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Hepatic microsomal drug-metabolizing enzyme activity in the opossum*

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THE LEVEL of hepatic microsomal drug-metabolizing enzyme activity is species as well as strain dependent.[1–3] The activity of microsomal azo and nitro reductase in livers from fish, amphibia, reptiles, birds, and mammals was studied by Adamson *et al.*,[4] and they suggested that microsomal reductive pathways were more "primitive" (were detected earlier in the phylogenic scheme) than were the oxidative ones. The importance of an aquatic or amphibian versus terrestial habitat of animals in regard to oxidative liver microsomal drug-metabolizing enzyme activity was suggested in a review by Brodie and Maickel.[5] Hepatic drug metabolism, according to these investigators, increased as the phylogenic scale was ascended from fish and mammals. Hepatic drug-metabolizing enzymes in birds and mammals were located in the microsomal fraction of the cell. The metabolism of aminopyrine in the toad, however, was qualitatively different from that of the mammal, and several oxidative drug-metabolizing enzyme systems in the toad were found in the soluble fraction of the liver cell.

The opossum, a marsupial, is considered to be an offshoot or terminal branch of the main phylogenic line of mammals as the toad is an offshoot of the line of amphibia. Since some differences in hepatic drug metabolism between toads and other animals were found by Brodie and Maickel,[5] the amount of enzyme activity and the location of hepatic enzymes that metabolize drugs were studied in the opossum.The effect of phenobarbital or benzpyrene pretreatment on these enzyme activities in opossum liver was also investigated.

METHODS

Animals. Male and female opossums (2–2·5 kg) were used. These animals were obtained from local trappers. "Treated" animals received an intraperitoneal (i.p.) injection of phenobarbital sodium 15, 25, or 35 mg/kg twice daily for 4 days, or one injection of benzpyrene, 25 mg/kg. All determinations were made 12 hr after the last dose of phenobarbital or 48 hr after benzpyrene pretreatment.

Enzyme assays. Livers were excised and homogenized (1 g liver and 2 ml of 1·15% KCl) in the cold with a glass homogenizer having a plastic pestle, or with a Waring Blendor. The liver homogenate was centrifuged at 9000 *g* for 20 min at 4° to obtain the 9000-*g* supernatant fraction. The liver 9000-*g* fraction was centrifuged at 104,000 *g* for 1 hr to produce the pellet of microsomes and a supernatant designated as the "soluble" fraction of the cell. The liver microsomes were washed once in 1·15% KCl, centrifuged at 104,000 *g*, and resuspended in 1·15% KCl such that each ml of suspension contained microsomes from $\frac{1}{3}$ g of liver. Each ml of liver 9000-*g* fraction or soluble fraction was considered as equivalent to 0·33 g liver.

One ml of the liver fraction (e.g. 9000-*g* fraction) was added to an incubation mixture containing cofactors [triphosphopyridine nucleotide (NADP) $1{\cdot}1 \times 10^{-4}$ M, glucose 6-phosphate 5×10^{-3} M, nicotinamide 2×10^{-2} M, and $MgSO_4$ 5×10^{-3} M] and substrate. The substrates used and their

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concentrations in μmoles/5 ml incubation mixture were: aminopyrine, 40; *p*-nitrobenzoic acid, 12; aniline, 7·2; benzpyrene, 0·6; and zoxazolamine, 3. The final volume of the incubation mixture was adjusted to 5 ml with 0·1 M potassium phosphate buffer, pH 7·4. When formaldehyde was measured, semicarbazide (50 μmoles/5 ml incubation mixture) was included in the reaction mixture as a trapping agent. All incubations were carried out in a Dubnoff shaking incubator at 37° under oxygen or nitrogen for 1 hr (except for benzpyrene which was incubated for 30 min).

