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DIFFERENCES BETWEEN SMALL AND LARGE INTESTINE AND LIVER IN THE INDUCIBILITY OF MICROSOMAL ENZYMES IN RESPONSE TO STIMULATION BY PHENOBARBITONE AND BETANAPHTHOFLAVONE IN THE DIET

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Abstract—Rats were fed either sodium phenobarbitone (PB) or betanaphthoflavone (BNF) for seven days. Deethylation of 7-ethoxyresorufin (7ERR) and 7-ethoxycoumarin (7EC) was measured in small and large intestine and liver, and cytochrome P-450 in liver. Our semi-purified diet was shown to produce minimal levels of intestinal deethylation activity. BNF was added to the semi purified diet and fed at levels from 0.1 to 100 mg BNF/kg of diet. Significant ($P < 0.05$) induction of deethylation in small intestine was seen at all dose levels, ranging from 2-fold at 0.1 mg/kg diet to greater than 100-fold at 100 mg/kg diet. A 3-fold increase was also seen in the large intestine at 50 mg/kg. A significant increase in hepatic deethylation was only seen at 100 mg/kg. PB was administered in drinking water at 50, 100 and 1000 mg PB/l. Significant ($P < 0.05$) induction of hepatic deethylation was seen at all dose levels, ranging from 2-fold at 50 mg/l to 5-fold at 1000 mg/l. Hepatic cytochrome P450 was also increased. No significant increase in intestinal deethylation was seen at any of the doses used.

Man is exposed to a variety of natural and synthetic chemicals, many of which are not used in metabolic processes. The importance of extrahepatic tissues in the metabolism of these foreign chemicals is becoming increasingly apparent [1, 2]. A major source of exposure to foreign compounds is by ingestion in food, in which case intestinal mixed function oxidase (MFO) activity is of particular significance.

Intestinal MFO activity is minimal in rats which have been starved for one to three days or fed a purified diet, and it has been suggested that the major part of drug metabolising enzyme activity in the gut is due to exogenous inducers in the diet [3]. The pioneering work in this field was done by Wattenberg who showed that feeding rats stock diet or purified diet supplemented with various vegetables, particularly the Brassicas, greatly increased benzo(*a*) pyrene hydroxylase activity in the intestine [3, 4].

Since this time a range of both natural dietary components and foreign chemicals have been shown to increase drug metabolising activity in the gut. These include phenobarbitone, β-naphthoflavone, 1,2-benzanthracene, benzo(*a*)pyrene, cholesterol, isosafrole, piperonyl butoxide, charcoaled meat and cigarette smoke. This work has been reviewed by Wollenberg and Ullrich [5]. Results of these studies show great variation, and it would appear that induction of intestinal drug metabolising enzymes varies with tissue, species and strain of experimental animal and route of administration of inducer. However, overall, intestinal MFO activity has been shown to be highly inducible by compounds commonly ingested in the diet, and is therefore likely to play a significant role in activation and inactivation of foreign chemicals.

It has been suggested that whereas cytochrome P-450 is the major subspecies of haemprotein in the liver, cytochrome P-448 may prodominate in the intestine [6]. In our studies, therefore, we have compared the inducing effects of phenobarbitone (PB)† and beta naphthoflavone (BNF), representatives of two distinct classes of inducing agents which stimulate *de novo* synthesis of different cytochrome P-450 species [7]. We have paid particular regard to the relative effects of these two inducers in liver and intestine. Two substrates were employed to measure MFO activity, 7-ethoxyresorufin (7ERR) which is more readily metabolised by cytochrome P-448 type enzymes, and 7-ethoxycoumarin (7EC) wich is believed to be more specific for cytochrome P-450 haemoproteins [7, 8].

Various groups have compared the effect of PB [9–16] and BNF [17–20] on drug metabolism in liver and intestine. Results vary considerably depending on route of administration, animal species and substrate employed. In our studies we have compared the effect of dietary administration of PB and BNF, as this is probably the main route of exposure of the intestine to exogenous inducers.

