Biochemical Pharmacology, Vol. 25, pp. 1659–1663. Pergamon Press, 1976. Printed in Great Britain.

HEXOBARBITAL SLEEPING TIME AND DRUG METABOLISM IN RATS WITH LIGATED BILE DUCTS—A LACK OF CORRELATION

ROGER DREW and BRIAN G. PRIESTLY
Department of Human Physiology and Pharmacology, University of Adelaide, Adelaide, South Australia 5001

(*Received* 3 *June* 1975; *accepted* 14 *November* 1975)

Abstract—Hexobarbital sleeping time is commonly used as an index of the activity of hepatic microsomal drug-metabolizing enzymes in animals. This report describes anomalies between hexobarbital sleeping time and the rate of metabolism *in vitro* by microsomal enzymes in rats after bile duct ligation (BDL). The duration of hexobarbital sleeping time, 2–24 hr after BDL, was approximately twice that of sham-operated controls. No significant decrease in the activity of microsomal aminopyrine demethylase, aniline hydroxylase, hexobarbital oxidase or the amount of cytochrome P-450 was detected during this period. A further prolongation of hexobarbital sleeping time was observed 48–72 hr after BDL, and this was accompanied by a significant impairment of drug metabolism *in vitro*. The effect of BDL on hexobarbital sleeping time was independent of the route of administration. Thiopental sleeping time was prolonged at 12 and 72 hr after BDL. Zoxazolamine paralysis time was prolonged at 72 hr after BDL, but not at 12 hr. Plasma protein binding of hexobarbital and thiopental was not altered by hyperbilirubinemia. These data suggest that changes in drug metabolism are not responsible for the prolongation of hexobarbital sleeping time during the early phase of cholestasis caused by BDL.

Hexobarbital is a moderately short-acting barbiturate whose hypnotic action is thought to be terminated by metabolism rather than by tissue redistribution [1]. The genetic and environmental factors affecting the metabolism of hexobarbital by rodents and responsiveness of rodents to hexobarbital have been studied in detail [2–4], and in most cases there is good correlation between the rate of metabolism of hexobarbital and the duration of loss of righting reflex after hexobarbital administration [5–7]. Hexobarbital sleeping time, therefore is used by a large number of laboratories as a simple adjunct to their methods of assessing liver microsomal mixed-function oxidase activity. However, factors other than metabolism may contribute toward termination of the action of a drug. Changes in hexobarbital sleeping time, therefore, should be interpreted with this in mind.

A gradual decline in the activity of hepatic microsomal mixed-function oxidase has been reported [8] in extrahepatic cholestasis produced experimentally by bile duct ligation (BDL). This study reports a lack of correlation between hexobarbital sleeping time and drug metabolism *in vitro* during the early stages of BDL-induced cholestasis.

MATERIALS AND METHODS

Male Wistar rats (200–300 g) were used throughout this study and were allowed food and water *ad lib.* After abdominal midline incision, the common bile duct was doubly ligated while under ether anaesthesia. Sham-operated animals served as controls. At various times after surgery, drug metabolism was measured *in vitro*, and the pharmacological activity of barbiturates was assessed *in vivo*.

Assessment in vitro of drug metabolism. Animals were stunned, and then killed by exsanguination. Livers were perfused *in situ* via the portal vein with ice-cold saline. After removal, the livers were homogenized with two to three times their weight of cold 0.25 M sucrose containing 0.05 M Tris–HCl buffer, pH 7.4, in a motor-driven Potter–Elvehjem homogenizer. The homogenate was centrifuged for 20 min at 10,000 *g* and the resulting supernatant was centrifuged for 1 hr at 105,000 *g*. The microsomal pellet was resuspended in cold 1.15% KCl (buffered with 0.01 M phosphate buffer, pH 7.4) and resedimented for 1 hr at 105,000 *g*. The washed microsomes were finally resuspended in 5.0 ml of cold 1.15% KCl. Microsomal protein was measured by the method of Lowry *et al.* [9]. Microsomal cytochrome P-450 content was determined from the carbon monoxide difference spectrum of dithionite-reduced microsomes assuming a molar extinction difference of 91 cm^{-1} mM^{-1} between 450 and 490 nm [10]. Incubation media used for the determination of aniline hydroxylase and aminopyrine demethylase activities were as described by Schenkman *et al.* [11], except that semicarbazide (4.1 mM) was used to trap formaldehyde produced by demethylation of aminopyrine. Formaldehyde was estimated by the method of Nash [12], and the metabolic product of aniline hydroxylation, *para*-aminophenol, was measured by a modified method of Imai *et al.* [13].

