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Microsomal Detoxication Enzyme Responses of the Marine Snail, *Thais haemastoma*, to Laboratory Oil Exposure

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The cytochrome P-450 monooxygenase or mixed function oxidase (MFO) system is a widely distributed enzyme system involved in the detoxication of foreign organic compounds (xenobiotics) taken up by organisms. It is present in marine animals such as fish, crustaceans and polychaetes (Lee, 1981; Stegeman, 1981) and has been identified more recently in bivalve and gastropod molluscs (Livingstone, 1985). Increases in the activities of the MFO system, and in the level of its protein components, occur with exposure of the organism to organic xenobiotics (Lee, 1981; Stegeman, 1981; Kato, 1982) and such responses in the field have been proposed as a means of identifying biological impact by organic pollution (Lee et al., 1980). A number of successful studies have been carried out with fish (e.g. Kurelec et al., 1977; Davies et al., 1984), but information is considerably more limited for molluscs which are widely used organisms in environmental monitoring programmes (Goldberg et al., 1978; Moore et al., 1982; Livingstone, 1984).

The carnivorous marine gastopod Thais haemastoma, or southern oyster drill, is widely distributed along the Gulf coast of the United States where it preys on the American oyster Crassostrea virginica, mussels and barnacles (St. Amant, 1957; Butler, 1953; Belisle and Stickle, 1978). Like other gastropods, it rapidly accumulates polynuclear aromatic and other hydrocarbons from the environment, through both the food source and the water-column (Stickle et al., 1984).

In laboratory experiments T. haemastoma were exposed to the water-soluble fraction (WSF) of South Louisiana crude oil and the responses of the MFO system examined. The exposure conditions were determined from previous toxicity studies where the LC-50 was found to decline from 1332±430 ppb aromatic hydrocarbons after 7 days exposure to 250±10 ppb after 21 days exposure to the WSF of South Louisiana crude oil (values are means ± 95% confidence intervals). Preliminary characterization of the snail MFO system was carried out using methodology developed from studies on the common mussel Mytilus edulis (Livingstone and Farrar, 1984). Microsomal benz[a]pyrene hydroxylase (BPH), NADH- and NADPH-

dependent cytochrome c reductase (NAD(P)H-CYTCRED) and NADH-dependent ferricyanide reductase (NADH-FERRIRED) activities were measured but it was not possible to determine cytochrome P-450 or b_5. Chemical analysis of tissue hydrocarbons was carried out by methods outlined in Stickle et al. (1985).

MATERIALS AND METHODS

Snails were collected from rock jetties in the vicinity of Caminada Pass, La., U.S.A. in April, 1983 and transported to Baton Rouge. Snails were maintained in 10-gal aquaria containing Instant Ocean artificial seawater at 20°/$_{oo}$S at room temperature (22-25°C). The temperature and salinity approximated conditions in the field. Oysters, C. virginica, were provided as food.

Snails were exposed to the WSF of South Louisiana crude oil in a flow-through bioassay system. An extraction apparatus similar to that developed by Nunes and Benville (1978) produced stock water-soluble petroleum hydrocarbon effluent which was then diluted serially to the desired dose concentrations. The total aromatic hydrocarbon concentrations of the water was monitored daily using ultraviolet spectrophotometry (Neff and Anderson, 1975) and the concentration of individual hydrocarbons was analyzed at regular intervals with gas chromatography. The average dosages of hydrocarbons were 0, 270 ± 6, and 867 ± 29 (mean ± SEM) ppb. The flow rate through each tank was maintained at approximately 140 ml min^{-1}

