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THE EFFECT OF PROLONGED SODIUM PHENOBARBITONE TREATMENT ON HEPATIC XENOBIOTIC METABOLISM AND THE URINARY EXCRETION OF METABOLITES OF THE D-GLUCURONIC ACID PATHWAY IN THE RAT

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Abstract—Male Sprague–Dawley rats were fed a 0.1% (w/w) sodium phenobarbitone (PB) diet for periods of 4, 12 and 24 weeks. Phenobarbitone treatment resulted in a marked increase in liver size which was accompanied by the induction of several parameters of hepatic xenobiotic metabolism including mixed function oxidase enzyme activities, UDP-glucuronyltransferase, cytochrome P450 and the microsomal protein content. In addition, treatment with the barbiturate led to the stimulation of the urinary excretion of D-glucaric acid, L-gulonic acid, free D-glucuronic acid, L-ascorbic acid and xylitol. The levels of stimulation of both the hepatic and urinary parameters remained relatively constant throughout the period of treatment. On cessation of PB treatment the parameters of hepatic xenobiotic metabolism reverted to control levels at a faster rate than the excretion of the D-glucuronic acid metabolites. The results demonstrate a correlation between the urinary excretion of metabolites of the D-glucuronic acid pathway and hepatic xenobiotic metabolizing enzyme activities during the prolonged administration of PB.

A multitude of chemicals of diverse structure have been shown to elicit the induction of hepatic xenobiotic metabolism both in experimental animals and in man [1, 2]. Furthermore, the induction of hepatic microsomal mixed function oxidase enzymes is often accompanied by the stimulation of enzymes of the D-glucuronic acid pathway [3, 4] resulting in the enhanced urinary excretion of metabolites of this pathway such as D-glucaric acid [5]. It has been suggested that the measurement of the urinary excretion of D-glucaric acid and other D-glucuronic acid metabolites may provide an index of the activity of the hepatic microsomal cytochrome P450 dependent mixed function oxidase complex [6–10]. For example, we were able to correlate the induction of hepatic xenobiotic metabolism in the rat by a number of foreign compounds with the stimulation of the combined urinary excretion of D-glucaric acid, free D-glucuronic acid, L-gulonic acid and xylitol [11].

Although a number of animal studies have been performed on the stimulation of the hepatic D-glucuronic acid pathway by foreign compounds [3, 4, 6, 8, 11–15], the agents employed have normally been administered over relatively short time periods. In this paper we have studied the effect of prolonged administration of a mixed function oxidase enzyme inducing agent on the urinary excretion of some D-glucuronic acid metabolites. Sodium phenobarbitone (PB) was selected as the inducing agent as the continued administration of this barbiturate to rats is known to result in the prolonged stimulation of hepatic xenobiotic metabolism [16]. Furthermore, PB also stimulates hepatic xenobiotic metabolizing enzymes [17] and urinary D-glucaric acid excretion [17, 18] in man and is used in the long term treatment of human epilepsy [19].

MATERIALS AND METHODS

Chemicals. NADP, DL-isocitric acid, isocitrate dehydrogenase (EC 1.1.1.42, from pig heart), *Tris*, 4-methylumbelliferyl-β-D-glucuronide and β-glucuronidase (EC 3.2.1.31, from bovine liver) were purchased from the Sigma Chemical Co., U.K. PB (laboratory reagent grade) was obtained from BDH Chemicals, Poole, and 4-methylumbelliferone from Koch–Light Lab. Ltd., Colnbrook, U.K. All other reagents were of the highest purity available.

Animals and treatment. Male Sprague–Dawley rats (40–60 g) were purchased from OLAC (Laboratory animal Centre Accredited) 1976 Ltd., Blackthorn, U.K. and were caged in groups of six in accommodation maintained at 20 ± 1° with a relative humidity of 50 per cent. They were allowed free access to Spratts' (Barking, U.K.) Laboratory Diet 1 containing 1% (w/w) maize oil and water. After acclimatizing to the experimental regime for 10 days the test animals were then fed the basic diet containing 0.1% (w/w) PB for periods of up to 24 weeks. The body wt gain, food and water consumption of both control and barbiturate treated rats were determined at either 1 or 2-week intervals (see Fig. 1).