Microsomal hexobarbital oxidase activity was determined in a medium containing 0.05 M phosphate buffer, pH 7.4, 1.33 mM hexobarbital, 0.33 mM NADP, 6.67 mM nicotinamide, 8 mM isocitrate and isocitric dehydrogenase (Sigma type IV) such that 0.4 μmole NADPH was generated/min/incubation at 37°.

B.P. 25/14 G

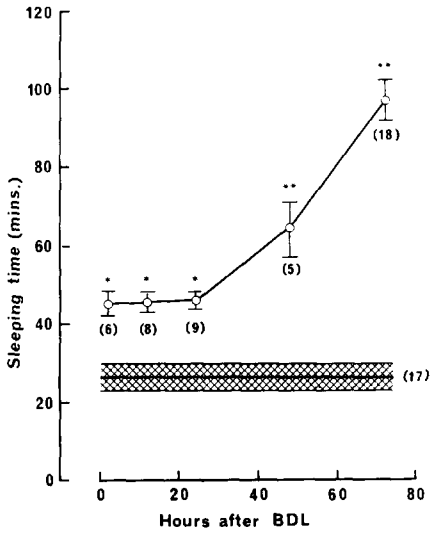

Fig. 1. Hexobarbital sleeping time in BDL rats. The shaded area represents the mean ± S. E. M. of control rats at various times after sham operation. Figures in parentheses are the numbers of rats. A single asterisk indicates significant difference from controls ($P < 0.001$). A double asterisk indicates significant difference from controls ($P < 0.001$) and from other groups ($P < 0.01$).

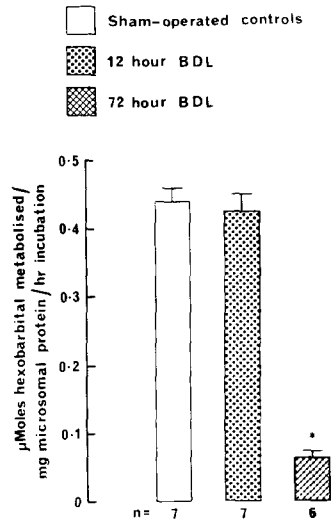

Fig. 2. Effect of BDL on microsomal hexobarbital oxidase activity. Bars represent the mean ± S. E. M. An asterisk indicates significant difference from controls ($P < 0.001$).

The amount of unchanged hexobarbital was determined by the method of Cooper and Brodie [14].

Assessment in vivo of pharmacological activity. Hexobarbital-, thio-pental-, and zoxazolamine-induced loss of righting reflex was measured according to the technique described by Fouts [15]. Hexobarbital (125 mg/kg) and zoxazolamine (80 mg/kg) were administered intraperitoneally. Intravenous injections of hexobarbital (50 mg/kg) and thiopental (20 mg/kg) were done via a tail vein. The time between the loss and spontaneous regaining of the righting reflex was regarded as being the duration of pharmacological activity.

RESULTS

The effects of BDL on hexobarbital sleeping time after intraperitoneal injection are shown in Fig. 1. Loss of righting reflex occurred within 4 min, and there was no difference between sham-operated and BDL rats in the onset of hexobarbital-induced narcosis.

However, as early as 2 hr after BDL, there was a significant increase in hexobarbital sleeping time which remained at a constant level for the first 24 hr. At 48 and 72 hr after BDL, there was a further significant increase in sleeping time.