For biochemical analyses, tissues of 3 snails were used for each sample. Three samples were taken per sex in the preliminary characterization study and 4 samples (2 male and 2 female in most cases) for each condition in the oil exposure experiment. Tissues were removed from snails by cracking the shell with a hammer and rapidly dissecting out the digestive gland/gonad complex (DG) and the remaining visceral and muscular mass (RVM) with the foot being discarded. The sex of the snail was determined by the presence or absence of the penis and the morphology of the accessory reproductive tract. The damp-dried tissues were immediately frozen in liquid-nitrogen and stored at -70°C prior to biochemical analysis. Biochemicals were obtained from Sigma and [H^3]-benzo[a]pyrene from New England Nuclear. Mitochondrial, microsomal and cytosolic subcellular fractions were prepared and characterized by marker enzymes (pyruvate kinase (E.C.2.7.1.40), β-N-acetylglucoseaminidase (E.C.3.2.1.30), succinic dehydrogenase (E.C.1.3.99.1) and glucose-6-phosphatase (E.C.3.1.1.9)) as described by Livingstone and Farrar (1984). Only microsomal and cytosolic fractions were prepared in the oil exposure experiment. The marker and reductase enzyme activities were assayed spectrophotometrically, BPH activity radiometrically using [H^3]-benzo[a]pyrene, cytochromes P-450 and b_5 by difference spectroscopy and protein by the Lowry method (see Livingstone and Farrar, 1984). The BPH reaction was linear for about 30 minutes using RVM microsomes.

Snail tissues were processed for aliphatic and aromatic

hydrocarbon analyses according to methods outlined in Stickle et al. (1985). Briefly, snail tissues (9 snails per sample) were digested in 10 N NaOH and hexane at 75°C for three hours. The ratio of NaOH to hexane used was 5:1 and the total volume used for the digestion was adjusted to the tissue weight at a ratio of 10:1 (V:W). After centrifuging, the petroleum hydrocarbons in the supernatant were extracted three times with 10 ml hexane. Any water in the extract was removed with Na_2SO_4 and the volume was reduced to approximately 1 ml in a rotary evaporator. Aliphatic hydrocarbons were then separated from the aromatic hydrocarbons using silica gel column chromatography. Hexane and hexane + mehylene chloride (4:1, V:V) were used respectively, to elute the aliphatic and aromatic hydrocarbons from the column. Samples were concentrated down to approximately one ml by gently vaporizing the solvent with nitrogen and were then analyzed by gas chromatography. All organic solvents used were nano-grade and redistilled. Internal standards were added before tissue digestion in order to adjust for any hydrocarbon loss during sample preparation.

Goups of values were compared by one-way analysis of variance.

RESULTS AND DISCUSSION

Results of the preliminary subcellular characterization study were similar to those obtained for M. edulis (Livingstone and Farrar, 1984) and are not shown here. The distribution of glucose-6-phosphatase indicated that some microsomes co-sedimented with the mitochondrial fraction but that substantial amounts of endoplasmic reticulum were still obtained at 100,000 g in the microsomal pellet. The lysosomes were broken open by the homogenization procedure and the activity of released β-N-acetylglucoseaminidase was considerably higher in DG (16.9 ± 2.0 μmoles min^{-1} g^{-1} wet weight) than in RVM tissues (0.83 ± 0.07 μmoles min^{-1} g^{-1} wet weight)(mean ± SEM, n = 6).

Table 1 Tissue yields of microsomal reductase and BPH activities in male and female T. haemastoma

Enzyme activity	DG		RVM	
	Male	Female	Male	Female
NADH-FERRIRED[a]	6146 ± 375	4090 ± 963	1215 ± 270	671 ± 268
NADH-CYTCRED[a]	783 ± 101	730 ± 131	80.7 ± 4.3	62.3 ± 23.0
NADPH-CYTCRED[a]	113 ± 24	128 ± 11	30.5 ± 4.2	21.9 ± 4.3
BPH[b]	13.1 ± 1.3	1.4 ± 1.3	11.0 ± 2.2	11.3 ± 5.5

a nmoles min^{-1} g^{-1} wet wt. b pmoles total metabolites min^{-1} g^{-1} wet wt. Values are means ± SEM (n=3)

