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HEPATIC DRUG-METABOLISING ENZYME ACTIVITY AND DURATION OF HEXOBARBITONE ANAESTHESIA IN BARBITONE-DEPENDENT AND WITHDRAWN RATS

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Abstract—The activity of several hepatic drug-metabolising enzymes and the duration of hexobarbitone anaesthesia were determined in barbitone-dependent and withdrawn rats. Rats were made dependent on barbitone sodium by the administration of increasing amounts in the drinking water over a 32-day period. Abrupt withdrawal was accompanied by a characteristic withdrawal syndrome. The body weight, liver weight and body weight/liver weight ratio were found to be increased during barbitone treatment and to fall after withdrawal. The liver protein and glycogen content did not change during barbitone treatment. Withdrawal produced a transient reduction in hepatic glycogen content but did not affect protein levels.

The capacity of hepatic microsomal preparations to metabolise hexobarbitone, aminopyrine and prontosil and to conjugate *o*-aminophenol was increased by barbitone treatment. Following withdrawal, the enzyme activity fell below that of control animals Four months after withdrawal the liver enzyme activity was still subnormal.

The acute administration of phenobarbitone to 4-month withdrawn animals was found to restimulate hepatic microsomal drug-metabolising enzymes but the level attained was not as high as that found in phenobarbitone-treated control rats.

The same degree of tolerance to injected hexobarbitone, as indicated by reduction in sleeping time, was found at all stages of barbitone treatment. A hypersensitivity to hexobarbitone was produced by withdrawal. It is concluded that both the tolerance in barbitone-dependent rats and the hypersensitivity of withdrawn animals can be adequately explained by altered hepatic drug-metabolising enzyme activity.

IT IS now well established that the administration of many drugs may stimulate the activity of hepatic microsomal drug-metabolising enzymes. (For review, see Conney, 1967[1]). The most studied of such stimulatory drugs are the barbiturates, which, on continuous administration to rats, produce maximum effect on these microsomal enzymes within 3–4 days[2]. More prolonged treatment has been shown to have no further effect and the drug-metabolising enzyme activity rapidly falls to normal when barbiturate administration ceases.[3] The enhanced hepatic drug metabolism in barbiturate-treated animals may result in decreased duration of drug action and has been shown by Remmer[4] to be responsible for the tolerance which develops to several barbiturates. Although a few studies[3, 5] have been made of the effect of chronic administration of barbiturate on hepatic drug-metabolising enzymes, none have been performed under conditions where development of physical dependence on the drug occurs. The relative contribution of stimulated metabolism and central nervous adaptation in accounting for drug tolerance in drug-dependent animals is unknown.

Crossland and co-workers[6, 7] were the first to describe physical dependence on barbiturate in rats chronically treated with barbitone sodium, administered in increasing dose in drinking water over a period of four to five weeks. Abrupt withdrawal, effected by replacing the barbitone solution with tap water, produced a characteristic withdrawal syndrome. In such barbitone-treated animals, tolerance, measured by a reduction in sleeping time, occurred with several other depressants including a number of barbiturates. The present work was undertaken to provide information on the mechanism of hexobarbitone tolerance occurring in barbitone-dependent rats. This paper reports the effect of this treatment on several hepatic drug-metabolising enzymes and on the duration of hexobarbitone anaesthesia.

MATERIALS AND METHODS

Female Wistar rats (weighing approximately 50 g at the beginning of the experiment), purchased from A. Tuck and Son, Rayleigh, Essex, were used throughout. All rats were fed *ad libitum* on normal laboratory diet.

