Effect of Development on the Activity of Microsomal Drug-Metabolizing Enzymes in Rat Liver

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1. The activity of rat liver microsomal drug-metabolizing enzymes was determined at various ages between 6 and 100 days *post natum*. The enzymes studied were: aromatic hydroxylases by using as substrate biphenyl, which is metabolized by oxidation to 2- and 4-hydroxybiphenyl; nitroreductase by using *p*-nitrobenzoate as substrate, which is metabolized by reduction to *p*-aminobenzoate; glucuronyl synthetase by using 4-methylumbelliferone as the substrate, which is conjugated to give 4-methylumbelliferone glucuronide, and cytochrome *P*-450, which is regarded as the major terminal mixed-function oxidase in hepatic microsomal hydroxylations. 2. The activity of biphenyl 2-hydroxylase reached a peak at 21 days, biphenyl 4-hydroxylase and 4-methyl glucuronyl transferase at 24 days, cytochrome *P*-450 at 31 days, and *p*-nitrobenzoate reductase at 38 days of age. After the peak activity had been reached, the activity of each enzyme decreased with age, and in the case of biphenyl 2-hydroxylase the activity fell to a negligible value at 52 days of age. 3. Neither the addition of Triton X-100 to the incubation medium nor the treatment of the animals with phenobarbital resulted in any increase in the activity of biphenyl 2-hydroxylase at 52 days of age. 4. The activity of biphenyl 2-hydroxylase was threefold higher in rats fed on a synthetic diet than in rats fed on a commercial stock diet. 5. These findings are discussed.

The development of the components of rat liver has been studied extensively, but less information is available on the development of the activity of the hepatic drug-metabolizing enzymes. The liver microsomal fraction of many newborn animals, including mice and guinea pigs, is deficient in certain drug-metabolizing enzymes (Jondorf, Maickel & Brodie, 1959). The activities of several hepatic enzyme systems that metabolize drugs by hydroxylation or by reduction have been shown to be absent in newborn rabbits, with some activity appearing at 14 days after birth, and full adult activity at 28 days (Fouts & Adamson, 1959). Similarly, newborn rats have been shown to have little capacity to metabolize a number of drugs, but both the total activities and the specific activities of a number of these enzymes progressively increase with age up to 30 days, then afterwards decrease gradually (Kato, Vassanelli, Frontino & Chiesara, 1964). A study in the rat of the development of hepatic aniline hydroxylase and ethylmorphine demethylase showed that both the total activities and the K_m values of these enzymes increased during the first few weeks after birth, but there was no correlation between the two enzyme activities nor between either of these and the

hepatic cytochrome *P*-450 content (Gram, Guarino, Schroeder & Gillette, 1969). Other workers have studied the development of microsomal glucuronyl-transferase in the liver of newborn rabbits (Flint, Lathe, Ricketts & Silman, 1964), the conjugation with GSH of sulphobromophthalein in mouse liver (Krasner, 1968), the hydroxylation of 7,12-dimethylbenz[*a*]anthracene in rat liver (Sims & Grover, 1967), and a number of oxidative, reductive, hydrolytic and synthetic drug-metabolizing enzymes in the liver of the pig (Short & Davis, 1970). All of these studies have shown a characteristic development of these enzymes during the first 4–6 weeks after birth.

The present work was undertaken to investigate further the correlation of the development of a number of hepatic drug-metabolizing enzyme activities with the concentration of hepatic cytochrome *P*-450 in the rat. The enzymic activities include aromatic hydroxylation, measured by the 2- and 4-hydroxylation of biphenyl, nitro-reduction, measured by the reduction of *p*-nitrobenzoate, and glucuronide synthesis, with the substrate 4-methylumbelliferone. In the present investigation the activity of the liver microsomal enzymes has been measured *in vitro* from 6 days of age to maturity,

in animals of known age reared under controlled conditions. The effect of feeding a purified diet during gestation, lactation and the post-weaning period, on the activity of the hepatic microsomal enzymes has also been studied. Further, the development and the nature of the activity of the latent microsomal enzyme biphenyl 2-hydroxylase (Creaven, Parke & Williams, 1965) has been investigated. This enzymic activity, although increasing after birth, contrasts with other hepatic drug-metabolizing enzymes in being absent from the livers of adult rats. A preliminary account of this work has been given (Basu, Dickerson & Parke, 1970).

