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THE EFFECT OF ORAL PHENOBARBITONE ON HEPATIC MICROSOMAL CYTOCHROME P-450 AND DEMETHYLATION ACTIVITY IN RATS FED NORMAL AND LOW PROTEIN DIETS

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Abstract—The use of oral phenobarbitone to stimulate microsomal hydroxylation is described. A 0·1% solution of sodium phenobarbitone instead of normal drinking water causes maximal stimulation of enzyme activity and an increase in cytochrome P-450, of 7-fold over controls; there are also increases in liver weight and microsomal protein.

The time course of this stimulation is found to be similar to that after injection of phenobarbitone.

Feeding a 3% casein diet causes a fall in cytochrome P-450. When rats fed a low protein diet are given phenobarbitone, the level of P-450 rises, but is still only a quarter of that found in phenobarbitone treated rats given a stock diet.

IT IS well established that treatment of animals with phenobarbitone causes a massive increase in the activity of hepatic microsomal drug-metabolising (hydroxylating) enzymes.[1,2] Microsomal cytochrome P-450, which is involved in oxygen activation and substrate binding in these enzyme systems, is also increased.[3,4]

In most of the experiments in which phenobarbitone has been used to stimulate drug metabolism in rats it has been given by intraperitoneal injection. The present paper describes a more convenient way of giving the drug, as a constituent of the drinking water, and presents data relating dose to response, and the level of stimulation achieved to the period of administration. The effects of a low protein diet on hepatic cytochrome P-450 and drug-metabolising enzymes are also discussed.

The effect of oral phenobarbitone on the mouse liver has been investigated by Kunz *et al.*,[15,16] and our findings are similar to theirs in the time course of phenobarbitone induced increase of liver weight.

A preliminary report of this work has already appeared.[5]

MATERIALS AND METHODS

Preparation of animals. Male Carworth rats were used throughout. Stock pellets or 3% casein diets[11] were given *ad libitum* up to the time of death. Drinking water was fully available to all animals, but for those receiving oral phenobarbitone an aqueous solution of the drug in tap water was the only source of water. Sodium phenobarbitone was purchased from British Drug Houses Ltd., (Poole, Dorset).

When phenobarbitone was given by i.p. injection, it was dissolved in 0·15 M sodium chloride. Regimes of drug administration were started between 10 and 11 a.m.

Preparation of tissue samples. Rats were killed by exsanguination from the carotid artery under ether anaesthesia. The livers were excised, rinsed in ice-cold isotonic saline, blotted dry and weighed. 2 g of liver was homogenised in 18 ml ice-cold 0·15 M potassium chloride for 8 sec using an Ultra-Turrax type TP 18/2 blendor (Janke & Kunkel K.G., Staufen i. Br., Germany.) Homogenates were centrifuged for 10 min at 9000 *g* in a refrigerated centrifuge. Part of the post-mitochondria supernatant was retained for measuring Pyramidon demethylating activity and the remainder was centrifuged at 105,000 *g* for 60 min at 4°. The supernatant was discarded and the microsomal pellet rinsed with distilled water. Lipid adhering to the walls of the centrifuge tubes was wiped off with tissue paper, and the microsomal pellet was resuspended in ice-cold 0·15 M potassium chloride using a glass homogeniser of the Dounce type (Blaessig Glass Specialities, 645 Atlantic Avenue, Rochester 9, New York.)

Assays in vitro. Cytochrome P-450 was measured by the method of Omura & Sato[6] on a Unicam SP 800 split beam recording spectrophotometer in conjunction with an external recorder. For this determination, microsomes were diluted with sodium phosphate buffer (final concentration 0·05 M, pH 7·4) such that 1 ml of the suspension contained the microsomes from 0·05 g of liver (1–2 mg protein/ml).

For the measurement of Pyramidon demethylation, 1 ml of the 10% post-mitochondrial supernatant was added to 2 ml of medium containing the following substances: Na_2HPO_4 buffer (made to pH 7·4 with HCl), 300 μmole; Na-isocitrate, 10 μmole; $MgCl_2$, 25 μmole; Pyramidon, 15 μmole; NADP, 1·5 μmole. The mixture was incubated at 37° in air for 20 min in a metabolic shaker. After the period of incubation the reaction was stopped by pouring into 1·5 ml of 18% trichloracetic acid, and 4-amino antipyrene was estimated by the method of La Du *et al.*[7] Microsomal protein was determined by the method of Lowry *et al.*[8]

RESULTS

Table 1 shows the changes in liver weight, hepatic microsomal protein and cytochrome P-450, produced by oral and injected phenobarbitone. There were striking increases in all three parameters, but the route of administration of the drug had no significant effect. The water intake for rats given either water or a 1 mg/ml solution of sodium phenobarbitone to drink was approximately 10 ml/100 g/rat/day (allowing a dose of 100 mg/kg/day). Rats fed 3% casein diet drank about 40 per cent less water.

