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# Benzo[a]pyrene oxidation and microsomal enzyme activity in the mussel (*Mytilus edulis*) and other bivalve mollusc species from the Western North Atlantic *

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## Abstract

Analysis of subcellular fractions revealed a complement of microsomal electron transport components including reductases and heme proteins in several organs of the three bivalve species *Mytilus edulis, Macrocallista maculata* and *Arca zebra.* Dithionite difference spectroscopy of CO-treated microsomes yielded spectra typical of cytochrome P-450 in digestive gland and gill, with absorption maxima at 450 nm. A time-dependent reduction of cytochrome P-450 was also observed. The levels of these components and rates of microsomal benzo[a]pyrene (BP) metabolism were highest in the digestive gland, and were very similar between species. In *M. edulis* there was a suggested seasonal variation in BP metabolism but no population differences in this activity or in levels of other components. Digestive gland microsomal metabolites of BP identified by HPLC retention and UV spectroscopy included BP-1.6-quinone, BP-3,6-quinone and BP-6,12-quinone, which comprised 65% of the total metabolites, and dihydrodiols and phenols, the latter products consistent with cytochrome P-450 monooxygenation and epoxide hydrolase function. However, the inconsistent dependence of BP metabolism on NADPH, and inconsistent inhibition by CO suggest that catalyst(s) additional to cytochrome P-450 may be acting in BP metabolism. Based on these results and the prominent quinone formation, we speculate that peroxidative mechanism(s) may be involved. The role of peroxidative as well as monooxygenase reactions in the *in-vivo* disposition and effects of foreign chemicals in bivalves, and also the major function of cytochrome P-450 in these bivalves, remain to be established.

## Introduction

Cytochrome P-450-dependent monooxygenase reactions in many species function in the metabolism of regulatory molecules, such as steroid hormones, and in the oxidative biotransformation of lipophilic xenobiotics. Such metabolism of many xenobiotics, including polynuclear aromatic hydrocarbons (PAH), is involved in both their detoxication and in their activation to toxic derivatives. The components and catalytic functions of microsomal cytochrome P-450-dependent monooxygenase systems are generally similar in vertebrates from teleost fish to mammals, and the involvement of these systems in metabolism of PAH in vertebrates has been clearly established (for reviews see Bend and James, 1978; Stegeman, 1981). Microsomal cytochrome P-450 systems also catalyze xenobiotic monooxygenase activity and PAH metabolism in some marine invertebrate groups, studied principally in crustaceans. However, the occurrence of oxidative xenobiotic metabolism and hydrocarbon metabolism in particular in some other marine invertebrate groups, notably bivalve molluscs, has until recently been a matter of some controversy.

Understanding hydrocarbon metabolism and its characteristics in bivalves may be important due to the use of these organisms in monitoring programs (Goldberg *et al.,* 1979) and the uncertain relationships between environmental PAH and neoplasms in bivalves (Mix, 1983). In earlier studies with the mussel *Mytilus edulis* (Lee *et al.,* 1972; Payne, 1977) and the clam *Mercenaria mercenaria* (Vandermeulen and Penrose, 1978), neither *in-vitro* nor *in-vivo* oxidative metabolism of aromatic hydrocarbons could be demonstrated, prompting some authors to suggest that bivalve molluscs did not possess such a capacity. However, azoreductase activity (Hanzel and Carlson, 1974) and low rates of aldrin epoxidation, p-nitroanisole O-demethylation, antipyrine hydroxylation and benzo[a]pyrene (BP) hydroxylation have been reported in microsomal preparations of tissue from one or another of the mussels *Mytilus californianus* (Krieger *et al.,*

* Some of these results have appeared in preliminary form; J. J. Stegeman, Sea Grant Annual Report, Words Hole Oceanographic Institution, p 15, 1981

1979; Trautman *et al.*, 1979), *Mytilus edulis* and *Modiolus modiolus* (Stegeman, 1980), *Mytilus galloprovincialus* )Ade *et al.*, 1982), and the oyster *Crassostrea virginica* (Anderson, 1978). The nature and characteristics of these activities or their catalysts in molluscs were not fully established, partly due to the low rates observed. However, these activities are known to be catalyzed by cytochromes P-450 in other groups. Recently Livingstone and Farrar (1984) described the levels of microsomal electron transport components, including cytochrome P-450 and several marker enzymes in *Mytilus edulis*, from the coast of England, implicating cytochrome P-450 in catalysis and establishing some characteristics of microsomal systems in one population of *M. edulis*.

Given the potential significance of xenobiotic metabolism to biological effects and the fact that environmental factors, particularly chemistry, are important regulators of this process in some species, it is important that the characteristics of such metabolism be defined in different groups and regions, so as to establish the general features of the oxidative systems in molluscs. In this report we consider the capacity of Mytilus edulis from the east coast of North America to carry out oxidative metabolism of PAH, describing the characteristics of microsomal systems in these bivalves. These systems are also described in two additional bivalve species. Factors contributing to variability of BP metabolism in the mussel *M. edulis* are considered, and the pattern and identity of BP metabolites formed *in vitro* by *M. edulis* tissue preparations are described. The results with *M. edulis* are compared to results obtained for this same species from a site in Great Britain.

## Materials and methods

*Mytilus edulis* were obtained from several localities around Cape Cod, Massachusetts and from the Island of Nantucket, Massachusetts. The mussels were sampled at various times of the year during 1978–1981. The individuals sampled ranged in size from 6 to 8 cm in length and were generally held in flowing seawater for a period of 2 to 24 h prior to dissection. Calico clams (*Macrocallista maculata*) and Bermuda mussel (*Arca zebra*), each about 8 cm in length, were obtained during August 1982, from Harrington Sound, Bermuda, held in ambient water, and dissected within hours after collection. Dogfish shark (*Squalus acanthias*) were obtained from local Cape Cod waters and liver was dissected within hours after capture.