Tissue yields (Table 1) and specific activities (Table 3) of microsomal enzymes of DG tissues were similar to those for M. edulis (Livingstone and Farrar, 1984), and other marine gastropods such as Littorina littorea (Livingstone et al., 1985), with the exception of BPH which was considerably lower in the oyster drill. The reductase activities and microsomal protein yields (Table 3) were higher in DG than in RVM tissues but BPH specific activities were highest in the latter (Table 3). No sex differences were seen with the exception of BPH which was higher in DG tissues of male snails (Table 1), in contrast to M. edulis where the highest BPH activities occurred in female mussels (Livingstone and Farrar, 1984). Attempts were made to measure cytochromes P-450 and b_5 but were unsuccessful, due largely to spectophotometric interference from particulate and lipid material. In the absence of such data it is not possible to conclude that the BPH activity is due to a cytochrome P-450 monooxygenase system, although this is indicated to be the case for other molluscs such as M. edulis and L. littorea (Livingstone, 1985).

The digestive gland is one of the main sites of hydrocarbon uptake in bivalve and gastropod molluscs and the tissue of highest MFO activity in M. edulis (Livingstone, 1985). However, from the limited data, the situation is not clear for the oyster drill. Microsomal protein yields and reductase activities were highest in DG tissues but total BPH activities (Table 1) were similar to or less than those in RVM tissues. The low BPH activity in the DG tissues may be due to seasonal variability of the enzyme activity, as occurs in M. edulis, or to the presence of inhibitors, possibly from the gonad tissue (Livingstone, 1985). The substantial proportion of muscular material in the RVM tissue fraction may indicate that another organ is high in BPH activity. The responses of both tissue fractions were therefore examined in the oil exposure experiment.

Aliphatic, monoaromatic and polyaromatic hydrocarbons were readily taken up into the DG tissues on exposure of oyster drills to WSF of South Louisiana crude oil, and the tissue hydrocarbon concentrations were greater at the higher oil level (Table 2). In contrast, there was very little or no apparent deposition of hydrocarbons in the RVM tissues, presumably reflecting the higher muscular and lower lipid content (Belisle and Stickle, 1978) of this tissue fraction. The Barataria Bay / Caminada Bay system is the site of the Louisiana Offshore Oil Platform (LOOP) terminal, and ongoing oil drilling and other hydrocarbon activities have existed there for several decades to the extent that oysters are found to be contaminated with aromatic hydrocarbons (Miland and Whelan, 1979) and locations within the vicinity of the Barataria Bay - LOOP terminal transect may have water column levels of petroleum hydrocarbons of 20 - 1000 ppb (Whelan, pers. commun.). These facts may account for the relatively high concentration of certain hydrocarbons, such as benzopyrene, in the tissues of control oyster drills (Table 2).

Table 2 The concentration of aliphatic and aromatic hydrocarbons in the tissues of T. haemastoma exposed to WSF of South Louisiana crude oil

Molecule	0ppb WSF (day 0)		270ppb WSF (day 20)		876ppb WSF (day 12)	
	DG	RVM	DG	RVM	DG	RVM
Total aliphatics	205	86	1725	69	6876	74
($C_{8-12'15-16'18'20'25'28}$)						
Monoaromatics						
Toluene	751	3348	8263	46	5763	2945
Ethylbenzene	681	76	1274	24	1084	-
m+p-Xylene	2268	354	5121	128	6964	295
o-Xylene	721	130	2241	530	3556	150
n-Propylbenzene	177	53	319	254	705	49
Mositylene	431	70	1134	35	4633	149
Polynuclear aromatics						
Naphthalene	128	63	1122	101	4081	599
1-Methylnaphthalene	-	-	642	317	4451	402
2-Methylnaphthalene	220	12	1640	396	4960	582
2,6-Dimethyl-naphthalene	232	14	642	95	4466	265
Acenaphthalene	65	21	544	28	220	42
Dibenzothiophene	-	10	158	19	462	-
Anthracene	256	18	120	88	1168	7
Fluoranthrene	-	-	200	-	62	9
Pyrene	50	110	33	136	-	115
Benzanthracene	87	85	142	74	769	40
Benzopyrene	3880	595	1839	451	1591	338
Total aromatics	9884	4959	25434	2722	44935	5987