The changes in microsomal cytochrome P-450 content, aniline hydroxylase and aminopyrine demethylase activities (Table 1) are similar to those reported by Schaffner *et al.* [8] and contrast the results *in vivo* in Fig. 1. There was a gradual decline in metabolism *in vitro* and cytochrome P-450 up to 24 hr after BDL, but it was not until 48 hr that the decrease became significant. At 48 hr metabolism was approximately 70 per cent of the respective controls; this decreased to 35 per cent 8 days after BDL. To ensure that the early change in sleeping time was not due to a specific decrease in hexobarbital metabolism, the microsomal oxidation of hexobarbital was measured 12 and 72 hr after BDL (Fig. 2). There was no significant difference between the hexobarbital oxidase activity of microsomes obtained from sham-operated control animals and those that had been ligated for 12 hr. Seventy-two hr after BDL, the microsomal activity was significantly lower than control values. Hence, the initial increase in hexobarbital sleeping time cannot be attri-

Table 1. Effect of BDL on microsomal drug metabolism*

Time after BDL (hr)	Cytochrome P-450 (nmoles/mg microsomal protein)			Aniline hydroxylase (nmoles PAP formed/mg microsomal protein/min)			Aminopyrine demethylase (nmoles HCHO formed/mg microsomal protein/min)		
	Control	BDL	(%)	Control	BDL	(%)	Control	BDL	(%)
2	1.05 ± 0.03	1.03 ± 0.06	98.5	1.06 ± 0.02	1.09 ± 0.03	102.6	7.69 ± 0.41	7.83 ± 0.39	101.8
12	1.02 ± 0.03	0.95 ± 0.02	92.9	0.87 ± 0.04	0.79 ± 0.03	91.7	6.30 ± 0.16	5.77 ± 0.10	91.6
24	0.96 ± 0.02	0.88 ± 0.02	91.9	0.87 ± 0.03	0.78 ± 0.02	90.2	6.49 ± 0.07	5.99 ± 0.20	92.3
48	0.94 ± 0.01	0.73 ± 0.05†	77.0	0.83 ± 0.04	0.57 ± 0.05‡	69.1	6.83 ± 0.32	5.11 ± 0.45†	74.8
72	1.09 ± 0.04	0.61 ± 0.0‡	56·1	0.82 ± 0.01	0.45 ± 0.02‡	55.0	6.01 ± 0.19	3.30 ± 0.22‡	54.9
8 Days	1.25 ± 0.04	0.47 ± 0.07‡	37.8	0.98 ± 0.08	0.36 ± 0.06‡	36.7	7.72 ± 0.74	2.55 ±0.64‡	33.0

* Values are mean ± S. E. M. for groups of four rats in each case.
† Significantly different from control ($P < 0.05$).
‡ Significantly different from control ($P < 0.01$).

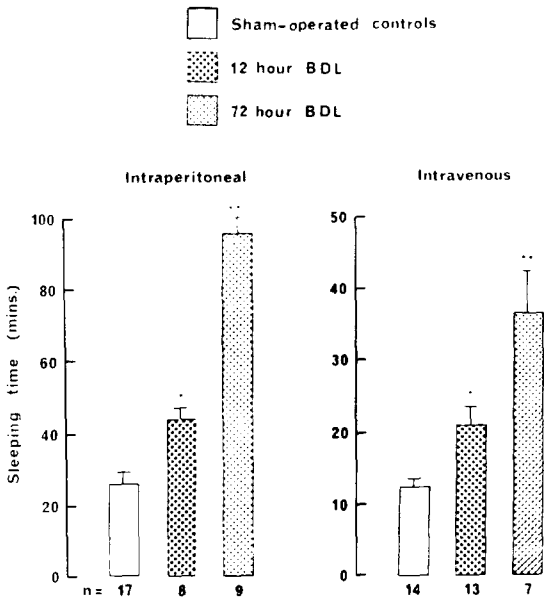

Fig. 3. Effect of BDL on hexobarbital sleeping time after intra-peritoneal (125 mg/kg) or intravenous (50 mg/kg) administration. Bars represent the mean ± S. E. M. A single asterisk indicates significant difference from controls ($P < 0.001$). A double asterisk indicates significant difference from controls ($P < 0.001$) and from 12-hr BDL ($P < 0.05$).

buted to a decrease in microsomal enzyme activity.