MATERIALS AND METHODS

Wistar albino rats were used throughout and maintained on a stock pellet diet (Spiller), and water *ad lib*, except where otherwise stated. Pregnant animals were separated from the stock and caged individually. In the first experiment litter size was maintained at three pups throughout the suckling period. Animals were weaned at 21 days, separated by sex and maintained in groups. Up to 21 days of age animals of both sexes were used, but after 21 days males only were used. In the second experiment, pregnant rats were reared throughout pregnancy on a synthetic diet containing 21% casein, 60% starch, 10% corn oil, 5% vitamin mixture (Cooper) and 4% mineral mixture. Litters were adjusted to eight pups at birth and the young were weaned at 21 days of age on to the same diet as that given to the mother.

In the first experiment, animals were killed by decapitation at intervals between 6 and 100 days and in the second experiment at intervals between 12 and 52 days of age. The livers were quickly removed, blotted, weighed and homogenized in ice-cold 1.15% (w/v) KCl in a glass tube with a Teflon pestle, to give a final concentration of liver of 25% (w/v). Other 12-, 21- and 52-day-old animals were treated with phenobarbital (10 mg/kg) intraperitoneally for 3 successive days and killed 24 h after the last dose. Liver homogenates of these animals were prepared as above.

Homogenates were centrifuged at 10000g for 10 min and the supernatant fractions centrifuged at 100000g for 1 h to sediment the microsomal fraction. The 10000g supernatant was used to measure biphenyl 2-hydroxylase and biphenyl 4-hydroxylase (Creaven *et al.* 1965), *p*-nitrobenzoate reductase (Bratton & Marshall, 1939) and 4-methylumbelliferone glucuronyltransferase (Bollet, Goodwin & Bron, 1959). DNA was separated from liver (Zamenhof, Bursztyn, Rich & Zamenhof, 1964) and determined by the diphenylamine reaction (Burton, 1956). Total and microsomal protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951). The content of cytochrome *P*-450 in the microsomal suspension was measured on a Unicam SP. 800 spectrophotometer (Sladek & Mannering, 1966). To ensure that the biphenyl 2-hydroxylase and the biphenyl 4-hydroxylase were liberated completely from membrane sites, various concentrations of Triton X-100 in KCl were added to the 10000g supernatant of the liver homogenates and also to the resuspended microsomal preparations.

The incubation medium used for the microsomal suspension was supplemented by the addition of an NADPH-generating system [glucose 6-phosphate (10 μ mol in 0.20 ml) and glucose 6-phosphate dehydrogenase (1.0 unit in 0.10 ml)].

RESULTS

Effect of growth on liver weight and the activity of drug-metabolizing enzymes. Fig. 1 shows that the liver weight increased rapidly until 70 days of age and showed no change after 85 days. The body weight, however, increased until 100 days of age. The absolute amount of liver protein increased progressively until the age of 70 days and thereafter it remained more or less constant. Total microsomal protein followed a similar pattern of development with age. The progressive increase in the absolute amount of DNA, however, continued until 85 days of age.

The activity of 4-methylumbelliferone glucuronyltransferase, expressed per g liver weight, per 100g body weight and per unit of DNA, attained a maximum value at 24 days, and subsequently declined to about 50% of this value at 52 days (Fig. 2). The activity of *p*-nitrobenzoate reductase expressed in all three parameters showed a peak activity at 38 days, which was followed by a slow decline to 60% of this value at 100 days (Fig. 3).

Fig. 1. Effect of age on body weight and liver weight, protein content and DNA content. Values for the first four ages are the means for four to 14 pooled samples of two to five livers. Values for later ages are the means of five to nine individual livers. ▲, Body weight (g); △, liver weight (g); ■, total protein (mg); ●, microsomal protein (mg); ○, DNA (mg).