Hepatic studies. Rats were killed by cervical dislocation and the livers immediately excised into ice-cold 0.154 M KCl containing 50 mM Tris–HCl buffer, pH 7.4. All subsequent operations were performed at 0–4°. Whole liver homogenates (0.25 g

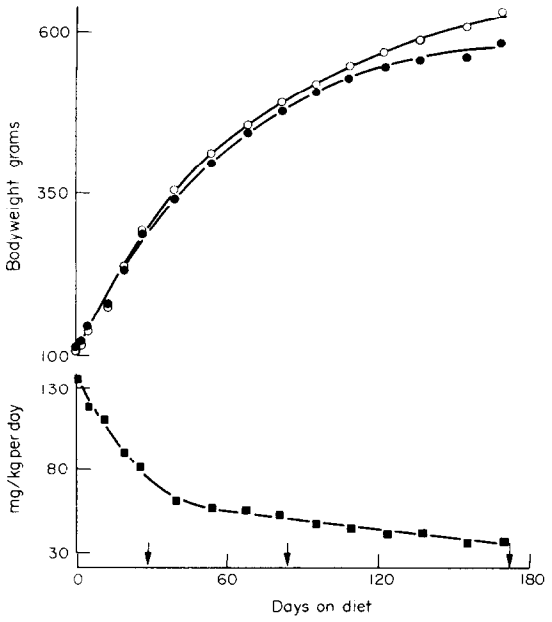

Fig. 1. Upper plot, the effect of sodium phenobarbitone treatment on the body wt gain of male rats. Each point represents the mean of 12–30 animals fed either the control diet (○) or a 0.1% (w/w) sodium phenobarbitone diet (●). Lower plot, the daily sodium phenobarbitone intake (■) of rats fed the barbiturate diet. The determinations of the hepatic and urinary parameters after 4, 12 and 24 weeks of treatment are indicated by arrows.

fresh tissue/ml) were prepared employing a Potter-type, Teflon–glass, motor-driven homgenizer (A. J. Thomas and Co., Philadelphia, PA, U.S.A.). Homogenates were centrifuged at 10,000 *g* average for 20 min and the postmitochondrial supernatant fractions used for the determinations of ethylmorphine N-demethylase [20], biphenyl 4-hydroxylase [21], aniline 4-hydroxylase [22] and 4-methylumbelliferyl glucuronyltransferase [23]. Portions of the 10,000 *g* average supernatant fractions were further centrifuged at 105,000 *g* average for 60 min to sediment the microsomal fractions which were assayed for cytochrome P450 [24] and protein [25] contents.

Hexobarbitone sleeping time. The duration of sodium hexobarbitone anaesthesia was determined following a 100 mg/kg i.p. dose of the barbiturate. The time taken between the loss of and the recovery of the righting reflex was taken as a measure of the hexobarbitone induced sleeping time.

Urine analysis. For the collection of 24 hr urine samples animals were caged singly in all-glass "Metabowls" (Jencons Ltd., Hemel Hempstead Herts, U.K.). A small quantity of NaF was added as a preservative to the urine collection flasks. The urinary concentrations of D-glucaric acid, L-gulonic acid, free (unconjugated) D-glucuronic acid, L-ascorbic acid and xylitol were determined by a gas chromatographic procedure previously described [11].

RESULTS

The administration of a 0.1% PB diet for 24 weeks had no significant effect on the body wt gain of the barbiturate treated animals compared to the controls (Fig. 1). In addition, there were no differences in either food or water consumption between the control and treated groups (data not shown). The mean daily intake of PB over the experimental period was 66 mg/kg/day, but this ranged from 34 to 137 mg/kg/day depending on the age of the animals (Fig. 1).

Hepatic effects. PB administration for periods of 4,

Table 1. Effect of treatment with PB on some parameters of rat hepatic xenobiotic metabolism

Parameter	Control activity	Week on 0.1% (w/w) PB diet*				
		4 weeks	12 weeks	24 weeks	20 weeks control diet then 4 weeks of feeding	4 weeks reversal after 24 weeks of feeding*
Relative liver wt (g liver/100 g body wt)	2.7–4.4†	140‡	145‡	150‡	145‡	105
Microsomal protein content (mg protein/g of liver)	30	140‡	120§	120§	135‡	95
Cytochrome P450 content (ΔE 450–490 nm/g of liver)	3.9	355‡	235‡	200‡	230‡	110
Ethylmorphine *N*-demethylase (μmoles product/hr/g of liver)	27	240‡	215‡	230‡	285‡	130§
Biphenyl 4-hydroxylase (μmoles product/hr/g of liver)	3.8	255‡	215‡	260‡	275‡	105
Aniline 4-hydroxylase (μmoles product/hr/g of liver)	2.3	240‡	185‡	190‡	195‡	105
4-Methylumbelliferyl glucuronyl-transferase (μmoles product/hr/g of liver)	94	570‡	375‡	310‡	300‡	95

* All results are expressed as percentages of control values, each group comprising six control and six PB-treated animals.

