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FURTHER STUDIES OF THE MICROSOMAL MIXED-FUNCTION OXIDASE SYSTEM OF THE LITTLE SKATE, *Raja erinacea*, INCLUDING ITS RESPONSE TO SOME XENOBIOTICS

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A previous report from our laboratory demonstrated that hepatic microsomes from the little skate contained significant levels of some xenobiotic and drug-metabolizing enzymes (Bend, Pohl and Fouts, Bull. MDIBL 12: 12, 1972). In the present study the distribution of several of these enzymes in extrahepatic organs of the little skate and some additional properties of the liver microsomal system including its response to the administration *in vivo* of foreign organic chemicals such as phenobarbital, Aroclor 1254 (a polychlorinated biphenyl mixture), and 3-methylcholanthrene, were examined.

Little skates (500-1,400 g) were caught locally and stored in live cars until use. Some extrahepatic tissues including gill, kidney, pancreas, heart, spleen, and stomach lining were passed through a tissue press prior to homogenization. Stomach lining and spiral valve mucosa were removed from the supporting tissue by scraping. Otherwise, microsome preparation was as described for liver (Bull. MDIBL 12: 12, 1972). Benzpyrene hydroxylase, 7-ethoxycoumarin deethylase and N,N-dimethylaniline demethylase activities were assayed as described by Pohl, Bend, Devereux and Fouts (Bull. MDIBL 13: 1973). Aniline hydroxylase and benzphetamine demethylase activities were determined by the quantitation of p-aminophenol and formaldehyde formed respectively (Bull. MDIBL 12: 12, 1972). Cytochrome P_{450} content of microsomes was measured by the dithionite difference technic (Bend, Hook, Easterling, Gram and Fouts, J. Pharmacol. Exp. Ther. 183: 206, 1972).

As shown in Table 1 hepatic microsomes from the little skate possessed the highest specific activities for all four substrates studied. However renal microsomes also exhibited significant activity towards each substrate. The fluorometric assays used for quantitation of 7-ethoxycoumarin and benzpyrene metabolisms are about an order of magnitude more sensitive than the spectrophotometric assays used for tests of the metabolism of aniline and benzphetamine. With 7-ethoxycoumarin, no extrahepatic tissue monitored (except kidney) had any apparent activity although there was benzpyrene hydroxylase activity in microsomes from all tissues studied except spiral valve mucosa. This extrahepatic activity was substantially lower than that found in liver in all cases (expressed per mg microsomal protein).

Several characteristics of the hepatic microsomal xenobiotic metabolizing system of the little skate were also investigated. It was found to be very similar to that of mammals. Thus reduced NADP or NADP with an NADPH generating system was required for maximum activity and NADH or NADP without an NADPH generating system substituted very poorly. Activity was drastically reduced when incubations were carried out in CO plus air or nitrogen atmospheres. Assays of skate liver microsomal suspensions by difference spectroscopy demonstrated the presence of both cytochrome P_{450} and cytochrome b_5. The effect of known *in vitro* inhibitors of the mammalian drug-metabolizing system upon skate liver microsomal metabolism was also measured. Both SKF 525-A

TABLE 1

DRUG-METABOLIZING ACTIVITY OF MICROSOMES PREPARED FROM SEVERAL ORGANS OF THE LITTLE SKATE, *Raja Erinacea*

Organ	Specific activity (nmoles[a] or Units[b] product formed/min/mg microsomal protein)			
	Aniline Hydroxylase[a]	Benzphetamine Demethylase[a]	Benzpyrene Hydroxylase[b]	7-Ethoxycoumarin Deethylase[a]
Liver	0.97*	1.25	0.17	0.48
Kidney	0.52	0.35	0.03	0.23
Gill	0.10	0.12	0.02	<0.001**
Spleen	<0.01**	<0.01**	0.03	<0.001
Spiral valve	<0.01	<0.01	<0.003**	<0.001
Stomach lining	0.04	0.12	0.02	<0.001
Pancreas	---- ***	----	0.03	<0.001
Heart	----	----	0.02	<0.001

* Results are averages of duplicate determinations on pooled tissues from 3 little skates.
** Limits of detection of activity using this procedure.
*** Not measured due to insufficient microsomal protein.