Cytochrome *P*-450 activity followed a similar pattern (Fig. 4) except that the peak was found to be at the age of 31 days, and this was followed by a sharp decrease at 38 days almost to the value for mature animals. The activity of biphenyl 4-hydroxylase showed a maximum at 24 days of age, but unlike *p*-nitrobenzoate reductase and cytochrome *P*-450, the activity then decreased markedly to 40% of this maximum value by 85 days (Fig. 5). Biphenyl 2-hydroxylase activity was maximal at 21 days of age, but unlike the other four enzymes, fell to a negligible value by 52 days, and thereafter disappeared completely (Fig. 6).

Fig. 2. Effect of age on the activity of 4-methylumbelliferone glucuronyltransferase in rat liver. Enzyme activity is expressed per 100g body wt. (○), per g liver wt. (●) and per mg of DNA (▲).

Fig. 3. Effect of age on the activity of *p*-nitrobenzoate reductase in rat liver. Enzyme activity is expressed per 100g body wt. (○), per g liver wt. (●) and per mg of DNA (▲).

Fig. 4. Effect of age on the concentration of cytochrome *P*-450 in rat liver: per 100g body wt. (○), per g liver wt. (●) and per mg of DNA (▲).

Fig. 5. Effect of age on the activity of biphenyl 4-hydroxylase in rat liver. Enzyme activity is expressed per 100g body wt. (○), per g liver wt. (●) and per mg of DNA (▲).

Fig. 6. Effect of age on the activity of biphenyl 2-hydroxylase in rat liver. Enzyme activity is expressed per 100g body wt. (○), per g liver wt. (●) and per mg of DNA (▲).

Table 1 shows that the percentage induction of enzyme activity by phenobarbital decreased with age. In 12-day-old animals the induction was greatest for biphenyl 2-hydroxylase and least for *p*-nitrobenzoate reductase. In 52-day-old animals, however, the induction was greatest for biphenyl 4-hydroxylase and negligible for *p*-nitrobenzoate reductase. The doses of phenobarbital used were below the maximum (50–75 mg/kg body wt.) of the dose-response curve. However, other work in our laboratory has shown that after induction by phenobarbital the K_m of the enzymes studied is unchanged.

Effect of Triton X-100 on the activity of liver biphenyl 2- and 4-hydroxylases. It was considered that the observed loss of activity of biphenyl 2-hydroxylase with increasing age might be due to increased binding of this membrane-bound enzyme. However, the addition of Triton X-100, a detergent that has been used to activate membrane-bound enzymes (Lueders & Kuff, 1967) did not increase the activity of biphenyl 2-hydroxylase, but at 0.1% Triton there was about 30% inhibition of the activity of biphenyl 4-hydroxylase.

Comparison of synthetic and stock diets. The offspring of pregnant rats that had been fed throughout gestation and lactation on the synthetic diet weighed less and grew more slowly than the offspring of female rats fed throughout on the stock diet. The liver weight relative to body weight of the rats on the synthetic diet followed the same pattern as that of those on the stock diet. Biphenyl 2-hydroxylase showed a consistently higher activity in rats fed on the synthetic diet. The activity of this enzyme was three times that in those rats that were weaned on to the synthetic diet, even when this

enzyme activity fell at 52 days of age (see Table 2). The amount of hepatic cytochrome *P*-450 was lower at all ages after weaning (18 days) in the rats fed on the synthetic diet. Activities of the other enzymes showed no significant changes caused by diet.

DISCUSSION

The liver grew at a faster rate than the whole body up to 38 days of age, but after 52 days the body weight increased faster than the liver weight. Between 70 and 100 days of age the absolute weight and protein content of the liver remained constant. The body weight, however, continued to increase until 100 days of age, partly because of the accretion of body fat. The nuclei of rat liver cells show polyploidy (Davidson, 1953), but as the rat liver cell with a tetraploid nucleus is twice as large as that with a diploid nucleus the ratio cytoplasm weight/unit weight of DNA remains constant (Epstein, 1967). It is therefore possible in this case to use DNA for the expression of cellular constituents on a unit cell basis (Enesco & Leblond, 1962).