The early prolongation of sleeping time was independent of the route of administration of hexobarbital (Fig. 3). BDL for 12 hr increased the duration of sleeping time 74 and 87 per cent after intraperitoneal and intravenous injections of hexobarbital; BDL for 72 hr caused an increase of 368 and 223 per cent respectively.

Hexobarbital-, thiopental- and zoxazolamine-induced loss of righting reflex was measured in animals whose drug metabolism had been induced with phenobarbitone or inhibited by SKF-525A (Table 2). As anticipated, phenobarbitone pretreatment significantly decreased the duration of action of hexobarbital and zoxazolamine, while SKF-525A significantly prolonged their action. Although SKF-525A had no effect on thiopental anaesthesia, phenobarbitone pretreatment produced a small but significant decrease; this was probably due to changed distribution of thiopental caused by increased liver size and increased hepatic blood flow. These data support the hypothesis that the pharmacological action of thiopental is terminated mainly by tissue redistribution and is relatively independent of the activity of the mixed-function oxidase system [16].

The effects of 12 and 72 hr BDL on thiopental sleeping time and zoxazolamine paralysis time are shown in Fig. 4. Changes in thiopental sleeping time closely followed the changes seen in hexobarbital sleeping time. There was a significant increase 12 hr after BDL, which was followed by a further increase at 72 hr. The effect of BDL on zoxazolamine paralysis time was different from the effects on barbiturate sleeping time. Paralysis time in animals that had been ligated for 12 hr was the same as in sham-operated

Table 2. Effect of phenobarbitone and SKF-525A on hexobarbital-, zoxazolamine- and thiopental-induced loss of righting reflex*

	Hexobarbital	Zoxazolamine	Thiopental
Control	26.7 ± 2.1 (5)	229 ± 9 (23)	25.8 ± 3.0 (12)
SKF-525A	131.7 ± 17.3† (8)	508 ± 12† (5)	34.2 ± 3.7 (12)
Phenobarbitone	12.5 ± 0.9† (6)	74 ± 10† (5)	17.2 ± 2.0‡ (12)

* Animals were pretreated with phenobarbitone (60 mg/kg, i.p.) for 3 days or given SKF-525A (25 mg/kg, i.p.) 40 min before hexobarbital (125 mg/kg, i.p.), zoxazolamine (80 kg/kg, i.p.) or thiopental (25 mg/kg, i.v.). Values represent the mean loss ± S. E. M. of the righting reflex in min for each treatment group. Figures in parentheses represent the number of animals in each group.

† Significantly different from controls ($P < 0.001$).

‡ Significantly different from controls ($P < 0.05$).

controls, but was significantly prolonged by 72-hr BDL.

During BDL, there was a marked rise in serum bilirubin levels. Bilirubin is extensively protein bound, and the possibility of competitive displacement of the barbiturates from binding sites was investigated. The results are presented in Table 3. Plasma protein binding of thiopental (approximately 90 per cent) or hexobarbital (approximately 60 per cent) was unaffected by the bilirubin which accumulated after BDL. The lack of displacement of barbiturate by bilirubin may be due to the large number of available binding sites [17].

An increase in brain sensitivity to barbiturates could be another factor in prolonging sleeping time. Blood and brain levels of hexobarbital and thiopental, therefore, were measured at the time of awakening in sham-operated controls and BDL rats (Table 4). Thiopental blood and brain levels at the time of awakening were not altered by BDL. BDL rats (12