MATERIALS AND METHODS

Chemicals

Sodium phenobarbitone (Analar) was purchased from BDH, Poole, Dorset. β-Naphthoflavone and 7-

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† Abbreviations: PB, sodium phenobarbitone; BNF, betanaphthoflavone; 7ERR, 7-ethoxyresorufin; 7EC, 7-ethoxycoumarin; ICDH, isocitrate dehydrogenase; MID, minimal inducing diet; MFO, mixed function oxidase; i.p., intra peritoneal.

ethoxycoumarin were purchased from Aldrich Chemical Company Ltd, Gillingham, Kent, and 7-ethoxyresorufin was from Molecular Probes Inc., Plano, Tx. Resorufin was from Pfaltz and Bauer, Stamford, CT.

Diets

The semipurified diet was based on cornflour, with 10% corn oil, 20% casein, plus vitamins and minerals. It was prepared as previously described [21].

β-Naphthoflavone (BNF) was dissolved in the corn oil component of the diet (i.e. 100 ml corn oil/kg diet) and thoroughly mixed with the semi purified diet to give final levels of 0.1–100 mg BNF/kg diet.

PB, as the sodium salt, was dissolved in the drinking water (tap water) at 50, 100 and 1000 mg/l and given *ad libitum*.

All diets were thoroughly mixed in a commercial food mixer and stored at +4°.

Animal treatment

Male rats of a Wistar strain, weighing between 100 and 150 g were purchased from A. Tuck and Sons, Battlesbridge, Essex. Animals were housed in groups of four, in plastic cages with wire mesh covers and bases.

All animals were fed a semi-purified diet for three days prior to feeding the experimental diets.

All experimental diets were mixed to a stiff paste with tap water and fed for seven days before sacrifice.

Preparation of homogenates

Liver. Rats were killed by cervical dislocation under ether. The livers were removed into ice-cold isotonic saline, blotted dry, weighed and two 1 g portions taken from the median lobe. One portion was frozen under water for subsequent cytochrome P-450 estimation (see below). The remaining portion was diluted with 9 ml of ice-cold KCl–Tris (150/25 mM) buffer (pH 7.5 at 5°) and homogenised, at full speed for 8 sec, on a Turrax blender. This 10% homogenate was further diluted to 1% in KCl–Tris for assay.

Small intestine. The 25 cm portion of small intestine, distal to the pylorus, was removed and flushed through with 40 ml isotonic KCl, using a plastic syringe. The gut was then slit open and pinned flat onto a cork board. After removing excess KCl with a paper tissue, the mucosa was separated from underlying layers of tissue by scraping with a preweighed glass microscope slide. The slide and mucosa were then weighed, and the mucosa diluted in an appropriate volume of KCl–Tris and homogenised using 10 strokes at 600 rpm, in a Potter type glass–Teflon homogeniser. A 2% homogenate in KCl–Tris was used for the assays.

Large intestine. The entire length of intestine from caecum to anus was removed and a 2% homogenate of mucosa prepared as described for small intestine.

Enzyme assays

7-Ethoxyresorufin. 7ERR deethylation was measured using a modification of the method of Pohl and Fouts [22]. The incubation mixture, in a total volume of 2 ml, consisted of 1 ml of homogenate and 1 ml of 0.1 M phosphate buffer (pH 7.8) containing 10 μmoles sodium isocitrate, 10 μmoles magnesium chloride, 0.76 μmoles NADP and 3.5 mg of bovine serum albumin. This mixture, in a 8 ml plastic tube (Luckham, Burgess Hill, Sussex) was pre-incubated at 37° for 2 min and the reaction started by adding 10 μl of 0.25 mM 7 ERR in dimethyl sulphoxide. The reaction was stopped after 5 min by the addition of 2.5 ml of methanol. After standing for 30 min at −20°, the tubes were centrifuged at 3000 rpm for 20 min, and the fluorescence in the supernatant read at excitation wavelength 550 nm and emission wavelength 585 nm, using an Aminco Bowman fluorimeter. Each homogenate was assayed in duplicate with its own blank, to which methanol was added before incubation. Resorufin was used as a fluorescent standard.

7-Ethoxycoumarin. 7EC deethylation was measured by a modification of the method of Greenlee and Poland [23]. Using 10 ml conical polypropylene test tubes, the incubation mixture, in a total volume of 1 ml, contained 700 μl of tissue homogenate, 5 mg bovine serum albumin, 0.77 units of isocitrate dehydrogenase, 3 μmoles magnesium chloride, 3 μmoles sodium isocitrate, 0.3 μmoles NADP and 125 μmoles ethoxycoumarin.