† The relative liver wt is a function of age and decrease as the animal matures.

‡ Significantly different (Student's '*t*' test) from control: $P<0.01$.

§ Significantly different (Student's '*t*' test) from control: $P<0.05$.

Table 2. Effect of treatment with PB on hexobarbitone sleeping time in male rats

Week of treatment	Sleeping time, min* Control diet	0.1% (w/w) PB diet	Per cent of control
4	12.7±1.9†	4.3±0.7‡	34
12	19.7±3.3	7.8±1.3§	40
24	29.5±2.5	9.5±1.0‡	32
3 weeks reversal after 24 weeks of feeding	25.7±1.9	21.7±1.9	85

* Sodium hexobarbitone (100 mg/kg) was administered by i.p. injection and the time taken between the loss of and the recovery of the righting reflex was taken as a measure of the hexobarbitone induced sleeping time.

† All results are expressed as the mean±S.E.M. of six animals.

‡ Significantly different (Student's '*t*' test) from control: $P<0.001$.

§ Significantly different (Student's '*t*' test) from control: $P<0.01$.

12 or 24 weeks markedly stimulated the activities of all the parameters of hepatic xenobiotic metabolism measured (Table 1). Whilst the increases in liver size and mixed function oxidase enzyme activities remained relatively constant from 4 to 24 weeks the stimulation of UDP-glucuronyltransferase activity and the microsomal contents of cytochrome P450 and protein were highest at week 4 of treatment, when the daily intake of the barbiturate was greater than at either 12 or 24 weeks (Fig. 1). The induction of hepatic xenobiotic metabolism *in vivo* by PB treatment was confirmed by measurement of the hexobarbitone induced sleeping times in control and barbiturate treated rats. PB administration for 4–24 weeks markedly reduced the hexobarbitone sleeping time of the treated animals to 32–40 per cent of the controls (Table 2).

To determine if the magnitude of induction of hepatic xenobiotic metabolism by PB was similar in young and in mature animals a group of rats was fed the control diet for 20 weeks and subsequently the barbiturate diet for 4 weeks. The degree of induction of all the parameters of hepatic xenobiotic metabiolism measured was found to be comparable in animals of the same age fed the barbiturate diet for either 4 or 24 weeks (Table 1).

To determine whether the induction of hepatic xenobiotic metabolism by PB treatment was reversible a group of rats was fed the barbiturate diet for 24 weeks and subsequently the control diet for 4 weeks. With the exception of the activity of ethylmorphine *N*-demethylase, which was still elevated to 130 per cent of control levels, the activities of all the other parameters measured had returned to control levels by 4 weeks after the cessation of PB treatment (Table 1). Furthermore, the hexobarbitone induced sleeping times of control and PB treated rats were not significantly different after 3 weeks of feeding both groups the control diet (Table 2).

Urinary studies. PB treatment markedly stimulated the urinary excretion of all five metabolites of the D-glucuronic acid pathway measured (Table 3). The induction of excretion of D-glucaric acid, free D-glucuronic acid and xylitol was fairly constant throughout the period of PB administration. However, the induction of L-gulonic acid excretion greatly exceeded that of any of the other urinary parameters measured and reached peak levels at 4 weeks of treatment. In contrast to the other urinary parameters measured the excretion of L-ascorbic acid was not significantly elevated after 4 weeks of PB administration. As a measure of the overall stimu-

Table 3. Effect of treatment with PB on the urinary excretion of some metabolites of the D-glucuronic acid pathway

Metabolite	Control excretion†	Week on 0.1% (w/w) PB diet* 4 Weeks	12 Weeks	24 Weeks	20 weeks control diet then 4 weeks of feeding	4 weeks reversal after 24 weeks of feeding*
D-Glucaric acid	0.8	200‡	240‡	295‡	250‡	90
Free D-glucuronic acid	3.0	235‡	250‡	250‡	355‡	205‡
L-Gulonic acid	1.4	2900‡	1250‡	1600‡	1500‡	255‡
Xylitol	0.6	260‡	240‡	285‡	380‡	95
L-Ascorbic acid	5.3	130	335‡	225‡	225‡	155‡
Totals§	5.8	340‡	610‡	490‡	540‡	195‡

* All results are expressed as percentages of control values, each group comprising six control and six PB-treated animals.