Rats injected with 100 mg/kg sodium phenobarbitone slept for about 6 hr after the injections but were active during the night and gained weight at approximately the same rate as controls. Rats given phenobarbitone in the drinking water at a concentration of 1 mg/ml, were lethargic for the first 2 days, but also gained weight at approximately the same rate as controls during the 4 days of the experiment. In experiments on the behaviour of animals on the oral regime, exploratory activity, as measured in a Y-shaped maze,[9] was unaltered, even on the first day.*

Table 2 shows that increasing the concentration of the sodium phenobarbitone solutions from 0·01 mg/ml to 2 mg/ml increases the liver weight microsomal, protein,

* A.E.M. McLean unpublished observations.

cytochrome P-450 and Pyramidon demethylation observed after 14 days up to a maximum at 1 mg/ml. There was no significant difference in the growth rate of the rats in different groups in this experiment.

Table 3 shows the time course of the stimulatory effects observed. Stimulation is maximal after 7 days and thereafter a constant level is maintained. Withdrawal of phenobarbitone causes an abrupt decrease in the elevated levels, and control values are reached in 7 days.

As we have previously reported, feeding a 3% casein diet for 14 days reduces cytochrome P–450 to 33 per cent of control values.[5] Giving phenobarbitone in the drinking water at the same time leads to an increase in cytochrome P-450 although

TABLE 1. THE EFFECTS OF ORAL AND INJECTED PHENOBARBITONE ON LIVER WEIGHT, HEPATIC MICROSOMAL PROTEIN AND CYTOCHROME P-450

	n	Liver weight (% body wt.)	P-450 (mμ moles/g liver wet wt.)	Microsomal protein (mg/g liver wet wt.)
Controls	4	4·7 ± 0·3	19 ± 1	23 ± 3
Saline injected	4	4·8 ± 0·2	23 ± 4	26 ± 2
Oral phenobarbitone	8	6·1 ± 0·4	95 ± 9	42 ± 5
Injected phenobarbitone	8	6·0 ± 0·5	96 ± 21	41 ± 5

Results are expressed as means ± S.D.

Injected sodium phenobarbitone was given i.p. on 4 successive days at a dose of 100 mg/kg and the rats were killed 24 hr after the last injection. Equivalent volumes of saline were injected at the same time into the 'Saline' control group. Orally treated rats were given Na phenobarbitone 1 mg/ml in the drinking water for 96 hr before they were killed.

The rats weighed 200–300 g at death.

TABLE 2. CHANGES IN LIVER WEIGHT, HEPATIC MICROSOMAL PROTEIN, CYTOCHROME P-450 AND THE DEMETHYLATION OF PYRAMIDON IN RESPONSE TO VARYING DOSAGE OF ORAL PHENOBARBITONE

Na phenobarbitone concentration (mg/ml)	n	Liver weight (% body wt.)	P-450 (mμ moles/g liver wet wt.)	Microsomal protein (mg/g liver wet wt.)	Pyramidon demethylation (μmoles/g liver wet wt./hr)
2·0	4	—	*144 ± 21	*44 ± 2	—
1·0	5	*8·3 ± 0·7	*135 ± 23	*45 ± 4	*2·6 ± 0·6
0·5	5	—	*108 ± 25	*47 ± 4	—
0·1	5	*6·4 ± 0·3	*82 ± 21	*36 ± 2	*1·7 ± 0·1
0·03	5	5·6 ± 0.1	*40 ± 5	*36 ± 4	1·0 ± 0·1
0·01	5	5·4 ± 0·3	26 ± 7	30 ± 6	0·6 ± 0·1
0·00	5	5·3 ± 0·5	20 ± 3	26 ± 3	0·6 ± 0·1

Results are expressed as means ± S.D.

Groups marked * differ significantly from the control group at a level of $P < 0·02$ as obtained by Student's test.

Rats were given Na phenobarbitone in the drinking water for 14 days before death. Body weight at death was 160–200 g for all groups except the 2 mg/ml and 0·5 mg/ml which weighed 250–300 g. Liver weight for these two groups were 5·6 ± 0·4 and 5·5 ± 0·2 respectively, which are significantly different from the value for controls of comparable body weight (4·5 ± 0·4).