Barbitone administration

Animals were habituated to barbitone by the administration, as sole drinking fluid, of aqueous barbitone sodium, containing 0·02% (w/v) saccharin to reduce the bitter taste. The dose administered was 100 mg/kg/day during the 1st week, and was increased at weekly intervals by 100 mg/kg/day to a maximum intake of 400 mg/kg/day. After 32 days of barbitone administration, withdrawal was effected by replacing the drug solution by saccharin solution. During the whole of the experimental period, control animals had 0·02% saccharin solution as sole drinking fluid. Saccharin administration alone was found to have no effect on the duration of hexobarbitone anaesthesia nor on the hepatic oxidation of hexobarbitone assayed *in vitro*. Measurement of hexobarbitone sleeping time and of hepatic drug metabolising activity was carried out on the 4th, 19th and 32nd days of barbitone treatment and on the 2nd, 6th and 22nd days after withdrawal.

Phenobarbitone treatment of withdrawn animals

In a few experiments on 4-month withdrawn animals, re-stimulation of hepatic drug-metabolising enzyme activity was attempted. Withdrawn and control animals were injected twice daily with phenobarbitone (60 mg/kg i.p.) for 4 days, and sleeping time and metabolism assays carried out on day 5.

Determination of hexobarbitone sleeping time

Sleeping time was measured as the time interval between intraperitoneal injection of hexobarbitone and restoration of the righting reflex, the same animals being used on each of the 6 assay days. Two dose levels of hexobarbitone were used—150 mg/kg in barbitone-treated animals and in one group of controls, and 100 mg/kg in a second group of control rats.

Animals used in the assay of liver enzyme activity and liver glycogen and nitrogen content were similarly dosed with 150 mg/kg hexobarbitone, and on the day of assay had received the same number of injections of hexobarbitone as those to be used in the sleeping time determination.

Determination of hepatic drug-metabolising enzyme activity

For *in vitro* metabolism experiments, rats were killed by stunning and subsequent cervical dislocation, the liver being rapidly excised and transferred to ice-cold isotonic KCl. 30% (w/v) liver homogenates were prepared (by homogenisation for 30 sec at 14,000 rpm) in an MSE homogeniser, and aliquots then spun at 0–4° for 30 min at 9000 *g*.

UDP-glucuronyltransferase activity in whole liver homogenates was determined by the method of Dutton and Storey[8] with *o*-aminophenol as substrate, 0·1 ml of 10% (w/v) homogenate being used in a total incubation volume of 0·6 ml. The other pathways studied were side-chain oxidation of hexobarbitone, *N*-dealkylation of aminopyrine and azoreduction of prontosil, the substrate concentrations being $3{\cdot}1 \times 10^{-4}$ M, $3{\cdot}5 \times 10^{-6}$ M and $6{\cdot}9 \times 10^{-5}$ M respectively. In each case, 0·5 ml 9000 *g* supernatant was used in a total incubation volume of 2·5 ml, containing cofactors in the concentrations described by McLuen and Fouts.[9] The metabolism of hexobarbitone was followed by measuring the disappearance of substrate,[10] aminopyrine dealkylation by estimation of the 4-aminoantipyrine formed[11] and prontosil reduction by determination of the reduction product, sulphanilamide.[12]

Determination of liver glycogen and nitrogen

Total nitrogen was measured by a micro-Kjeldahl technique[13] after digestion of 0·5 ml 10% (w/v) liver homogenate in 2·0 ml conc. sulphuric acid, in the presence of a copper-selenium catalyst.

For glycogen analysis, 1 ml of 10% (w/v) liver homogenate was digested in 2·0 ml 30% (w/v) KOH at 100° for 15 min. After precipitation with 4·0 ml ethyl alcohol, the glycogen was hydrolysed by boiling with 2·0 ml 2 N H_2SO_4 for 2 hr. An aliquot of the hydrolysate was assayed for glucose by the glucose-oxidase method[14] with the modification that after allowing the oxidation to proceed for 10 min, the reaction was stopped by adding 5 ml 9*N* H_2SO_4 and absorption at 546 mμ determined.

