in agreement with our own observations, a preliminary account of which has been previously published (Parke, 1972; Ioannides, 1973).

Acknowledgements

The authors wish to thank May and Baker Limited, (Dagenham, Essex) for their generous gifts of barbiturates.

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