TABLE 3. CHANGES WITH TIME IN LIVER WEIGHT, HEPATIC MICROSOMAL PROTEIN AND CYTOCHROME P-450 DURING TREATMENT WITH PHENOBARBITONE IN THE DRINKING WATER

Time (days)	n	Liver weight (% body weight)	P-450 (mμ moles/g liver wet wt.)	Microsomal protein (mg/g liver wet wt)
0	32	4·5 ± 0·4	20 ± 3	26 ± 6
1	4	4·8 ± 0·6	26 ± 1	29 ± 2
2	4	4·9 ± 0·4	72 ± 6	35 ± 2
4	8	6·1 ± 0·4	95 ± 9	42 ± 5
7	4	6·3 ± 0·1	124 ± 5	43 ± 4
14	12	6·3 ± 0·6	129 ± 27	47 ± 6
21	8	5·9 ± 0·6	116 ± 18	47 ± 5
28	4	6·2 ± 0·2	127 ± 11	43 ± 3
Time off phenobarbitone (days)				
4	4	5·2 ± 0·1	66 ± 22	39 ± 2
7	4	3·8 ± 0·2	20 ± 7	29 ± 3
14	4	4·1 ± 0·4	16 ± 8	24 ± 5

Results are expressed as means ± S.D.

Rats of initial body weight 240–260 g were given Na phenobarbitone (1 mg/ml) in the drinking water. After 28 days, the remaining rats were given tap water to drink.

TABLE 4. THE EFFECT OF A 3% CASEIN DIET AND ORAL PHENOBARBITONE ON LIVER WEIGHT, HEPATIC MICROSOMAL PROTEIN, CYTOCHROME P-450 AND THE DEMETHYLATION OF PYRAMIDON

	n	Liver weight (% body wt.)	P-450 (mμ moles/g liver wet wt.)	Microsomal protein (mg/ liver wet wt.)	Pyramidon demethylation μmoles/g liver wet wt./hr.)
Controls (Stock diet)	4	5·3 ± 0·5	20 ± 3	26 ± 3	0·6 ± 0·1
3% casein	9	5·4 ± 0·6	6·4 ± 1·9	18 ± 3	0·1 ± 0·01
3% casein + phenobarbitone	9	7·0 ± 0·8	31 ± 14	26 ± 10	0·5 ± 0·6
Stock diet + phenobarbitone	5	8·3 ± 0·7	135 ± 23	45 ± 4	2·6 ± 0·6

Results are expressed as means ± S.D.

The diets were fed for 14 days before death, and animals given phenobarbitone had 1 mg/ml. Na phenobarbitone in the drinking water throughout this period.

Stock fed animals weighed 150–180 g at death.

The animals given 3% casein diet weighed 120–130 g initially and 100–120 g after 2 weeks on diet ± phenobarbitone.

the level attained is only 25 per cent of that achieved by animals on stock diet given phenobarbitone. The ratio between the phenobarbitone-treated and controls on each diet is approximately the same, representing a 5- to 7-fold increase in 14 days.

DISCUSSION

Our results show that giving phenobarbitone in the drinking water gives rise to an

increase in cytochrome P-450 and microsomal protein entirely comparable to that observed when the drug is given by injection. The stimulation achieved with an oral dose of about 100 mg/kg/day is maximal. Giving the drug in the drinking water is a convenient technique and should obviate the need to perform daily injections when a long period of phenobarbitone administration is required.

The time course of cytochrome P-450 induction by oral phenobarbitone is in agreement with the data of other workers who have given the drug by injection.[4,10]

The 0·03 mg/ml concentration of sodium phenobarbitone in the drinking water used in the dose–response experiment corresponds to a dose of about 3 mg/kg/day and leads to a 100 per cent increase in cytochrome P-450. This dose is within the range of use for man.

The 3% casein diet leads to reduced levels of cytochrome P-450 in comparison with stock fed rats. Because of their lower water intake, the 3% casein-fed rats receive a smaller dose of phenobarbitone but the results in Table 2 show that the difference in P-450 levels obtained in the two groups cannot be explained by a 40 per cent reduction in dose. The reduction in P-450 levels produced by feeding a 3% casein diet is present whether untreated or phenobarbitone treated groups are compared, and might be due to a reduction in the levels of endogenous inducers and/or a reduced response to inducers by liver cells. Adrenal and gonadal steroids, and substances derived from the gut have been suggested as endogenous inducers and a low protein diet may well lead to a reduction in any of these. However, the reduced response to both phenobarbitone and DDT[11] suggests that the mechanism of induction has been disturbed by the low protein diet.[12]

Since the P-450 enzyme system is of considerable importance in determining the effect of environmental toxins on animals, the alterations in the system produced by drugs and low protein diets may well be of importance for humans, especially in places where populations live on diets deficient in protein and liable to contamination with substances like aflatoxin.[13,14]

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