The protein/DNA ratio, which may be used as a measure of cell size, increased steadily until 32 days of age and showed a broad peak between 38 and 85 days. This suggests that the cells of the liver may be larger during the period of anabolism caused by puberty. The total amount of DNA increases at different rates in different organs and reaches its adult value at different ages, and in the rat it is considered to reach its adult value in the liver at 70 days of age (Winick & Noble, 1965). In the liver of the rats used in our experiments, the total amount of liver DNA appears to reach the mature value at 85 days of age.

Table 1. *Effect of phenobarbital on the activity of liver microsomal enzymes at three different ages in the growing rat*

The rats were treated with phenobarbital (10 mg/kg body wt. intraperitoneally) for 3 successive days, the last dose being given 24 h before death. The results are given as the mean $\pm$ S.D. for at least six rats. The values in parentheses indicate the percentage increase in activity caused by phenobarbital.

	Phenobarbital treatment	Activity		
		Age (days) ... 12	21	52
Biphenyl 4-hydroxylase (μ mol/h per g liver wt.)	—	2.6 $\pm$ 0.24	4.9 $\pm$ 0.03	3.0 $\pm$ 0.8
	+	6.8 $\pm$ 0.27 (161%)	8.0 $\pm$ 1.2 (63%)	4.5 $\pm$ 0.7 (50%)
Biphenyl 2-hydroxylase (μ mol/h per g liver wt.)	—	0.15 $\pm$ 0.05	0.30 $\pm$ 0.05	0.04 $\pm$ 0
	+	0.54 $\pm$ 0.12 (260%)	0.40 $\pm$ 0.05 (30%)	0.05 $\pm$ 0 (25%)
<i>p</i> -Nitrobenzoate reductase (μ mol/h per g liver wt.)	—	0.42 $\pm$ 0.02	0.90 $\pm$ 0.06	1.2 $\pm$ 0.34
	+	0.67 $\pm$ 0.06 (60%)	1.06 $\pm$ 0.05 (18%)	1.3 $\pm$ 0.28 (8%)
Cytochrome <i>P</i> -450 (nmol/g liver wt.)	—	13.0 $\pm$ 1.0	23.0 $\pm$ 2.5	21.0 $\pm$ 4.1
	+	24.0 $\pm$ 1.9 (84%)	38.0 $\pm$ 6.0 (65%)	27.0 $\pm$ 3.8 (30%)

Table 2. Comparison of the activities of liver microsomal enzymes in rats whose parents were fed throughout gestation and lactation on a stock diet and on a synthetic diet (21% casein), and who were themselves fed on the same diet from weaning

Values given for the synthetic diet are means for four animals and for the stock diet are the means of the number of animals shown in Fig. 1. The results are given as the mean $\pm$ s.d.

Diet	Age (days)	Activity						
		12	18	21	24	31	38	52
Biphenyl 2-hydroxylase (μ mol/h per 100 g body wt.)	Synthetic	0.70 $\pm$ 0.04	1.30 $\pm$ 0.03	2.20 $\pm$ 0.02	2.20 $\pm$ 0.04	2.00 $\pm$ 0.03	1.9 $\pm$ 0.02	0.53 $\pm$ 0
	Stock	0.41 $\pm$ 0.05	—	1.10 $\pm$ 0.03	0.80 $\pm$ 0.05	0.78 $\pm$ 0.05	0.66 $\pm$ 0.06	0.14 $\pm$ 0.01
Biphenyl 4-hydroxylase (μ mol/h per 100 g body wt.)	Synthetic	7.90 $\pm$ 0.07	16.0 $\pm$ 0.3	20.0 $\pm$ 0.2	26.0 $\pm$ 2.2	23.1 $\pm$ 0.5	22.0 $\pm$ 0.1	17.4 $\pm$ 0.4
	Stock	8.50 $\pm$ 1.1	11.5 $\pm$ 1.7	20.5 $\pm$ 3.0	26.8 $\pm$ 4.0	18.6 $\pm$ 2.7	19.7 $\pm$ 4.5	13.6 $\pm$ 3.0
Cytochrome P-450 (nmol/100 g body wt.)	Synthetic	35.0 $\pm$ 3.0	51.0 $\pm$ 4.2	70.0 $\pm$ 2.2	76.0 $\pm$ 2.0	86.0 $\pm$ 8.2	74.0 $\pm$ 3.0	78.0 $\pm$ 4.0
	Stock	39.0 $\pm$ 3.3	49.0 $\pm$ 4.5	93.0 $\pm$ 9.3	92.0 $\pm$ 11.5	125.0 $\pm$ 7.8	86.0 $\pm$ 18.0	96.0 $\pm$ 18.0
<i>p</i> -Nitrobenzoate reductase (μ mol/h per 100 g body wt.)	Synthetic	0.9 $\pm$ 0.02	1.6 $\pm$ 0.2	3.0 $\pm$ 0.04	3.3 $\pm$ 0.5	4.8 $\pm$ 0.2	5.1 $\pm$ 0.06	4.4 $\pm$ 0.05
	Stock	1.2 $\pm$ 0.1	3.1 $\pm$ 0.4	3.3 $\pm$ 0.8	3.9 $\pm$ 0.5	5.4 $\pm$ 1.4	6.8 $\pm$ 0.5	5.4 $\pm$ 0.5