Assays were tested for linearity with both time and homogenate concentration, using induced and non-induced animals.

Cytochrome P-450. Hepatic cytochrome P-450 was measured as previously described [24].

RESULTS

The purified diet (minimal inducing diet, MID) fed to control animals for seven days, was shown to produce minimal levels of deethylation activity in rat intestine and low levels of activity in the liver.

β-Naphthoflavone induction (Table 1). At all dose levels of BNF tested, deethylation in small intestine was significantly increased ($P < 0.05$) when compared to rats fed a semi-purified diet alone. Deethylation in small intestine increased in a dose-dependent fashion from 2-fold at 0.1 mg/kg to over 100-fold at 100 mg/kg. At 100 mg BNF/kg diet, specific activity in small intestine was more than ten times that in liver (per mg total protein) and at least twice that of liver in terms of total enzyme activity found in the whole tissue.

Deethylation in the large intestine was significantly increased at 5 and 50 mg/kg of diet, although the large intestine appeared to be less sensitive than small intestine to BNF induction.

There was no significant increase in hepatic deethylation activity, except with 7EC at 100 mg/kg BNF. Hepatic cytochrome P-450 and liver weights were not significantly altered at any dose level of BNF tested.

The total amount of BNF ingested in diet, over the dose range, was approximately 0.01–10 mg/kg body weight/day.

Phenobarbitone induction (Table 2). At all dose levels hepatic deethylation activity was significantly increased ($P < 0.05$) in a markedly dose dependent fashion. Hepatic cytochrome P-450 was similarly increased, although the response was considerably reduced in comparison with that found in rats fed

Table 1. Mixed function oxidase (MFO) activity in homogenates of liver, small and large intestine from rats fed minimal inducing diet (MID) or MID + β-naphthoflavone (BNF)

BNF mg/ kg diet	7ERR deethylation			7EC deethylation	
	Small intestine	Large intestine	Liver	Small intestine	Liver
0.1	1.7*		1.1	1.6*	1.0
0.5	8*	1.2	1.2		
5.0	57*	2.3*	0.8	44*	1.0
50	83*	3.2*	1.2	102*	1.2
100	149*		1.4	131*	1.6*

Results expressed as a ratio of induced/control values for groups of four animals.
* Significantly different from control ($P < 0.05$).
Control values for 7EER deethylation (pmoles/mg protein/min) were mean (± standard deviation) 9(±5.4), 8(±5.6) and 40(±5.3) for small intestine, large intestine and liver respectively.
Control values for 7EC deethylation (pmoles/mg protein/min) were 1.9(±0.1) and 51(±12) for small intestine and liver respectively.
Hepatic cytochrome P-450 and liver weights were not significantly altered at any dose level.

stock pellet diet [25]. There was no significant change in intestinal deethylation at any dose level.

The total amount of PB ingested in drinking water, over the dose range, was approximately 5–100 mg/kg body weight/day.

DISCUSSION

From the above work there are two main findings. Firstly, that intestinal MFO activity is minimal in rats fed a semi-purified diet, thus providing a suitable control diet for the investigation of enzyme inducers in the gut. Secondly, the cytochrome P-448 inducer, β-naphthoflavone, is far more active in the intestine than the liver when administered in the diet.

In previous studies on the effect of PB and BNF on the MFO activity in liver and intestine, results have shown great variation [9–20]. One of the main reasons for this discrepancy may be that appropriate control diets were not used. To assess the effect of inducing compounds, particularly in the intestine, it is important to use a non-inducing control diet which gives minimal levels of MFO activity. Stock animal diets, similar to those used by previous workers [9, 11, 13, 15, 17, 19, 20] have been shown in our laboratory and by others to induce considerable levels of benzo(*a*)pyrene hydroxylase activity [3] and 7EC and 7ERR deethylation activity (McDanell and McLean, in press) in the small intestine, and would not therefore seem to be appropriate control diets for this type of study.