RESULTS

In confirmation of previous observations[6, 7] physical dependence on barbitone was found to have developed during the course of barbitone administration. This was indicated, on stopping drug treatment after 32 days, by the occurrence of a characteristic withdrawal syndrome accompanied by a dramatic fall in body weight.

The effect of barbitone administration and withdrawal on total body and liver weights is shown in Table 1. As previously found[7], the growth rate of barbitone-treated rats was greater than that of control animals, the extra weight gained being rapidly lost on withdrawal of the barbitone. Liver weight was also increased by the drug treatment but to a greater degree than the body weight, such that the liver weight/body weight ratio was significantly higher in rats receiving barbitone. After withdrawal, there was a decrease in liver weight, with a corresponding fall in the liver weight/body weight ratio to normal levels.

The results in Table 2 indicate that total liver nitrogen content per unit weight was unaffected by drug treatment and subsequent withdrawal. Liver glycogen content was also unaltered by barbitone treatment but, 2 days after withdrawal, was found to have decreased by 60 per cent, the level then gradually returning to normal.

The effect of barbitone treatment and withdrawal on hexobarbitone sleeping time and on the ability of liver preparations to oxidise hexobarbitone *in vitro*, is shown in Fig. 1. It is apparent that by the 4th day of barbitone treatment considerable tolerance to hexobarbitone had developed. There was an appreciable decrease in the duration of hexobarbitone anaesthesia, the sleeping time of barbitone-treated animals

TABLE 1. THE EFFECT OF BARBITONE ADMINISTRATION AND WITHDRAWAL ON BODY WEIGHT, LIVER WEIGHT AND LIVER WEIGHT/BODY WEIGHT RATIO OF FEMALE RATS

Days of barbiturate habituation	Body wt. (g) Control	Body wt. (g) Treated	Liver wt. (g) Control	Liver wt. (g) Treated	Liver Wt./body wt. Control	Liver Wt./body wt. Treated
4	74 ± 7	81 ± 7	3·5 ± 0·6	4·1 ± 0·6	0·0467 ± 0·0055	0·0508 ± 0·0045
19	135 ± 9	163 ± 7‡	5·9 ± 0·5	9·7 ± 0·7‡	0·0441 ± 0·0026	0·0594 ± 0·0028‡
32	166 ± 8	192 ± 5‡	6·8 ± 0·3	9·7 ± 0·3‡	0·0409 ± 0·0023	0·0506 ± 0·0022‡
Days after withdrawal						
2	177 ± 17	172 ± 6	7·3 ± 0·9	6·4 ± 0·4*	0·0412 ± 0·0027	0·0373 ± 0·0026*
6	173 ± 13	179 ± 9	7·5 ± 0·9	7·2 ± 0.7	0·0434 ± 0·0033	0·0403 ± 0·0035
22	200 ± 11	226 ± 10†	7·7 ± 0·7	8·4 ± 0·2*	0·0385 ± 0·0022	0·0373 ± 0·0011

Figures denote mean ± S.D. of six observations. For details of drug administration and withdrawal, see Methods.

* Results significant at $P < 0·05$.

† Results significant at $P < 0·01$.

‡ Results significant at $P < 0·001$.

All other results not signficant at the 0·05 level.

TABLE 2. THE EFFECT OF BARBITONE ADMINISTRATION AND WITHDRAWAL ON LIVER GLYCOGEN AND NITROGEN CONTENT OF FEMALE RATS

Days of barbiturate habituation	Glycogen mg/g wet wt. liver Control	Glycogen mg/g wet wt. liver Treated	Nitrogen mg/g wet wt. liver Control	Nitrogen mg/g wet wt. liver Treated
4	50 ± 5	49 ± 4	25 ± 5	25 ± 3
19	57 ± 6	53 ± 10	30 ± 1	28 ± 2
32	47 ± 4	53 ± 3	32 ± 2	32 ± 3
Days after Withdrawal				
2	55 ± 6	22 ± 10*	31 ± 2	31 ± 2
6	57 ± 3	48 ± 6	21 ± 1	21 ± 1
22	52 ± 8	50 ± 15	28 ± 3	28 ± 2

Figures denote mean ± S.D. of 3 observations. For details of drug administration and withdrawal and glycogen and nitrogen analyses see Methods. Liver extracts were prepared from pairs of animals and estimated in duplicate.