TABLE 2

EFFECT OF SKF 525-A ON THE METABOLISM *In Vitro* OF ANILINE, BENZPHETAMINE AND 7-ETHOXYCOUMARIN BY HEPATIC MICROSOMES OF THE LITTLE SKATE

SKF 525-A (Conc., M)	% Inhibition* of		
	Aniline Hydroxylase	Benzphetamine Demethylase	7-Ethoxycoumarin Deethylase
0	0 (0.99)**	0 (1.12)	0 (0.21)
10^{-3}	49 (0.49)	50 (0.56)	67 (0.07)
5×10^{-4}	----	----	28 (0.16)
10^{-4}	23 (0.76)	13 (0.98)	18 (0.18)
10^{-5}	0 (1.07)	0 (1.12)	0 (0.21)

* Data are from a single experiment that was repeated with similar results.
** (nmoles product formed/min/mg protein)

TABLE 3

EFFECT OF METYRAPONE ON THE METABOLISM *In Vitro* OF ANILINE, BENZPHETAMINE AND 7-ETHOXYCOUMARIN BY HEPATIC MICROSOMES OF THE LITTLE SKATE

Metyrapone (Conc., M)	% Inhibition* of Aniline Hydroxylase	Benzphetamine Demethylase	7-Ethoxycoumarin Deethylase
0	0 (0.99)**	0 (1.12)	0 (0.41)
10^{-3}	88 (0.12)	62 (0.43)	85 (0.06)
10^{-4}	65 (0.35)	23 (0.86)	51 (0.20)
10^{-5}	25 (0.74)	11 (1.00)	23 (0.32)
10^{-6}	13 (0.86)	0 (1.11)	0 (0.40)

* Data are from a single experiment that was repeated with similar results.
**(nmoles product formed/min/mg protein).

(Table 2) and metyrapone (Table 3) were effective inhibitors of skate microsomal xenobiotic metabolism. Taken collectively the above data demonstrate that the skate liver xenobiotic metabolizing system is a cytochrome P_{450}-dependent microsomal mixed-function oxidase system.

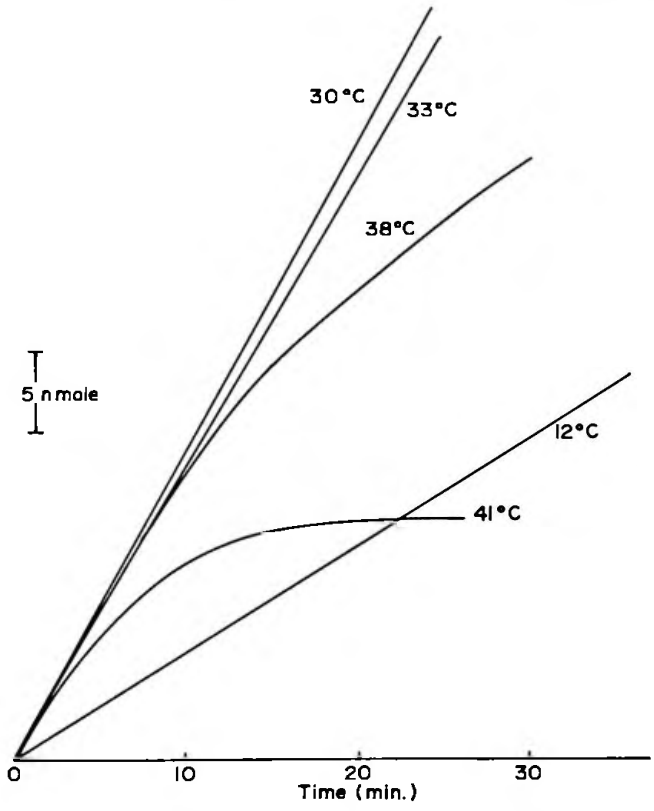

Figure 1. Time course of little skate liver microsomal 7-ethoxycoumarin deethylation at various temperatures.

The effect of incubating fortified skate liver microsomes at several temperatures upon 7-ethoxycoumarin metabolism is illustrated in Figure 1. This substrate was chosen since product formation is monitored continuously. At the approximate summer temperature of Frenchman Bay, Maine (12°C) specific activity was only 35 percent that recorded at the *in vitro* temperature optimum (30°C). As incubation temperatures were increased above the optimum, product formation began to fall off. This was especially true at the highest temperature (41°C) studied where metabolism had virtually ceased after 15 minutes. This loss of activity at higher temperatures is probably related to denaturation of one or more components of the skate liver microsomal electron transport chain.