Concentrations are in ppb on a wet weight basis. DG digestive gland/gonad complex, RVM remaining visceral and muscle tissues less foot

Biochemical changes occurred in both DG and RVM tissues with 12 days exposure of oyster drills to WSF of South Louisiana crude oil (Table 3). Microsomal protein yield remained high in DG tissues but declined in RVM tissus. The most striking changes occurred in the reductase activities which tended to increase in both tissue fractions and more so at the higher oil level, although the high individual variability resulted in only the latter changes being statistically significant. In contrast, no changes ocurred in BPH activity. Total cytosolic protein also increased, indicating a dose-response: the biochemical nature of the protein species involved in this response was not investigated.

Table 3 Changes in microsomal protein, BPH and reductase specific activities and cytosolic protein of tissues of T. haemastoma with exposure to WSF of South Louisiana crude oil

Parameter	Tissue	Control	270ppb	876ppb
Microsomal protein [a]	DG	7.10 ± 0.64	5.82 ± 0.78	8.29 ± 2.40
	RVM	3.95 ± 0.74	1.67 ± 0.50 #	1.67 ± 0.32 #
NADH-FERRIRED [b]	DG	664 ± 80	847 ± 69	756 ± 377
	RVM	180 ± 16	595 ± 151 #	672 ± 116 #
NADH-CYTCRED [b]	DG	85.6 ± 12.2	96.8 ± 11.7	151 ± 51.7 #
	RVM	21.5 ± 3.4	34.3 ± 8.9	65.0 ± 15.0 #
NADPH-CYTCRED [b]	DG	13.8 ± 1.09	16.6 ± 1.9	22.2 ± 5.9 #
	RVM	11.7 ± 1.7	12.0 ± 1.5	13.8 ± 1.6
BPH [c]	DG	1.37 ± 0.46	0.29 ± 0.17	1.75 ± 1.09
	RVM	3.23 ± 0.56	6.30 ± 3.62	2.25 ± 0.56
Cytosolic protein [a]	DG	42.6 ± 5.4	54.5 ± 5.1	55.6 ± 1.8 #
	RVM	18.0 ± 1.9	22.8 ± 7.7	24.3 ± 1.4 #

a mg g^{-1} wet wt. b nmoles min^{-1} mg^{-1} protein
c pmoles total metabolites min^{-1} mg^{-1} protein
Data from preliminary characterization study are included in values for control aminals. Values are means ± SEM (n=10(control) or 4) # $p<0.1$

The responses of the oyster drill were similar to those of a number of other molluscs that have been studied (M. edulis, L. littorea and the cockle Cardium edule) which showed the general trend of response to hydrocarbon exposure of increases in microsomal reductase activity, in particular in NADPH-CYTCRED activity, and in concentrations of cytochromes P-450 and b_5, but no change in BPH activity (Livingstone, 1985). The NADPH-CYTCRED activity is thought to be some measure of the in situ catalytic activity of cytochrome P-450 reductase, a key component of the MFO system (Masters and Okita, 1982). The in vivo significance of increased NADPH-CYTCRED activity in molluscs is unknown at present. It could be important in relation both to detoxication resulting in increased metabolism of organic xenobiotics (either by the contribution of cytochrome-P450 reductase to flux regulation in the MFO system, or possibly by acting as a non-specific monooxygenase itself - Stier, 1976) and to toxication through the involvement of cytochrome P-450 reductase in lipid peroxidation and membrane breakdown (Kappus and Sies, 1982). However, what is clear is that the responses of the oyster drill are consistent with those of other gastropod and bivalve molluscs and that the changes in NADPH-CYTCRED activity, and in cytochromes P-450 and b_5, offer potential for development as specific indices of biological impact by organic pollution for this group of organisms. Particularly encouraging is the agreement between the responses of NADPH-CYTCRED activity and studies in molluscs on the

cytochemically measured NADPH-neotetrazolium reductase activity (Moore, 1980) which is another measure of cytochrome P-450 reductase activity (Masters and Okita, 1982).

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