* Results significant at $P < 0·01$.

following i.p. injection of hexobarbitone (150 mg/kg) being significantly less than that of similarly injected control rats. The extent of tolerance developed is further emphasised by the results (Fig. 1) showing the sleeping time of treated animals to be less than that of control animals given only 100 mg/kg hexobarbitone. More prolonged treatment, for up to 32 days, produced no further alteration in hexobarbitone response, and even in rats tested 2 days after withdrawal, the sleeping time was the same

as that in 4-day treated animals. At 6 days after withdrawal, the sleeping time had increased to a level higher than that in control animals given 100 mg/kg hexobarbitone, but was not significantly different from that in control animals given the higher dose of hexobarbitone. When tested 22 days after withdrawal however, the animals were found to be hypersensitive to hexobarbitone, the sleeping time being considerably longer than in control rats.

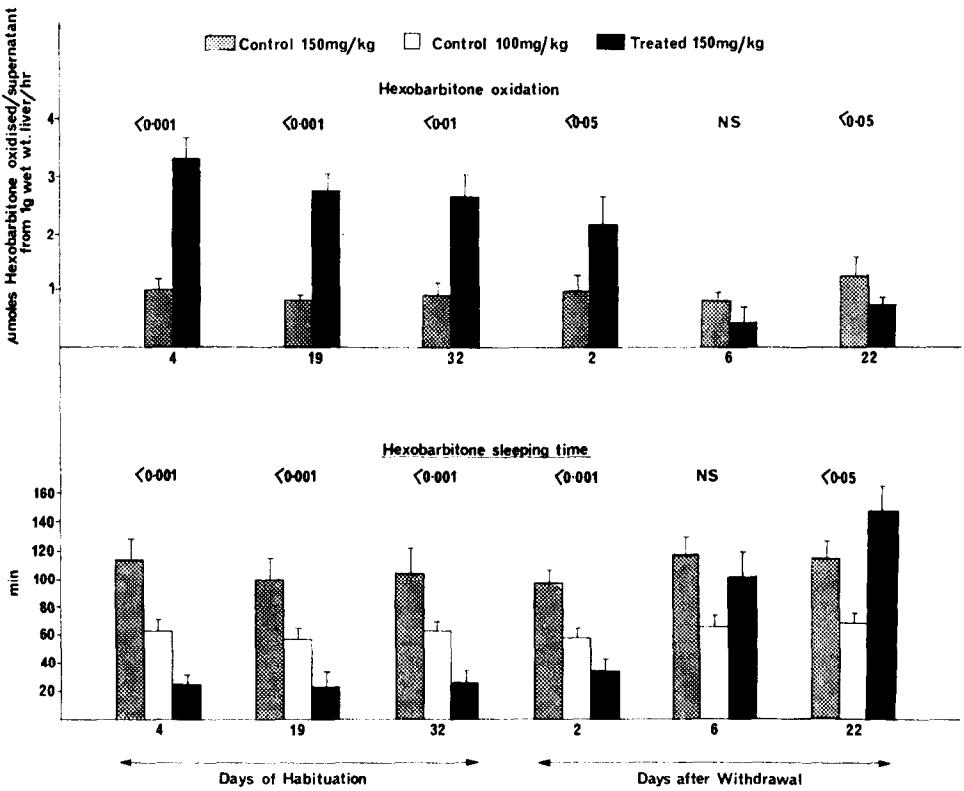

FIG. 1. The effect of chronic barbitone administration and withdrawal on hepatic oxidation of hexobarbitone *in vitro* and duration of hexobarbitone anaesthesia.