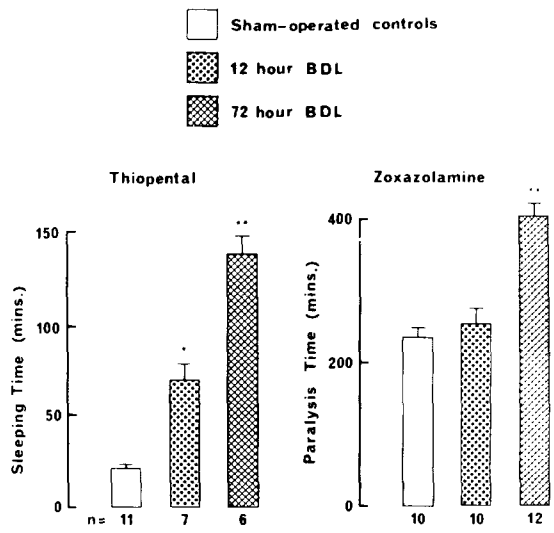

Fig. 4. Effect of BDL on thiopental sleeping time (25 mg/kg, i.v.) and zoxazolamine paralysis time (80 mg/kg, i.p.). Bars represent the mean ± S. E. M. A single asterisk indicates significant difference from controls ($P < 0.001$). A double asterisk indicates significant difference from controls ($P < 0.001$) and from 12-hr BDL ($P < 0.001$).

Table 3. Effect of BDL on serum protein binding of hexobarbital and thiopental*

	No. of rats	Serum bilirubin (mg/100 ml)	Serum concn (μg/ml)	Protein bound (%)
HEXOBARBITAL				
Controls	6	0.23 ± 0.04	99.9 ± 11.1	61.6 ± 3.8
12-hr BDL	4	2.64 ± 0.23	94.8 ± 13.7	61.2 ± 5.0
72-hr BDL	4	4.44 ± 0.08	124.0 ± 7.8	55.7 ± 0.9
THIOPENTAL				
Controls	5	0.27 ± 0.04	66.2 ± 4.3	91.6 ± 0.6
12-hr BDL	5	2.79 ± 0.27	59.8 ± 4.4	91.9 ± 0.8
72-hr BDL	4	6.86 ± 2.68	42.9 ± 5.2	88.4 ± 1.4

* Protein binding of hexobarbital and thiopental was determined by ultrafiltration of serum samples obtained approximately 5 min after administration of hexobarbital (125 mg/kg, i.p.) and approximately 2 min after thiopental (25 mg/kg, i.v.). Hexobarbital and thiopental were determined by the methods of Cooper and Brodie [14] and Goldstein and Aronow [18]. Values represent the mean ± S. E. M.

and 72 hr) receiving hexobarbital regained their righting reflexes at lower blood and brain levels of hexobarbital than did the sham-operated controls.

DISCUSSION

The duration of hexobarbital- and zoxazolamine-induced loss of righting reflex is sensitive to changes in microsomal enzyme activity. Bile duct ligation caused a gradual decrease in microsomal enzyme activity, but the increase in hexobarbital narcosis occurred at a time when the microsomal parameters were not significantly reduced. Twelve hr after BDL, hexobarbital sleeping time doubled, but zoxazolamine paralysis time and activities *in vitro* of hexobarbital oxidase, aniline hydroxylase and aminopyrine demethylase were unchanged. Hence, the changes in hexobarbital sleeping time during the initial stages of BDL-induced cholestasis do not correlate with the activity of the microsomal mixed-function oxidase system. This lack of correlation is accentuated by the differences in response of the barbiturates and zoxazolamine to BDL and known modifiers of microsomal enzyme activity. The enzyme inhibitor SKF-525A prolonged the effect of hexobarbital and zoxazolamine but not that of thiopental. Twelve-hr BDL prolonged the effect of hexobarbital and thiopental, but not zoxazolamine, while 72-hr BDL prolonged all three.

Intraperitoneal injection is the most common method of administering hexobarbital to small laboratory animals. By this route of administration the drug is mainly absorbed into the portal circulation and passes through the liver before reaching its systemic site of action [19]. After the common bile duct has been ligated, there is an increase in pressure within the bile canalicular tree due to the hepatocytes continuing to secrete bile for a short time. Under these circumstances, it is conceivable that an alteration in liver haemodynamics and/or the blood supply to the gut could occur which might influence the absorption of hexobarbital. However, it is unlikely that this is a major contributing factor to the increase in hexobarbital sleeping time 12 hr after BDL because (a) similar increases in hexobarbital sleeping time were observed using both the intraperitoneal and intravenous routes of administration; (b) there was no difference in the onset of action of hexobarbital in sham-operated or BDL rats; and (c) zoxazolamine was also given intraperitoneally and there was no change in the duration of its paralysis time 12 hr after BDL.