† In units of mg metabolite(s) per rat per 24 hr urine.

‡ Significantly different (Student's '*t*' test) from control: $P<0.05$.

§ The combined urinary excretion of D-glucaric acid, free D-glucuronic acid, L-gulonic acid and xylitol.

lation of the D-glucuronic acid pathway, the combined urinary excretion of D-glucaric acid, free D-glucuronic acid, L-gulonic acid and xylitol was significantly enhanced to 340–610 per cent of control levels by PB treatment for 4–24 weeks (Table 3).

In the study on PB induction in young and in mature animals the levels of urinary excretion of D-glucaric acid, L-gulonic acid, L-ascorbic acid and the combined excretion of all metabolites except L-ascorbic acid were generally similar in the animals fed the barbiturate diet for either 4 or 24 weeks (Table 3). However, the urinary excretion of both free D-gluconic acid and xylitol were somewhat higher in the mature animals fed the PB diet for only 4 weeks.

In contrast to the hepatic parameters (Table 1), the urinary levels of free D-glucuronic acid, L-gulonic acid and L-ascorbic acid were still significantly enhanced 4 weeks after the withdrawal of the barbiturate diet (Table 3). The prolonged induction of some urinary markers was also reflected in the combined excretion of D-glucaric acid, free D-glucuronic acid, L-gulonic acid and xylitol, which was still almost twice that of the controls. However, a partial reversal of the effects of PB on the hepatic D-glucuronic acid pathway was apparent as the levels of all urinary parameters were lower 4 weeks after the cessation of PB treatment than after 24 weeks of feeding the barbiturate diet (Table 3).

DISCUSSION

This report demonstrates that the prolonged administration of PB to rats results in a marked and sustained stimulation of both hepatic xenobiotic metabolism and the urinary excretion of some metabolites of the D-glucuronic acid pathway. In addition, PB treatment was found to have a similar inductive effect in studies with either young or mature rats. Thus, PB was equally effective in stimulating both the hepatic and urinary indices when administered to animals either for a period of 24 weeks or for just the last 4 weeks of treatment. As in our previous short-term studies on the induction of hepatic xenobiotic metabolism in the rat [11], the measurement of a spectrum of D-glucuronic acid metabolites provides a more sensitive index of induction than the estimation of only a single metabolite. For example, the stimulation of the combined urinary excretion of D-glucaric acid, free D-glucuronic acid, L-gulonic acid and xylitol always exceeded that of D-glucaric acid alone.

In the reversibility study it was apparent that the enzymes of the D-glucuronic acid pathway reverted more slowly to control levels than those of the mixed function oxidase complex, as the urinary excretion of several D-glucuronic acid metabolites was still elevated 4 weeks after the cessation of PB treatment. A similar observation was made by Hanninen *et al.* [26] who studied the effect of short term PB treatment on the hepatic levels of cytochrome P450 and D-glucuronalactone dehydrogenase (EC 1.1.1.70). Although the levels of both parameters were induced by PB, the activity of the D-glucuronic acid pathway enzyme returned to control levels at a much slower rate than that of cytochrome P450. These observations would suggest that either the enzymes of the D-glucuronic acid pathway have considerably longer half-lives than those of the mixed function oxidase complex or that the two pathways are under different regulatory mechanisms with respect to their response to foreign compounds. In addition, these findings would indicate that in order to ensure that the measurement of urinary D-glucuronic acid metabolites accurately reflects the stimulation of hepatic xenobiotic metabolism, determinations of the urinary parameters should at least be made at two time intervals. The results obtained would thus provide information on the state of hepatic enzyme induction, i.e. whether the induction of enzyme activities was either increasing, sustained or undergoing reversal.

In conclusion, the present findings with PB lend support to the use of the urinary measurement of a spectrum of D-glucuronic acid metabolites as an index of induction of hepatic xenobiotic metabolism in the rat. Furthermore, as PB is often used in the treatment of human epilepsy [19], the present observations in the rat may serve as a basis on which to assess hepatic microsomal enzyme activities in patients receiving PB and other anticonvulsant drugs.

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