

Table I
Maximum Response to Angiotensins I and II Before and After Converting Enzyme Inhibitor (SQ 20881)

TREATMENT	Pressure		Heart Rate/min	Resp. Rate/min
	Systolic	Diastolic		
1. a) Control	23.7±2.1	17.8±1.4	33.0±2.6	40.1±1.0
b) 20 µg A _I	31.9±2.6	24.6±1.4**	33.3±2.3	40.3±1.2
2. a) Control	22.7±2.0	17.8±1.5	34.0±2.3	36.7±3.8
b) 20 µg A _{II}	33.7±2.4**	26.2±1.4**	35.6±2.3	40.7±1.3
3. a) Control	24.0±1.6	18.3±1.1	33.1±2.4	41.3±1.2
b) After SQ 20881 (3.3 mg total)	23.8±1.5	18.9±1.1	33.3±2.7	40.6±1.1
4. a) Control (After SQ 20881)	22.4±1.2	17.9±0.9	33.9±2.7	41.0±1.3
b) 20 µg A _I	23.2±1.4	18.9±0.9	33.7±2.6	41.4±1.2
5. a) Control (After SQ 20881)	22.1±1.5	17.4±1.1	34.1±2.5	41.3±1.2
b) 20 µg A _{II}	34.3±2.6**	26.4±1.9**	34.6±2.3	41.4±1.4

N=10 ** P<.001

pressor action can be blocked at vascular receptor sites by an alpha adrenergic blocking agent. This research was supported by the Monmouth County Chapter, New Jersey Heart Association Affiliate.

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Solubilization, Separation and Partial Purification of Cytochrome P-450 and Cytochrome b₅ from Hepatic Microsomes of the Little Skate, *Raja erinacea*

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Investigations in our laboratory during the past few summers have shown that the little skate, *Raja erinacea*, has a hepatic drug-metabolizing system which is similar in many ways to analogous mammalian systems (*Bull. MDIBL* 12: 12, 1972; 13:9, 1973; 14:7, 1974). Thus, the little skate is capable of modifying lipophilic xenobiotics to more polar metabolites by a process known as mixed-function oxidation. The hydroxylation reactions carried out are catalyzed by cytochrome P-450, the terminal oxidase of the system. Our present understanding of mammalian mixed-function oxidations has been greatly increased by progress in the solubilization and purification of the membrane-bound components of the system and the reconstitution of hydroxylase activity from the purified components (see review, *Biochem. Biophys. Acta* 344: 205, 1974). We have initiated similar studies on the hepatic drug-metabolizing system of the little skate in order to identify the components of and study the mechanisms of mixed-function oxidation in a marine species. Our progress in solubilizing and partially purifying cytochrome P-450 and cytochrome b₅ from hepatic microsomes of the little skate is reported here.

Little skates were caught locally and stored in live cars for a maximum of three days before use.

Microsomes were prepared from livers (average weight 24.3 g) as previously described (*Bull. MDIBL* 12:12, 1972) and suspended in 0.25 M sucrose for storage under nitrogen at -5 to -10°C. The yield of protein in these preparations was 13.8 mg per g tissue; about 15% of microsomes was obtained in all. The samples were transported to NIEHS packed in dry ice and stored at -20°C until used.

For solubilization, thawed microsomes (about 200 mg protein in 80 ml) were suspended to a final volume of 200 ml in 100mM phosphate buffer, pH 7.7, containing 20% glycerol, 1 mM dithiothreitol (DTT) and 1 mM EDTA. Sodium cholate was then added to a final concentration of 1 mg detergent per mg protein and the suspension was stirred for 30 min at 4°C. The suspension was then centrifuged at 176,000g for 2 hr to precipitate the undigested material. The clear, yellow supernatant fraction was diluted 2-fold with a 20% glycerol solution containing 1 mM DTT and 1 mM EDTA and applied to a column of DEAE-cellulose (2.5 x 45 cm) previously equilibrated with 50 mM phosphate buffer, pH 7.7, containing 1 mM DTT, 1 mM EDTA, and 0.5% cholate (buffer I). Cytochrome P-450 was eluted from the column with buffer I containing 0.3% Emulgen 913, a nonionic detergent.

Fractions containing cytochrome P-450, obtained by ion exchange chromatography on DEAE-cellulose, were combined and diluted 2-fold with a 20% glycerol solution and applied to a column of hydroxylapatite (2.5 x 6 cm) either directly or after a following concentration by ultrafiltration (Amicon, PM 100 membrane). The hydroxylapatite was equilibrated with 25 mM phosphate buffer, pH 7.7, containing 0.5 mM DTT, 0.5 mM EDTA, and 0.25% cholate (buffer II). After application of the sample, the column was washed with 50-100 ml of buffer II and a protein-containing fraction (fraction I) obtained. The cytochrome P-450 (fraction II) was then eluted by increasing the buffer concentration to 140 mM and adding Emulgen 913 to 0.2%.