The fact that BDL increased thiopental and hexobarbital sleeping times by a similar amount suggests

Table 4. Effect of BDL on hexobarbital and thiopental blood and brain concentrations upon awakening*

	No. rats	Sleeping time (min)	Blood concn (μg/ml)	Brain concn (μg/g)
HEXOBARBITAL				
Controls	15	30.1 ± 1.6	38.4 ± 1.0	39.2 ± 1.9
12-hr BDL	10	54.3 ± 3.6†	27.2 ± 2.2†	25.1 ± 1.7†
72-hr BDL	10	109.2 ± 4.0†‡	30.7 ± 1.7†	29.2 ± 1.8§
THIOPENTAL				
Controls	10	19.4 ± 2.5	22.7 ± 1.0	17.5 ± 0.8
12-hr BDL	6	48.6 ± 11.6§	23.0 ± 1.2	19.1 ± 1.0
72-hr BDL	8	154.6 ± 17.1†‡	22.6 ± 1.4	19.1 ± 1.0

* Animals were decapitated upon regaining their righting reflex after hexobarbital (125 mg/kg, i.p.) or thiopental (25 mg/kg, i.v.). Whole blood and brain concentrations of hexobarbital and thiopental were determined by the methods of Cooper and Brodie [14] and Goldstein and Aronow [18]. Values represent the mean ± S. E. M.

† Significantly different from controls ($P < 0.001$).

‡ Significantly different from 12-hr BDL ($P < 0.001$).

§ Significantly different from controls ($P < 0.01$).

that the same factor may be altering the action of both drugs. This factor is unlikely to be a reduced rate of metabolism, since hexobarbital and thiopental clearly differed in their response to enzyme inhibition with SKF-525A. Redistribution from the brain is primarily responsible for the short duration of thiopental anaesthesia [18]; hence BDL-induced changes in the redistribution of the two barbiturates might be the common factor.

There are several ways in which BDL might result in altered barbiturate distribution. Firstly hepatic uptake might be reduced as a result of changes in liver haemodynamics or uptake mechanisms. It would have to be argued that this is more important for the barbiturates than for zoxazolamine in order to explain the lack of effect of 12 hr of BDL on zoxazolamine paralysis time. Reyes *et al.* [20] reported that 3 days of BDL decreased the hepatic content of Y and Z anion-binding proteins by approximately 40 per cent, and reduced the hepatic uptake of sulfobromophthalein. However, there was no effect on this uptake mechanism at the earliest time studied (25 min). One can only speculate on the effect that impaired binding of barbiturates to hepatic Y and Z proteins might have on the duration of their pharmacological action. It is unlikely that this factor can explain the prolongation of hexobarbital sleeping time seen 2–12 hr after BDL, although it might be of significance at subsequent times. Secondly, displacement from plasma protein binding sites by bilirubin might increase the amount of barbiturate available for distribution. The data of Table 3 suggest that this is unlikely. Thirdly, BDL might result in alteration in permeability of the blood–brain barrier. BDL animals woke up at lower blood and brain levels of hexobarbital than did sham-operated controls, indicating an increased brain sensitivity to hexobarbital. However, BDL did not increase the sensitivity of the brain to thiopental, and it is difficult to rationalize how such a selective change in the sensitivity of the brain to barbiturates could occur in obstructive jaundice. Fourthly, BDL might alter barbiturate redistribution into fat. It was observed that food consumption was reduced after BDL and that rats lost weight. However, in experiments where sham-operated rats were deprived of food for 12 or 72 hr, starvation for 12 hr failed to reproduce the prolongation of either hexobarbital or thiopental sleeping times seen after 12-hr BDL (Drew and Priestly, unpublished observations).