Chemical assays. The metabolite of aminopyrine, 4-aminoantipyrine (4AAP), was determined by diazotization and coupling with α-naphthol according to the method of Brodie and Axelrod[6] after precipitating the protein in the incubation mixture with trichloroacetic acid.[7] Formaldehyde formed from aminopyrine was estimated in a perchloric acid filtrate by the method of Nash[8] as modified by Cochin and Axelrod,[9] after precipitating 1 ml of the reaction mixture with 0·6 N perchloric acid. The method of Fouts and Brodie[10] was used to measure *p*-aminobenzoic acid (PABA) formed from *p*-nitrobenzoic acid (PNBA). The hydroxylation of aniline was estimated by measuring *p*-aminophenol (PAP) according to the method of Gillette as quoted in Dixon *et al.*[11] The method of Juchau *et al.*[12] as adapted from Conney *et al.*[13, 14] was used to determine benzpyrene hydroxylation. The hydroxylation of zoxazolamine was estimated by measuring the disappearance of zoxazolamine.[15] Hepatic glucose 6-phosphate dehydrogenase (G-6-PD) activity was determined in the manner described by Kornberg and Horecker.[16] A method described by Hart and Fouts[17] as adapted from Gillette *et al.*[18] was used to estimate NADPH oxidase activity of liver microsomes. The method of Klingenberg[19] was used to measure hepatic cytochrome CO-binding pigment. Results were usually expressed as micromoles of substrate metabolized or product formed per g liver per incubation period.

RESULTS

The amounts of 4AAP or formaldehyde formed from aminopyrine, PAP from aniline, PABA from PNBA, and the amount of benzpyrene metabolized by liver fractions from male or female opossums are shown in Table 1. The enzyme systems that metabolized aminopyrine, aniline, PNBA, and benzpyrene were located in the microsomal fraction of the liver cell. The metabolism of these

TABLE 1. PHENOBARBITAL STIMULATION OF HEPATIC DRUG-METABOLIZING ACTIVITY IN MALE AND FEMALE OPOSSUM

Substrate	Enzyme activity measured by	Exp. No.	Enzyme activity* in hepatic 9000-*g* fractions from: Male Control	Male Phenobarbital-pretreated†	Female Control	Female Phenobarbital-pretreated†
p-Nitrobenzoic acid	PABA formed	1	1·97	3·63	1·55	3·78
		2	0·99 ± 0·30‡	2·64	0·78	2·85
		3		2·88		
Aniline	PAP formed	1	1·19	1·59	0·91	1·70
		2	0·73 ± 0·10‡	1·32	0·51	1·20
		3		1·29		
Benzpyrene	Substrate disappeared	1	0·64	0·98	0·67	0·83
		2	0·39 ± 0·10‡	0·70	0·41	0·59
		3		0·79		
Aminopyrine	4AAP formed	1	0·47	1·27	0·39	1·09
		2	0·56 ± 0·10‡	0·90	0·17	0·37
		3		1·35		
Aminopyrine	Formaldehyde formed	1	4·62	12·00	4·08	10·92
		2	6·85 ± 1·00‡	9·48	2·19	12·12
		3		13·80		

* Enzyme activity in micromoles substrate metabolized or product formed per g (wet weight) liver per hr except with benzpyrene where time was 30 min.

† Animals were injected i.p. with phenobarbital sodium twice daily for 4 days as follows: exp. 1, 15 mg/kg; exp. 2, 25 mg/kg; exp. 3, 35 mg/kg.

‡ Mean ± S.E. with three animals per group. All other values in the table are from single animals.

compounds by hepatic enzyme systems seemed to be "stimulated" about two- to threefold by phenobarbital pretreatment. Both males and females responded to such phenobarbital treatment, and increasing the dose of phenobarbital above 15 mg/kg twice daily produced no further increase in the rate of metabolism of these compounds. The level of drug-metabolizing enzyme activity in control male opossum liver was similar to that in control female opossum liver for all pathways studied.

Glucose 6-phosphate dehydrogenase activity in the male opossum liver, and NADPH oxidase and cytochrome CO-binding pigment levels in both male and female opossum livers appeared to be slightly increased by phenobarbital pretreatment, as seen in Table 2.

TABLE 2. THE EFFECT OF PHENOBARBITAL PRETREATMENT ON HEPATIC MICROSOMAL CO-BINDING PIGMENT, NADPH OXIDASE AND GLUCOSE 6-PHOSPHATE DEHYDROGENASE ACTIVITY IN THE OPOSSUM*

Animal	CO-binding pigment†	NADPH oxidized‡	NADPH formed with G-6-PD§
Male			
Control	144	3·2	40·2
Phenobarbital-pretreated	191	4·8	156·0
Female			
Control	55	3·8	
Phenobarbital-pretreated	113	4·9	

* Opossums were injected with phenobarbital sodium 15 mg/kg twice daily for 4 days. Each value represents a single determination.