The values given are means ± S.D. For the estimation of hexobarbitone oxidation, groups of 6 animals were used, the activity being estimated in duplicate on 3 paired 9000 *g* supernatants. For sleeping time determination, groups of 12 animals were used, the dose of hexobarbitone being as indicated in the key to the figure. For details of barbitone habituation and withdrawal see Methods. In all instances the levels of significance shown refer to that between treated animals and controls given 150 mg/kg hexobarbitone.

Changes in the ability of 9000 *g* liver supernatants from treated animals to oxidise hexobarbitone *in vitro* complemented alterations in hexobarbitone sleeping time. Thus the activity was markedly increased after 4 days' treatment and remained approximately at this level until 2 days after withdrawal. Twenty-two days after withdrawal, the activity was significantly lower than in liver preparations from control rats (Fig. 1).

The same pattern of variation was found with the other three hepatic microsomal processes studied (Fig. 2). Thus livers from barbitone-treated animals had enhanced ability to reduce prontosil and dealkylate aminopyrine, and had higher UDP-glucuronyltransferase activity, the increase in transferase activity developing less rapidly

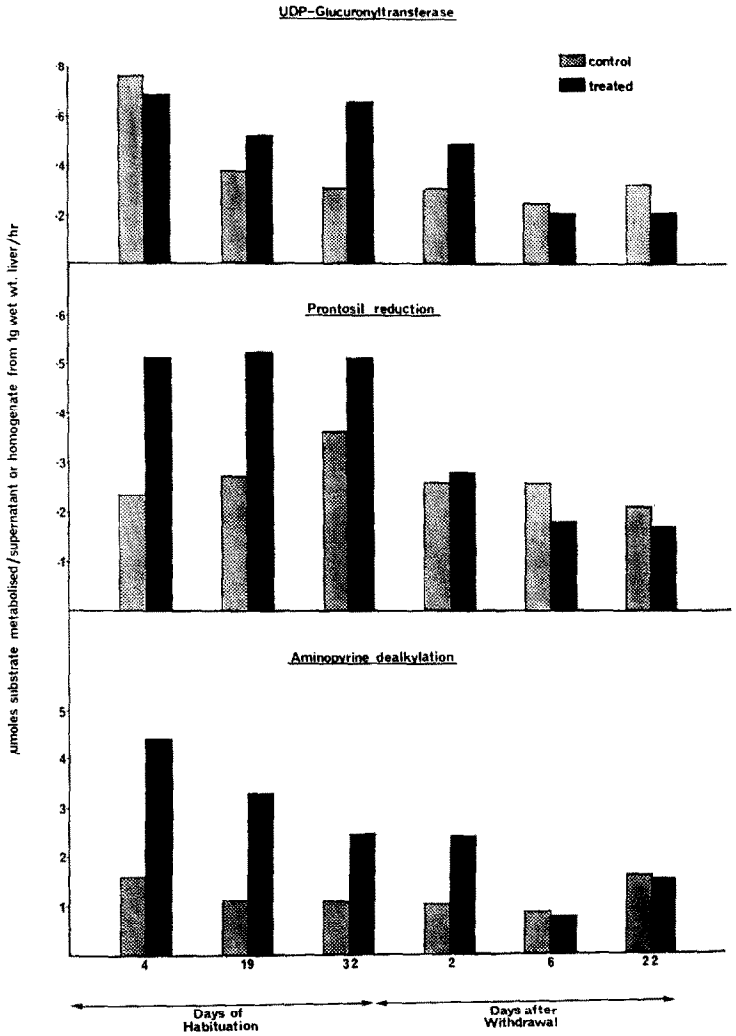

FIG. 2. The effect of chronic barbitone administration and withdrawal on liver microsomal drug-metabolising enzyme activity.