Cytochromes P-450 and b_5 concentrations were determined by the method of Omura and Sato (*J. Biol. Chem.* 239: 2370, 1964). Soluble cytochrome b_5 was assayed as previously described (*Chem.-Biol. Interactions* 169, 1974). All spectra were recorded with an Aminco DW2 spectrophotometer (American Instrument Co., Silver Springs, Md.).

The results from a typical purification experiment are shown in table 1. Cytochrome P-450 obtained by chromatography on DEAE-cellulose was purified 4- to 6-fold over the microsomal concentration. The CO/dithionite-reduced minus dithionite-reduced difference spectra obtained from these preparations showed the typical peak absorption for cytochrome P-450 (450 nm). These spectra also showed that the cytochrome was somewhat unstable in the presence of dithionite as indicated by a slowly developing peak of absorption near 420 nm. This was indicative of the formation of cytochrome P-450, a denatured form of cytochrome P-450. Examination of absolute spectra obtained from oxidized and dithionite-reduced samples of the preparation from DEAE-cellulose showed that it was contaminated with cytochrome b_5 as well as an unidentified pigment. Cytochrome b_5 is, like cytochrome P-450, a membrane-bound microsomal hemoprotein which may be associated with the mixed-function oxidase system. The presence of an unidentified contaminant was indicated by peaks of absorption at 465 and 490 nm in absolute spectra of the cytochrome P-450 preparations. These peaks are not associated with cytochromes P-450 or b_5 and have not been observed in spectra of cytochrome P-450 preparations from mammalian hepatic microsomes.

When the preparation from the DEAE-cellulose procedure was subjected to ultrafiltration immediately following chromatography, cytochrome b_5 was separated from cytochrome P-450 and was recovered in the filtrate. The filtration procedure was designed to pass through proteins with molecular weights of less than 100,000

daltons. If the samples were stored 2-3 days prior to ultrafiltration, the cytochrome b_5 remained in the concentrate. This result suggested that a time-dependent aggregation of the solubilized proteins was taking place. The unidentified contaminant was retained in the concentrate in either case indicating that it was either tightly bound to cytochrome P-450 or was a macromolecule with a molecular weight in excess of 100,000 daltons.

Chromatography of the concentrated preparations of cytochrome P-450 on hydroxylapatite separated the unknown contaminant from the cytochrome P-450. The pigment was not retained by the hydroxylapatite and could be obtained in a fraction (II) which contained only a trace of cytochrome P-450 (table 1). The absorption spectrum of this pigment had a major peak near 280 nm as well as minor peaks in the visible range at 465 and 490 nm. This material may be associated with the dark coloration (jet-black in some cases) of a number of the skate livers we processed and we suggest that it is probably melanin or a related substance.

The cytochrome P-450 fraction (fraction II) obtained by chromatography on hydroxylapatite contained up to 2.2 nmol cytochrome P-450 per mg protein (table 1). These preparations were free of cytochrome b_5 and contained only a trace of the unidentified pigment. Similar preparations were obtained when the cytochrome b_5 was not removed by ultrafiltration. In such cases the cytochrome b_5 was retained on the column of hydroxylapatite after removal of the cytochrome P-450.

The cytochrome P-450 concentrations reported for solubilized preparations from skate liver (table 1) may be lower than the actual values. Spectral evidence suggested that dithionite was unable to completely reduce the cytochrome in these preparations. The cytochrome P-450 assay procedure is based on the magnitude of the spectrum formed when carbon monoxide is completed with the reduced cytochrome and incomplete reduction will cause an apparent lowering of the cytochrome con-

Table I
Solubilization, Separation and Partial Purification of Cytochrome P-450, Cytochrome b_5 and an Unidentified Pigment from Hepatic Microsomes of the Little Skate, *Raja erinacea*^a

Fraction	Total Protein (mg)	Cytochrome P-450 (nmol)			Cytochrome b_5 (nmol)			Unidentified Pigment
		Total	per mg Protein	% Yield	Total	per mg Protein	% Yield	
Microsomes	2298	670	0.29	100	138	0.06	100	N.D. ^b
Soluble Fraction of the Microsomal Digest	1950	597	0.31	89	N.D.	—	—	N.D.
Deae Eluate	111	145	1.30	22	N.D.	—	—	Present
Filtrate from Ultrafiltration	56	0	0.00	0	97	1.73	70	Absent
Hydroxylapatite Fraction I	5	0	0.00	0	0	0.00	0	Present
Hydroxylapatite Fraction II	9.6	20.8	2.20	3	0	0.00	0	Trace

a. Results are given for a single experiment; similar results have been obtained in additional experiments.
b. N.D. = not determined.

centration. This has been reported to be the case in several mammalian cytochrome P-450 preparations (*Chem-Biol. Interactions* 9:169, 1974; *Pharmacologist* 17:185, 1975). Evidence for incomplete reduction of cytochrome P-450 in preparations devoid of cytochrome b₅ is a peak of absorption at 423 nm in the absolute spectrum of dithionite-reduced preparations complexed with carbon monoxide. A significant peak at 423 nm was observed in the majority of the spectra obtained for skate liver cytochrome P-450.

