the food chain. In order to estimate the half-life of this compound in the lobster it will be necessary to study tissue levels for a prolonged time period after administration.

1972 #4

SOME PROPERTIES OF THE MICROSOMAL DRUG-METABOLIZING ENZYME SYSTEM IN THE LITTLE SKATE, *Raja erinacea.*

John R. Bend, Roberta J. Pohl, and James R. Fouts, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina

The marine ecosystem is contaminated with pesticides, industrial chemicals, and other foreign organic chemicals. Consequently the ability of marine animals to metabolize xenobiotics is of interest. We studied xenobiotic metabolism in hepatic and gill tissues of some marine species indigenous to the Frenchman Bay area of Maine including the rock crab *(Cancer borealis)*, lobster *(Homarus americanus)*, little skate *(Raja erinacea)*, dogfish shark *(Squalus acanthias)*, and winter flounder *(Pseudopleuronectes americanus)* and found that aniline hydroxylase and benzphetamine demethylase specific activities were significantly higher in fortified liver microsomes (Table 1) from skate than for winter flounder or dogfish shark. Negligible activities for aniline and benzphetamine metabolisms were found in gill tissue of all species studied and in hepatopancreas from lobster and crab. Since the little skate possessed readily detectable levels of aniline hydroxylase and benzphetamine demethylase, some of the characteristics of these enzyme systems were investigated using the liver microsomal fraction.

TABLE 1

COMPARISON OF LIVER MICROSOMAL ANILINE HYDROXYLASE AND (d)-BENZPHETAMINE DEMETHYLASE ACTIVITIES IN THE LITTLE SKATE, WINTER FLOUNDER AND DOGFISH SHARK

Species	Specific Activity (nmoles product formed/min/mg microsomal protein)	
	aniline hydroxylase	(d)-benzphetamine demethylase
Little skate *(Raja erinacea)*	0.40*	1.41
Winter flounder *(Pseudopleuronectes americanus)*	0.03	0.11
Dogfish shark *(Squalus acanthias)*	0.07	0.27

*Data are from a single, representative experiment.

Skates (500-1, 200 g) were caught locally and stored in live-cars or tanks at MDIBL until used. After severing the spinal cord in several places the liver, minus the large gall bladder, was immediately removed and placed in ice-cold 0.15 M KC1 adjusted to pH 7.5 with Hepes buffer. All subsequent steps were carried out at 0-4° C. The livers were minced with scissors and homogenized in a glass Potter-Elvejhem homogenizer having a motor-drive teflon pestle. Four passes of the pestle (from top to bottom of the homogenizer) were used. The final homogenate was diluted to 33 1/3 percent (w/v) by addition of ice-cold 0.15 M KC1. Cell debris, nuclei, and mitochondria were removed by centrifugation at 10,000 g for 20 minutes. Microsomes were isolated from the 10,000 x g supernatant by sedimentation at 177,700 x g for 45 minutes (Beckman 60 Ti rotor, 50,000 rpm). The resulting pellet was washed by resuspension in 0.15 M KC1 and resedimentation at 177,700 g for 30 minutes. The washed microsomal pellets were resuspended in KC1 to a final protein concentration of 10-20 mg/ml. The Lowry procedure was used for protein measurement (Lowry, Rosebrough, Farr, Randall, J. Biol. Chem. 193, 265, 1951).

Aniline hydroxylase activity was measured by the quantitation of the p-aminophenol formed (Imai, Ito, Sato, J. Biochem (Tokyo) 60, 417, 1966) and d-benzphetamine demethylase activity by determination of the formaldehyde produced using a modified Nash procedure (Cochin and Axelrod, J. Pharmacol. Exp. Ther. 125, 105, 1959). Typical aniline hydroxylase incubation mixtures (2.5 ml) contained 5-10 mg microsomal protein, 0.1 M Hepes buffer, (pH 7.6), 8mM aniline HC1 and an NADPH generating system consisting of 1.0 mM NADP, 5 mM $MgSO_4$, 5 mM glucose-6-phosphate and 2 units glucose-6-phosphate dehydrogenase. Typical benzphetamine demethylase incubation mixtures (2.5 ml) varied only in that d-benzphetamine HC1 (3.0 mM) replaced aniline HC1, 0.67 mM NADP was used and 5 mM semicarbizide HC1 was included in the mixture. The cofactor solution was allowed to generate NADPH for 10 minutes before addition of substrate and microsomes in both cases. Incubations were generally carried out at 28-30° C for 20 minutes in air in a Dubnoff metabolic shaker whose shaking rate was at least 100 rpm.