The second observation from our study is that BNF, a cytochrome P-448 inducer, is very much more active towards the intestine than the liver, when administered in the diet. BNF, when administered by the i.p. route is a potent inducer of the rat hepatic monooxygenase system [7, 8]. However, when BNF is given in the diet we have shown an effect on hepatic deethylation only at the highest dose (100 mg BNF/kg diet). The effect in the intestine is very much more pronounced. At 100 mg BNF/kg diet, specific activity in the small intestine was ten times that of liver and at least twice that of liver in terms of total enzyme activity in the whole tissue. This could be explained in two ways. Either the BNF is not absorbed via the oral route, although in view of the high lipid solubility of the compound this explanation would seem unlikely. Alternatively, the BNF may be completely metabolised by the intestinal cells or gut microflora to products which are inactive or not absorbed.

Very little is known of the inducing effect of BNF in the gut. To our knowledge no other studies have looked at the effect of dietary BNF on intestinal MFO activity. Watanabe [17] showed that hepatic and intestinal benzo(*a*)pyrene hydroxylase activity varied with strain, after intragastric administration of BNF to mice. Niwa [18] showed a similar genetic

Table 2. Mixed function oxidase (MFO) activity in homogenates of liver and small intestine from rats fed minimal inducing diet (MID) or MID + phenobarbitone (PB) in the drinking water

PB (mg/l)	7ERR deethylation		7EC deethylation		Cyt P-450	Liver
	Small intestine	Liver	Small intestine	Liver	Liver	weight
50	0.5	1.8*	1.0	2.0*	1.1	1.2*
100	0.5	2.9*	1.5	3.4*	1.5*	1.3*
1000	0.6	5.1*	1.3	6.4*	1.8*	1.3*

Results are expressed as a ratio of induced/control values for groups of four animals.
* Significantly different from controls ($P < 0.05$).
Control values for 7ERR deethylation (pmoles/mg protein/min) were 4(±1.8) and 40(±6) for small intestine and liver respectively.
Control values for 7EC deethylation (pmoles/mg protein/min) were 1.6(±0.5) and 47(±14) for small intestine and liver respectively.
Control values for hepatic cytochrome P450 (nmoles/g wet weight) were 47(±4).

variation in mice after i.p. dosage of BNF. Strobel and his co-workers [19, 20] after i.p. administration of BNF to rats, showed a 35-fold increase in colonic benzo(*a*)pyrene activity and an 8-fold increase in cytochrome P-448 in the colon. It would appear that induction may be dependent on both route of administraton of inducer, and strain or species.

With phenobarbitone induction by the oral route, the effect is opposite to that of oral BNF, with a marked induction of both 7EC and 7ERR deethylation in the liver and no effect in the intestine. It is possible that the cytochrome sub species in the gut is predominantly P-448, and therefore is not affected by phenobarbitone which has its main effect on P-450. Our results are similar to those of Lake *et al.* [9] who showed that after dietary administration of phenobarbitone in rats there was no significant alteration in benzo(*a*)pyrene hydroxylase, biphenyl-4-hydroxylase or biphenyl-2-hydroxylase in small intestine. However, this effect may vary with species as Lehrmann [10] working with mice, found an 8-fold increase in 7EC deethylation in small intestine after oral phenobarbitone. When these workers [10] looked at the time course of induction with daily oral PB dosing, they showed maximum induction at four days, after which time activity decreased. As Lake *et al.* measured activity after seven days of dietary administration, it may be that they missed the peak of induction.

As we have observed before, the inducing effect of phenobarbitone in the liver of rats fed a purified diet is much reduced in comparison with the effect in rats fed stock pellet diet [25].

A somewhat surprising finding from our studies was that the two enzyme assays, 7EC and 7ERR deethylation, intended to distinguish between P-448 and P-450 type induction, gave similar results for the response of both liver and intestine to both PB and BNF. It would appear from our results that either PB is inducing hepatic P-448 as well as P-450, or that the 7ERR assay is not specific for P-448 as has been suggested. In retrospect it might have been better to use an alternative assay to 7EC deethylation, with a substrate more specific for P-450 haemoproteins. However, it seems likely that this is too simplistic and that as more sub species of P-450 are identified [26], the question of substrate specificity will become increasingly complex.

From the above studies there are two main conclusions. Firstly, the route of administration of inducer is likely to make a considerable difference to the relative activity produced in hepatic and extrahepatic tissues. Therefore, further studies, directly comparing different routes of administration would seem to be indicated.