The activity of each enzyme studied, when expressed per unit of body weight, rose to a peak soon after weaning (18–21 days) and then decreased with age. This decrease was only partly accounted for by the increase in the rate of growth of the body relative to that of the liver. With biphenyl 2-hydroxylase, the activity fell rapidly after 31 days and disappeared altogether at 70 days. The low activities of this enzyme were not due to decreased availability of enzyme sites caused by increased association of the enzyme with membranous elements of the endoplasmic reticulum, for there was no increase of activity when the detergent Triton X-100 was added. However, this result is not surprising because Tween 80, another membrane-activating detergent, is already present in the incubation mixture necessary for the determination of this enzyme activity (Creaven *et al.* 1965). This addition of Triton X-100 actually resulted in inhibition of the activity of the biphenyl 4-hydroxylase. The reason for the disappearance of biphenyl 2-hydroxylase activity with increasing age is therefore still obscure. The activity of biphenyl 4-hydroxylase in mature rats, on the other hand, was about 40% of the maximum value, and as with all other enzymes activities studied, with the exception of biphenyl 2-hydroxylase, remained constant after 52 days of age.

Phenobarbital induced the activity of the microsomal enzymes in rats of all ages, but the rate of induction was highest in the 12-day-old rats. This finding supports the work of Kato & Takanaka (1968) who demonstrated that the induction by

phenobarbital of aminopyrine *N*-demethylation, hexobarbital hydroxylation, aniline hydroxylation and *p*-nitrobenzoate reduction was much greater in 40-day-old rats than in older animals. Thus immature rats are intrinsically more responsive to enzyme induction.

The activity of biphenyl 2-hydroxylase was not increased at 52 days of age by the addition of Triton X-100 and treatment with phenobarbital failed to induce the enzyme. The induction of 4-hydroxylation by phenobarbital to some extent paralleled the increase in cytochrome *P*-450, whereas the induction of 2-hydroxylation at 12 days was greatly in excess, and at 21 days, was much less. Therefore there would appear to be no correlation between 2-hydroxylation of biphenyl and amount of cytochrome *P*-450, although there may be for 4-hydroxylation. Thus biphenyl 2-hydroxylase is not likely to be affected via cytochrome *P*-450, but it is a microsomal enzyme, and like glucuronyl-transferase may be expected to exist in different states of association with the endoplasmic reticulum and of availability to the substrate. If this is so, then it is far more difficult to establish than for glucuronyltransferase. Parke & Rahman (1970) have shown that the induction of 2-hydroxylase by safrole and isosafrole is due in part to protein synthesis. Similarly, the failure to induce biphenyl 2-hydroxylase activity after 52 days of age is probably due to loss of ability to synthesize this particular protein. Biphenyl 2-hydroxylase also differed from the other hepatic microsomal enzymes studied in being consistently more active in rats

fed on the synthetic diet. The reason for this higher activity may be that the synthetic diet contained a higher concentration of utilizable protein.

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