As shown in Figure 1, aniline hydroxylase activity of skate liver microsomes remained linear with increasing protein concentration up to a microsomal protein content of 10 mg/ml incubation mixture, the highest concentration studied. Similar results were found for benzphetamine demethylase activity (data not shown). This is an interesting species difference, since in rabbit and rat product formation from these activities tends to fall off at protein concentrations above 1-3 mg/ml incubation mixture (Bend, Hook, Easterling, Gram, Fouts, J. Pharmacol. Exp. Ther. 183, 206, 1972). Benzphetamine demethylase (Figure 2) and aniline hydroxylase (data not shown) activities also remained linear for up to 30 minutes and 45 minutes respectively at 20° or 30° C. The lower activities found at 40° C are probably due to protein denaturation. The period of linearity for product formation at the lower temperatures studied is considerably longer than the 15-20 minutes normally found for rabbit liver microsomal benzphetamine demethylase and aniline hydroxylase. Since the summer water temperature of Frenchman Bay is approximately 15° C, it is interesting that the highest specific activities of the microsomal drug-metabolizing enzymes studied occurred between 30° and 37° C.

Substrate concentration-activity curves were determined for both benzphetamine (Figure 3) and aniline (data not shown). Enzyme-saturating substrate concentrations were about 3 mM and 8-10 mM, respectively. The kinetic constants for benzphetamine demethylase activity were evaluated from Lineweaver-Burk plots of the activities recorded in the presence of increasing substrate concentrations. The apparent Km was approximately 0.4 mM and the apparent Vmax was 1.6 nmoles