Secondly, it appears that when administered in the diet the P-448 type inducer BNF is very much more active in the intestine than the liver. This is an important finding as it shows that the intestine is highly inducible and has the potential to play a significant role in the metabolism of foreign compounds, particularly those ingested with the diet. This has recently been demonstrated by Strobel [19, 20] who showed a 4-fold increase in benzo(*a*) pyrene activation and a 20-fold increase in 2-amino-anthracene metabolism by colonic microsomes isolated from BNF induced rats. Ioannou *et al.* [27] have also shown that in mice fed 3 mg BNF/g diet and given benzo(*a*)pyrene there is a large decrease in the formation of the DNA-diol epoxide adduct. The importance of this phenomenon in terms of human disease remains to be established, and further studies on activation and covalent binding of xenobiotics in different tissues of induced animals would be of interest.

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REFERENCES

1. M. D. Burke and S. Orrenius, *Pharmac. Ther.* **7**, 549 (1979).
2. E.T. Gram, in *Extrahepatic Metabolism of Drugs and Other Foreign Compounds*. MTP Press, Lancaster (1980).
3. L. W. Wattenberg, *Cancer* **28**, 99 (1971).
4. W. D. Loub, L. W. Wattenberg and D. W. Davies, *JNCI.* **54**, 985 (1975).
5. P. Wollenberg and V. Ullrich, in *Extrahepatic Metabolism of Drugs and Other Foreign Compounds*. MTP Press, Lancaster (1980).
6. N. G. Zampaglione and G. J. Mannering, *J. Pharmac. exp. ther.* **185**, 676 (1973).
7. D. W. Nebert, H. J. Elsen, M. Negishi, M. A. Lang and L. M. Hjelmeland, *Ann. Rev. Pharmac. Toxicol.* **21**, 431 (1981).
8. A. Y. U. Lu and S. B. West, *Pharmac. Rev.* **31**, 227 (1980).
9. B. G. Lake, R. Hopkins, J. Chakraborty, J. W. Bridges and D. V. W. Parke, *Drug Metab. Dis.* **1**, 342 (1973).
10. C. Lehrmann, V. Ullrich and R. Rummel, *Naunyn-Schmeid. Archs. Pharmac.* **276**, 89 (1973).
11. S. J. Stohs, R. C. Grafstrom, M. D. Burke, P. W. Moldeus and S. G. Orrenius, *Archs. Biochem. Biophys.* **177**, 105 (1976).
12. R. S. Chhabra, *Envir. Hlth Perspect.* **33**, 61 (1979).
13. R. J. Shirkey, J. Kao, J. R. Fry and J. W. Bridges, *Biochem. Pharmac.* **28**, 1461 (1979).
14. U. Koivusaari, M. Lang and E. Hietanen, *Acta Pharmac. Toxicol.* **46**, 37 (1980).
15. C. L. Miranda and R. S. Chhabra, *Biochem. Pharmac.* **29**, 1161 (1980).
16. R. S. Chhabra, *Toxic. Envir. Chem.* **3**, 173 (1981).
17. M. Watanabe, *Gann* **66**, 217 (1975).
18. A. Niwa, K. Kumaki and D. W. Nebert, *Archs. Biochem. Biophys.* **166**, 559 (1975).
19. W. Fang and H. W. Strobel, *Archs. Biochem. Biophys.* **186**, 128 (1978).
20. H. W. Strobel, W. Fang and R. J. Oshinsky, *Cancer* **45**, 1060 (1980).
21. A. E. M. McLean and P. A. Day, *Biochem. Pharmac.* **24**, 37 (1974).
22. R. J. Poul and J. R. A. Fouts, *Analyt. Biochem.* **107**, 150 (1980).
23. W. F. Greenlee and A. Poland, *J. Pharmac. exp. ther.* **205**, 596 (1978).
24. A. E. M. McLean and P. A. Day, *Biochem. Pharmac.* **23**, 1173 (1974).
25. W. J. Marshall and A. E. M. McLean, *Biochem. J.* **114**, 77P (1969).
26. D. W. Nebert and M. Negishi, *Biochem. Pharmac.* **31**, 2311 (1982).
27. Y. M. Ioannou, A. G. E. Wilson and M. W. Anderson, *Cancer Res.* **42**, 1199 (1983).