The values given are the means obtained from groups of 6 animals the activity being determined in duplicate on 3 paired liver preparations. For details of barbitone habituation and withdrawal see Methods.

however. Twenty-two days after withdrawal, the activity of all three metabolic processes appeared to be lower than in control animals.

A few results, obtained using animals withdrawn for 4 months, are shown in Table 3. It is apparent from the sleeping times that these withdrawn animals are still

hypersensitive to hexobarbitone. In addition, hexobarbitone oxidation and UDP-glucuronyltransferase activity measured *in vitro* were below normal. Considerable stimulation of these microsomal processes in the withdrawn rats could be brought about by phenobarbitone treatment (Table 3). The levels of activity attained however, appeared to be below that in phenobarbitone-treated control animals.

TABLE 3. HEPATIC DRUG-METABOLISING ACTIVITY AND DURATION OF HEXOBARBITONE ANAESTHESIA IN CONTROL AND 4-MONTH WITHDRAWN RATS—THE EFFECT OF PHENOBARBITONE TREATMENT

Animal	μ moles substrate metabolised/supernatant or homogenate from 1 g wet wt. liver/60 min				Hexobarbitone sleeping time (min)
	Hexobarbitone oxidation		UDP-glucuronyl transferase		
	A	B	A	B	A
Control	0·93(3)	0·72(3)	0·28(3)	0·32(3)	47 ± 9(6)
Withdrawn	0·59(4)	0·35(3)	0·26(3)	0·18(3)	62 ± 5(6)*
Phenobarbitone-treated control	—	2·70(4)	0·48(3)	0·50(4)	—
Phenobarbitone-treated withdrawn	—	0·91(4)	0·45(3)	0·43(4)	—

The values given are means with the number of animals used in parentheses. In the case of sleeping time, the S.D. is also given. Results from two series of experiments A and B are reported, the phenobarbitone treatment being as described in Methods. Hexobarbitone (100 mg/kg i.p.) was used for determination of sleeping time.

* Results significant at $P < 0.01$.

DISCUSSION

Crossland and Leonard[6] were the first to describe physical dependence on barbiturate in rats. Their method, involving the administration of increasing amounts of barbitone in drinking water over a period of weeks, enables large numbers of animals to be treated. That physical dependence had developed was indicated by the occurrence of a withdrawal syndrome on cessation of barbitone treatment. The withdrawal was characterised by hyperexcitability, susceptibility to audiogenic seizures, and a decrease in convulsant threshold to leptazol, picrotoxin and bemigride[6,7]. A barbitone habituation period of four weeks was found[7] to be necessary for physical dependence to develop to the extent that withdrawal produced susceptibility to seizures in all animals.

In contrast, it is apparent from the present work (Fig. 1) that tolerance, as measured by a decreased hexobarbitone sleeping time, has completely developed within 4 days of beginning barbiturate administration. The results show in addition that the decreased effect of hexobarbitone is associated with elevated liver microsomal drug-metabolising activity as assayed *in vitro*, both with hexobarbitone and with other substrates (Fig. 2). The hexobarbitone tolerance resulting, in the present experiments, from long term barbitone administration is adequately explained, therefore, in terms of stimulation of liver microsomal drug-metabolising enzymes and is of the type often referred to as metabolic tolerance.

The hexobarbitone sleeping time is the same on day 4 of the barbitone habituation period, at a time when there is no evidence of physical dependence, as it is on day 32,

by which time dependence has developed. The determination of hexobarbitone sleeping times under these conditions therefore provides no information as to the degree of barbitone dependence which has developed, the only indication being the withdrawal syndrome on stopping barbitone administration. The part played by central nervous adaptation in the development of tolerance to hexobarbitone in barbitone-dependent rats would appear from these results to be of minor importance. In the determination of sleeping time, however, no allowance was made for barbitone present in the tissues of treated animals. The extent to which this factor may have influenced the results is uncertain. The effect of chronic barbitone treatment therefore differs from that produced by morphine or codeine. The continuous administration of the latter drugs has been reported to lead to tolerance to their analgesic effect, presumably occurring through a central nervous adaptation, since such treatment is known to produce inhibition of hepatic drug-metabolising activity[15, 16].