Figure 1

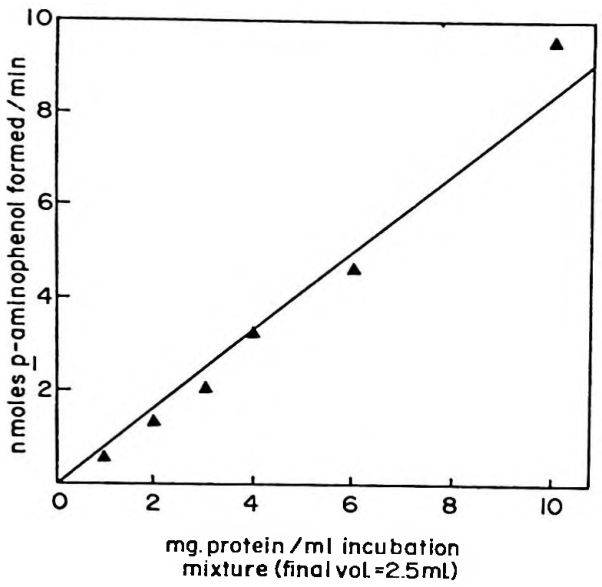


Figure 2

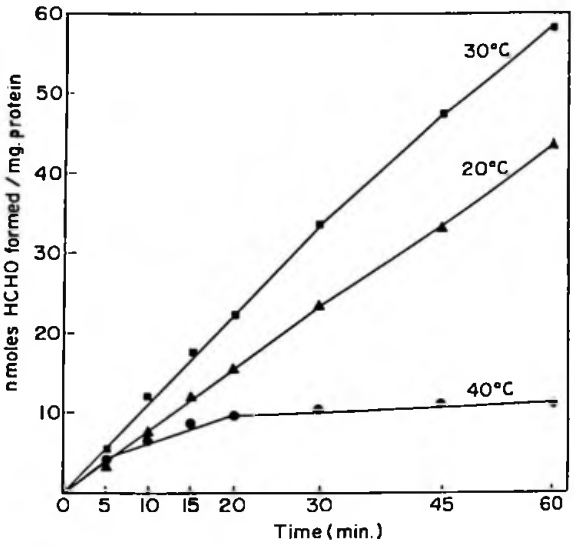


Figure 3

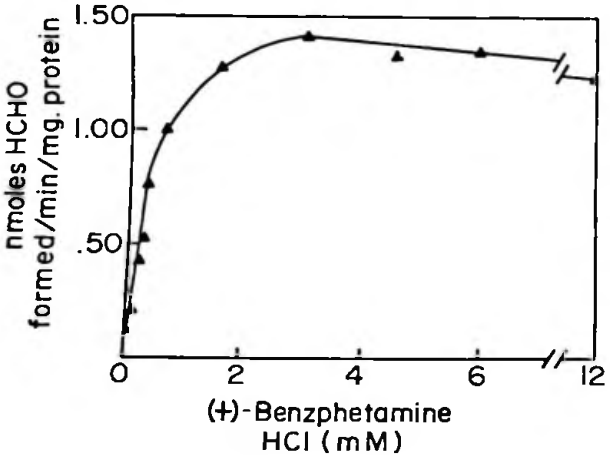
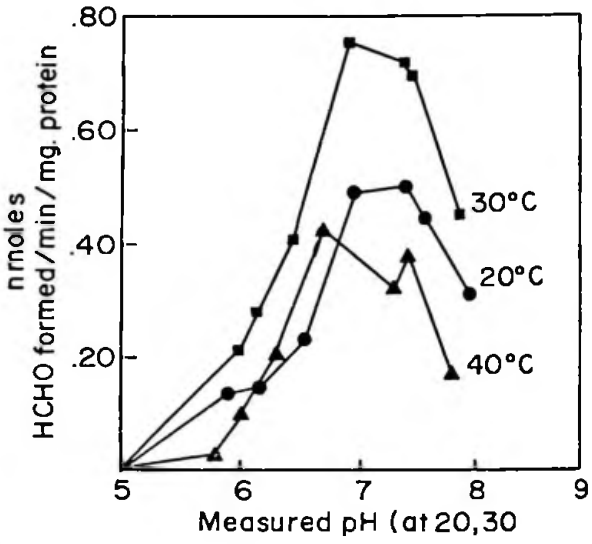


Figure 4



List of Figures

Figure 1. Protein concentration-activity curve of liver microsomal aniline hydroxylase in the little skate. Microsomes were prepared from the pooled livers of two skates.

Figure 2. Effect of incubation time upon liver microsomal benzphetamine demethylase activity in the little skate. Time-course experiments were conducted, in duplicate, at 20°, 30°, and 40°C. Microsomes were prepared from the pooled livers of four skates.

Figure 3. Substrate saturation curve for liver microsomal benzphetamine demethylase in the skate. Microsomes were prepared from a single liver.

Figure 4. pH optima of liver microsomal benzphetamine demethylase in the little skate at 20°, 30°, and 40°C. Microsomes were prepared from the pooled livers of four skates.

HCHO/min/mg protein. In comparison, the apparent kinetic constants for benzphetamine demethylase in male, New Zealand rabbit liver microsomes were 0.13 mM (Km) and 7.9 nmoles HCHO/min/mg protein (Vmax), (Bend, Hook, Easterling, Gram, Fouts, J. Pharmacol. Exp. Ther. 183, 206, 1972).

The effect of NADP concentration in the incubation mixtures upon product formation was also determined. The NADP concentrations normally used in our laboratory for rat liver microsomal incubations (0.67 mM for benzphetamine demethylase and 1.0 mM for aniline hydroxylase) were found to give maximum activities with skate liver. The effect of pH on product formation was also

TABLE 2

EFFECT OF CYTOCHROME c ADDED *IN VITRO* ON LITTLE SKATE LIVER MICROSOMAL ANILINE HYDROXYLASE AND BENZPHETAMINE DEMETHYLASE ACTIVITIES

Cytochrome c (conc., M.)	% Inhibition* of	
	aniline hydroxylase	(d)-benzphetamine demethylase
0	0 (0.36)**	0 (1.01)**
10^{-4}	96 (0.02)	83 (0.17)
10^{-5}	84 (0.06)	44 (0.57)
10^{-6}	8 (0.33)	11 (0.90)

* Data from a single experiment which was repeated with similar results.

** (nmoles product formed/min/mg protein)

measured at 20°, 30°, and 40° for benzphetamine demethylase (Figure 4) and aniline hydroxylase. In general the optimal pH for both activities occurred between pH 7.0 and 7.5 although the pH at 40° C appeared to shift slightly towards the acidic side in both cases. (All pH determinations were made at the incubation temperature.) The effects of cytochrome c, a known inhibitor of mammalian microsomal drug metabolism, on skate liver microsomal aniline hydroxylase and benzphetamine demethylase was also determined (Table 2). Significant inhibition was noted at the highest concentration (10^{-4}M) of cytochrome tested in each case. This data suggests that NADPH cytochrome c reductase is a component of the skate liver microsomal drug-metabolizing electron transport chain as is the case with mammals.

These experiments have demonstrated that the marine species *Raja erinacea* (little skate) has relatively high levels of aniline hydroxylase and benzphetamine demethylase activities in its liver microsomes and that some of the properties of these enzymes from the skate liver differ from those of rat and rabbit livers.