We have been able to solubilize and partially purify cytochrome b₅ from skate hepatic microsomes. These cytochromes appear to be spectrally identical to those found in mammalian hepatic microsomes. Thus, the little skate, *Raja erinacea*, not only contains a hepatic mixed-function oxidase system that is similar to the analogous system found in mammals, but at least one of the components of the skate and mammalian systems appear to be spectrally identical. Attempts are now underway in our laboratory to purify the other enzymatic component of the skate mixed-function oxidase system, i.e., NADPH-cytochrome c reductase, and to reconstitute skate hydroxylase activity from soluble components.

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Response of Hepatic Microsomal Mixed-Function Oxidases in the Little Skate, *Raja erinacea*, and the Winter Flounder, *Pseudopleuronectes Americanus* to Pretreatment with TCDD (2,3,7,8-Tetrachlorodibenzo-p-Dioxin) or DBA (1,2,3,4-Dibenzanthracene)

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Experiments conducted in our laboratory last summer were designed to measure the response of mixed-function oxidases in little skate liver and kidney to exposure to chemicals which increase benzpyrene hydroxylase (AHH) activities in rats. Interpretation of the data was complicated by wide variability in data from both control and treated skates (*Bull MDIBL* 14, 7-12, 1974). We report here experiments which confirm, statistically, the increase of hepatic AHH activity in little skates and winter flounder after exposure to TCDD and DBA as compared with control activity. Statistical analyses using the

two-sided Mann-Whitney U test were performed by Dr. Joseph Haseman of the Biometry Branch at NIEHS

Little skates (500-1000 gm) were caught locally and stored in live cars. TCDD and DBA were administered interperitoneally. Winter flounder (ca. 250 gm) were caught locally and kept in aquaria with running sea water. Flounder were given oral doses of TCDD by stomach intubation; control fish received vehicle only, i.e. CO₂ oil: acetone (10:2). Microsomes were prepared from liver as previously described (*Bull. MDIBL*, 12, 12-15, 1974). Aniline hydroxylase, *d*-benzphetamine N-demethylase, benzpyrene hydroxylase (BPH) and 7-ethoxycoumarin O-deethylase (7-EC) activities were assayed as previously described (*Bull. MDIBL*, 12, 12-15, 1972; 13, 94-98, 1973). Hepatic microsomal cytochrome P-450 content was assayed at NIEHS by dithionite difference procedure (microsomes were prepared from frozen tissue). The dithionite difference spectra correct for hemoglobin contamination which can be large when frozen livers are used to prepare microsomes (*Bull. MDIBL*, 14, 7-11, 1974). In one case, microsomes were gassed with CO, frozen, and transported to NIEHS (packed in dry ice). Hemoglobin contamination of these microsomes was sufficiently low so that the carbon monoxide-reduced minus reduced spectra could be used for cytochrome P-450 assay (*J. Biol. Chem.*, 239, 2370-2378, 1964). Degradation of cytochrome P-450 to cytochrome P-424 was also less evident in spectra from microsomes prepared from fresh liver and transported frozen than spectra from microsomes prepared from frozen liver segments.

Table 1 shows the effect of orally administered TCDD on microsomal AHH and 7-EC activities in liver of winter flounder. 7-EC activity was increased 2-fold by animal treatment with TCDD. Although AHH activity did not show statistically significant increases after TCDD examination of the four control values revealed one which was very high. If this value were eliminated, significant increases (2-fold) in AHH activity of the TCDD-treated group would be evident. A striking feature of this data is the 10-fold higher AHH control values as compared with data collected from flounder in 1973, i.e., 2.54 ± 1.0 units/min/mg protein (*Bull. MDIBL*, 13, 94-98, 1973; *Drug Metab. Disp.*, 2, 545-555, 1974). Comparison of the AHH control values of little skate seen in Tables 2 and

Table 1

Effect of TCDD Administration on Two Microsomal Mixed-Function Oxidase Activities in Liver of Winter Flounder (*Pseudopleuronectes americanus*)

Enzyme Activity Assayed	Control Fish	TCDD Fish	P
Benzpyrene Hydroxylase ¹	36.7±12.8 (4) ² (17.3,26.6,28.6,74.3)	59.2±4.6 (6)	N.S.
7-Ethoxycoumarin O-Deethylase ³	.199±.068 (4)	.418 ±.070 (6)	<.05

¹Fluorescence Units formed/min/mg microsomal protein

²Mean ± SEM (N)

³Nmoles product formed/min/mg microsomal protein

Flounder were treated orally with 4.5 µg 2,3,7,8-tetrachlorodibenzo-p-dioxin/kg on days 1 and 3. Animals were sacrificed on day 8.