† Expressed as O.D. units $\times 10^3$ per 3 ml volume containing microsomes from 100 mg liver with a 1-cm light path.

‡ Expressed as mμmoles NADPH oxidized/min by microsomes from 100 mg liver.

§ Expressed as mμmoles NADPH formed/min by microsomes from 100 mg liver.

The effect of benzpyrene pretreatment on the hepatic microsomal metabolism of benzpyrene and zoxazolamine was studied in the adult male opossum. The rate of metabolism of benzpyrene by 9000-*g* supernatants prepared from liver of control male opossum was 0·62 μmole/g liver per 30 min. Forty-eight hours after a single i.p. injection of benzpyrene (25 mg/kg), this enzymic activity rose to 1·22 μmole/g liver per 30 min. The rate of metabolism of zoxazolamine by hepatic 9000-*g* fractions from normal vs. benzpyrene-pretreated opossum was similarly affected (control = 0·96 μmole/g liver per hr; benzpyrene-pretreated = 2·52 μmole/g liver per hr). Thus, the hepatic metabolisms of benzpyrene and zoxazolamine were enhanced about twofold by benzpyrene pretreatment.

DISCUSSION

Unlike those of the toad, opossum liver enzymes for the metabolism of aminopyrine were found in the microsomal fraction of the cell. The other hepatic drug metabolisms studied appeared to be similar in opossum to those in other mammals. Phenobarbital pretreatment increased the activity of four hepatic drug-metabolizing enzyme systems in the adult opossum, and pretreatment with benzpyrene enhanced the metabolism of benzpyrene and zoxazolamine. This suggests that hepatic microsomal drug-metabolizing enzyme systems in the adult opossum are similar to those in rats, rabbits, and other mammals.

Since the intra-uterine gestation period for the opossum is short (13 days), and since young animals may be removed, treated with a drug, and replaced in the maternal pouch, it may be of interest to study the effects of enzyme-inducing drugs on young opossum liver metabolism. Such effects of drugs on the development of hepatic microsomal drug-metabolizing enzymes may be studied more easily in the opossum than in other mammals, since the absence of a placenta after 13 days' gestation in the opossum makes dosing of the young animal more accurate and may eliminate some maternal influences on enzyme development.

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Neurotoxic action of β-N-oxalyl-L-α,β-diaminopropionic acid

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β-N-OXALYL-L-α,β-DIAMINOPROPIONIC ACID (OX-Dapro) is a recently characterized neurotoxic amino acid, found in the seeds of *Lathyrus sativus*, prolonged consumption of which has been associated with human "neurolathyrism", a crippling disease.[1, 2] The structural requirements for the neurotoxic action of OX-Dapro in day-old chicks has also been recently reported.[3] Curiously enough OX-Dapro does not induce any neurotoxic effects in normal adult animals such as rats or mice.[1] We now report of our studies with this neurotoxin which could provide a new approach towards the study of this crippling disease, since preliminary studies reported here show that the innocuous nature of OX-Dapro to adult animals could be due to an effective blood brain barrier (BBB) system. It has been found that under certain experimental conditions OX-Dapro does induce neurotoxic effects in adult animals, showing that the BBB is altered under such conditions.

While day-old chicks (35–45 g) manifest typical neurological symptoms upon intraperitoneal administration of OX-Dapro* (10–20 mg/chick), adult birds do not respond to the neurotoxin at similar dosages but require higher dosages of the same. Thus a 25-day-old bird (60–70 g) requires a dosage of 1 mg/g to manifest clearcut neurotoxic effects. Adult rats and mice (1–3 months old) do not show any neurotoxic effects upon intraperitoneal administration of OX-Dapro at the level of 1 mg/g. Similar failures were also observed with the adult monkey.

* OX-Dapro used in these studies, isolated as described previously[1] had a melting point of 206°.