The hypersensitivity to hexobarbitone found some three weeks after withdrawal was associated with a decreased rate of hexobarbitone oxidation (Fig. 1). Several workers have reported that the activity of hepatic drug-metabolising enzymes returns to normal levels following the chronic administration of barbiturates[2, 3]. Aston has demonstrated a latent hypersensitivity to pentobarbitone and barbitone, which was not associated with reduced metabolism.[17, 18] The data in Figs. 1 and 2 shows that reduction of hepatic drug-metabolising enzyme activity occurs after barbitone withdrawal, but the possibility of a simultaneous hypersensitivity of the central nervous system is not ruled out. It is interesting to note in particular that the ability of liver preparations from withdrawn rats to form glucuronides was impaired (Fig. 2, Table 3). In support of these results, it has been reported[19] that at some time after their withdrawal from glutethimide, human patients may exhibit an impaired ability to conjugate this drug as a glucuronide. With barbitone-withdrawn rats, the impaired microsomal metabolism appeared to be a long-lasting effect, being demonstrable as long as four months after withdrawal (Table 3). Phenobarbitone treatment stimulated microsomal drug-metabolising enzyme activity in the withdrawn animals but to a lower level than in phenobarbitone-treated control rats. Together, these results may reflect possible liver damage resulting either from administration of barbitone in such large amounts or from its sudden withdrawal. Certainly, in rats given lower doses of barbiturate, hepatic drug-metabolising enzyme activity falls to normal on cessation of drug administration.[3] A species difference may also operate, since in both man[20] and the dog[21] microsomal enzyme activity may remain elevated for a considerable period after stopping administration of the stimulatory agent.

All four microsomal pathways studied, hexobarbitone oxidation, aminopyrine dealkylation, prontosil reduction and the conjugation of o-aminophenol, showed the same pattern of variation throughout barbitone administration and withdrawal. The finding that stimulation of UDP-glucuronyltransferase by barbitone administration occurred only after two weeks' treatment is consistent with a previous report that acute injection of barbitone does not elevate liver transferase activity.[22]

As with many agents[1] stimulating liver drug-metabolising enzymes, barbitone administration increased the liver weight/body weight ratio (Table 1). While no change was detectable in liver nitrogen content throughout the experiment, 2 days after withdrawal the liver glycogen content was markedly reduced (Table 2). The observation (Figs. 1 and 2), that hepatic drug-metabolising enzyme activity at this time was not

appreciably different from the elevated levels attained during barbitone administration, adds to previous evidence that glycogen depletion is not always associated with altered microsomal enzyme activity.[23] The fall in liver glycogen content occurred along with a considerable body weight loss in the treated animals (Table 1). Both changes are possibly related to the marked reduction in food intake shown[7] to result from barbitone withdrawal.

The present work demonstrates that chronic barbitone administration and withdrawal in rats results in appreciable change, both in hexobarbitone susceptibility and in the activity of several hepatic microsomal drug-metabolising enzymes. Of particular interest is the reduced microsomal enzyme activity following withdrawal. Increased rate of drug metabolism during drug administration is known to occur in man and may influence both drug dosage and the course of subsequent therapy.[1] The present results suggest that consideration ought also to be given to the possible effect on drug metabolism of the withdrawal of a chronically administered drug.

It is perhaps surprising that the development of physical dependence was not accompanied by obvious change in the duration of hexobarbitone response and that measurement of the latter provided no evidence of central nervous tolerance. Present experiments, to investigate the relationship between hexobarbitone response and brain barbitone and hexobarbitone levels in barbitone-treated and withdrawn rats, may provide information on the mechanism of barbitone dependence.

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