TABLE 4

ATTEMPTED INDUCTION WITH PHENOBARBITAL OF THE HEPATIC MICROSOMAL DRUG METABOLIZING SYSTEM IN THE LITTLE SKATE

ROUTE, DOSE	DRUG METABOLISM ACTIVITY*	EFFECT UPON CYTOCHROME P-450 CONTENT** (NMOLES/MG PROTEIN)
1. 40 MG/KG IP FOR 3 DAYS (SACRIFICED 60 HRS AFTER LAST DOSE)	SIGNIFICANTLY INHIBITED	CONTROLS: 0.32 ± .09 (4)*** TREATED: 0.31, 0.35
2. 7.5 MG/KG IV FOR 6 DAYS (SACRIFICED 48 HRS AFTER LAST DOSE)	SIGNIFICANTLY INHIBITED	CONTROLS: 0.26 ± 0.07 (4) TREATED: 0.26 ± 0.06 (4)
3. 100 MG/KG ORALLY FOR 2 DAYS (SACRIFICED 96 HRS AFTER LAST DOSE)	SIGNIFICANTLY INHIBITED	----------
4. 100 MG/KG ORALLY FOR 3 DAYS	LETHAL DOSE	LETHAL DOSE

* One or more of the following pathways were measured in each experiment: Aniline Hydroxylase, Benzphetamine Demethylase, 7-Ethoxycoumarin Deethylase and N,N-DI-Methylaniline N-Demethylase.
** There was no increase in Microsomal Protein yield with Phenobarbital administration.
*** Mean ± S.D. (N).

The ability of phenobarbital (PB) to induce protein synthesis and drug metabolism in mammalian liver has been well documented. The effect of PB pretreatment upon liver microsomal mixed-function oxidase activity was examined in the little skate (Table 4). Surprisingly, inhibition rather than induction was found in microsomes isolated from skates after IV, IP or oral doses of PB. This inhibition is likely related to unchanged PB or its metabolites in the microsomes at the time of assay. Cytochrome P_{450} synthesis is also induced by PB administration in mammals. For this reason the cytochrome P_{450} content of hepatic microsomes from pretreated and control skates was compared (Table 4). No increase in the amount of this cytochrome in microsomes from PB-treated skates (vs. controls) was found after IP or IV administration. Thus under our experimental conditions PB did not induce the hepatic microsomal drug-metabolizing system of the little skate.

Similar results occurred with Aroclor 1254. There was no difference in liver microsomal benzphetamine demethylase or aniline hydroxylase activity in skates that were treated orally with 100 mg/kg Aroclor 1254 (in corn oil) for three consecutive days and then examined 48 hours after the last dose as compared to control skates which were treated only with corn oil. Aroclor 1254 is a potent inducer of hepatic microsomal xenobiotic metabolism in several mammalian species (e.g., rats).

TABLE 5

INDUCTION WITH 3-METHYLCHOLANTHRENE OF THE HEPATIC DRUG METABOLIZING SYSTEM IN THE LITTLE SKATE

SUBSTRATE	NMOLES PRODUCT FORMED/MIN/MG PROTEIN* CONTROL	3-MC TREATED**	
ANILINE	0.43 ± 0.09	0.85 ± 0.20	($P < 0.05$)
BENZPHETAMINE	0.96 ± 0.14	1.99 ± 0.18	($P < 0.01$)
BENZPYRENE***	0.084 ± 0.024	0.650 ± 0.021	($P < 0.01$)
7-ETHOXYCOUMARIN	0.36 ± 0.15	0.56 ± 0.14	(N.S.)

* Mean ± S.D. (N = 3).
** Dosed orally, 50 mg/kg in DMSO on day 1 and day 3, assayed day 11.
*** Units product formed/min/mg protein

Polycyclic hydrocarbons such as 3-methylcholanthrene (3-MC) also induce mammalian hepatic and extrahepatic drug-metabolizing systems. Therefore the effect of 3-MC upon skate liver microsomal xenobiotic metabolism was determined (Table 5). In view of the inhibition that was observed following PB treatment (even when examined four days after the last PB dose), eight days were allowed to elapse between the terminal dose of 3-MC and testing. Using this treatment schedule statistically significant increases in hepatic microsomal aniline, benzphetamine, and benzpyrene metabolisms were recorded. Future experiments will characterize the response of the little skate hepatic xenobiotic metabolizing system to 3-MC in more detail including dose-response and treatment schedule-response relationships.

In conclusion these experiments have shown that the skate liver microsomal system differs from the characteristic mammalian system in its inability to respond to PB although it is induced by a polycyclic hydrocarbon, 3-methylcholanthrene.