A fifth possibility is that BDL prevents biliary excretion of the barbiturates. Although urinary excretion is generally considered to be the major route of barbiturate elimination, biliary excretion of [^{14}C]phenobarbitone (18 per cent) and [^{14}C]pentobarbitone (28 per cent) in the form of polar metabolites has been reported [21]. Significant biliary excretion of hexobarbital has not been clearly established. Thompson *et al.* [22] detected unchanged hexobarbital, and smaller amounts of the 3′-hydroxy metabolite and its glucuronide by gas–liquid chromatography-mass spectrometry in rat bile, but did not quantitate them. Holcombe *et al.* [23] recovered less than 1 per cent of a dose of [^{14}C]hexobarbital in the first 20 min of bile collection from an isolated perfused liver. Analysis by thin-layer chromatography (t.l.c.) of the 11 per cent of the dose recovered over 90 min bile collection indicated that the radioactivity was all in a form more polar than unchanged hexobarbital. Our own t.l.c. experiments have also failed to detect unchanged drug in the bile of rats injected with hexobarbital. The pharmacological activity of hexobarbital metabolites is unknown. If it can be assumed that the biliary metabolites are inactive, it is difficult to conclude that blockage of their biliary excretion by BDL should significantly alter hexobarbital sleeping time.

Our conclusions are that hexobarbital sleeping time is not a reliable index of the activity of hepatic microsomal drug-metabolizing enzymes after BDL. A change in brain sensitivity to hexobarbital may be as important as a change in its rate of metabolism, and this factor should not be ignored when interpreting a change in the duration of pharmacological response. Furthermore, since BDL changed the duration of response to thiopental without apparent alteration in brain sensitivity, altered drug distribution should also be considered as a factor in sleeping time experiments.

REFERENCES

1. M. T. Bush and W. Weller, *Drug Metab. Rev.* **1**, 249 (1972).
2. E. S. Vesell, *Pharmacology* **1**, 81 (1968).
3. J. Noordhoek, *Eur. J. Pharmac.* **3**, 242 (1968).
4. R. L. Turner, T. E. Gram and R. E. Stitzel, *Pharmacology* **2**, 181 (1969).
5. A. H. Conney, C. Davison, R. Gastel and J. J. Burns, *J. Pharmac. exp. Ther.* **130**, 1 (1960).
6. A. R. Hansen and J. R. Fouts, *Toxic. appl. Pharmac.* **13**, 212 (1968).
7. G. J. Mannering, in *Selected Pharmacological Testing Methods* (Ed. A. Burger), Vol. 3, p. 51. Marcel Dekker, New York (1968).
8. F. Schaffner, P. G. Bacchin, F. Hutterer, H. H. Scharnbeck, L. L. Sarkwzi, H. Denk and H. Popper, *Gastroenterology* **60**, 888 (1971).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
10. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
11. J. B. Schenkman, H. Remmer and R. W. Estabrook, *Molec. Pharmac.* **3**, 113 (1967).
12. T. Nash, *Biochem. J.* **55**, 416 (1953).
13. Y. Imai, A. Ito and R. Sato, *J. Biochem., Tokyo* **60**, 417 (1966).
14. J. R. Cooper and B. B. Brodie, *J. Pharmac. exp. Ther.* **114**, 409 (1955).
15. J. R. Fouts, in *Methods in Pharmacology* (Ed. A. Schwartz), Vol. 1, p. 287. Meredith Corp., New York (1971).
16. J. Bogan, *J. Pharm. Pharmac.* **22**, 709 (1970).
17. L. R. Goldbaum and P. K. Smith, *J. Pharmac. exp. Ther.* **111**, 197 (1954).
18. A. Goldstein and L. Aronow, *J. Pharmac. exp. Ther.* **128**, 1 (1960).
19. G. Lukas, S. D. Brindle and P. Greengard, *J. Pharmac. exp. Ther.* **178**, 562 (1971).
20. H. Reyes, A. J. Levi, Z. Gatmaitan and I. M. Arias, *J. clin. Invest.* **50**, 2242 (1971).
21. C. D. Klaasen, *Br. J. Pharmac. Chemother.* **43**, 161 (1971).
22. R. M. Thompson, N. Gerber, R. A. Seibert and D. M. Desiderio, *Drug Metab. Dispos.* **1**, 489 (1973).
23. R. R. Holcombe, N. Gerber and M. T. Bush, *J. Pharmac. exp. Ther.* **188**, 15 (1974).