The bivalves were opened and organs were dissected and placed in ice-cold buffer. Gonadal tissues, and in some cases other organs, from males and females were pooled separately. Otherwise, organs from several individuals were pooled without regard to sex. Crystalline style was removed from the digestive gland immediately upon dissection. Organs were weighed and homogenized in ice-cold TRIS-Cl buffer, 0.05 M, with 0.15 M KCl, ph 7.4, using a Potter-Elvehjem tissue grinder. Subcellular fractions were obtained by differential centrifugation of homogenates according to a protocol described earlier for preparation of subcellular fractions from teleost organs (Stegeman *et al.*, 1979). Microsomal fractions were resuspended in 50 m*M* Tris-Cl, pH 7.4, containing 1 m*M* EDTA, 1 m*M* dithiothreitol and 20% glycerol by volume. Blood was withdrawn from the foot, cells sedimented at 5 000×g for 10 min, evaluated microscopically, resuspended in three volumes of homogenizing buffer and homogenized in a Dounce hand-held mortar and pestle. Blood cell homogenates were analyzed without further fractionation. All preparations were used directly or were stored in liquid $N_2$ until use.

### Enzyme assays

Benzo[a]pyrene metabolism was assessed by analyzing fluorescence of phenolic derivatives (Nebert and Gelboin, 1968) or by determining the total production of water soluble derivatives of uniformly labelled $^3$[H]-BP (New England Nuclear). The latter was accomplished by a microscale radiometric procedure described previously (Binder and Stegeman, 1980), using a reaction mixture volume of 50 $\mu$l and substrate (BP) concentration of 30 $\mu M$. Incubations were carried out at 29 °C and pH 7.4, conditions that were determined in this laboratory to be optimal for *Mytilus edulis* digestive gland activity, and under conditions of linearity with time and protein. When employed, $\alpha$-naphthoflavone (ANF) was added in 2 $\mu$l of MeOH. Prostaglandin synthetase-dependent cooxygenation was evaluated using reaction conditions above, but with 100 $\mu M$ arachidonate or linoleic acid replacing NADPH. Blanks consisted of reaction mixtures minus cofactor or complete reaction mixtures with heat-inactivated enzyme, which was prepared in a way that avoided precipitation of protein or evaporation of buffer. Assays were carried out in triplicate under red light.

Ethoxyresorufin (ER) O-deethylase activity was measured by spectrophotometric assay (Klotz *et al.*, 1984) using a 0.5-ml reaction containing 2 $\mu M$ ER, 0.1 M NaCl, and 3 to 8 mg of microsomal protein per ml. Substrate concentration, pH and ionic strength used were optimal. The reaction at 25 °C was initiated with NADPH at a final concentration of 0.5 m*M* and the appearance of resorufin was monitored at 572 nm using a Cary 118C recording spectrophotometer. Activity was calculated using an extinction coefficient of 73 mmol $l^{-1}$ $cm^{-1}$ for resorufin at 572 nm.

NADPH-cytochrome *c* (P-450) reductase activity was assayed at 25 °C by a modification of the method of Phillips and Langdon (1962) with a reaction mixture containing 0.175 m*M* NADPH, 80 $\mu M$ horse heart cytochrome *c* and 1 m*M* KCN in 0.2 M potassium phosphate buffer, pH 7.7. NADH-cytochrome *c* ($b_5$) reductase activity was assayed using the conditions for NADPH-cytochrome *c* reductase, with 0.40 m*M* NADH replacing the NADPH. Succinate-cytochrome *c* reductase activity was assayed as before (Stegeman *et al.*, 1979). Reac-

tion mixtures of 0.50-ml volume contained 10 mg bovine serum albumin per ml, 80 $\mu M$ cytochrome *c*, and 1 m*M* KCN in 0.02 M phosphate buffer, pH 7.4. Reactions at 25 °C were initiated by addition of sodium succinate to a final concentration of 5 m*M*. Reduction of cytochrome *c* at 550 nm was followed using a Cary 118C recording spectrophotometer.

Cytochrome $b_5$ was estimated from NADH difference spectra obtained as before (Stegeman *et al.*, 1979). Cytochrome P-450 was estimated from $Na_2S_2O_4$-difference spectra of CO-treated samples that had also been balanced with respect to NADH, thus eliminating contribution of both cytochrome $b_5$ and hemoglobin to absorbance near 420 nm. Reduction was followed over 20 min. Extinction coefficient for putative cytochrome P-420, i.e. any peak of absorbance remaining at 420 to 428 nm, was 111 m$M^{-1}$ $cm^{-1}$. Resuspensions for analysis of both cytochrome $b_5$ and P-450 contained 0.3 to about 1 mg microsomal protein per ml.

Practical limits of detection for the assays as used, dependent on the amount of microsomal protein, were: cytochrome P-450, 0.005 nmol $mg^{-1}$ microsomal protein; cytochrome $b_5$, 0.005 nmol $mg^{-1}$; reductases, 0.25 nmol cytochrome *c* reduced $min^{-1}$ $m^{-1}$; ER O-deethylase, 1.0 pmol resorufin $min^{-1}$ $mg^{-1}$; BP metabolism, 0.3–1.2 pmol $min^{-1}$ $mg^{-1}$. Protein was determined according to the method of Lowry *et al.* (1951).

## Metabolite analysis

Metabolites of [$^3$H]-BP were obtained by incubation *in vitro* with digestive gland microsomes. Reaction mixtures of 2.0 ml final volume containing 30 $\mu M$ BP with a specific activity of about 3.22 GBq $mmol^{-1}$ (87 mC $mmol^{-1}$), 0.375 mg NADPH $ml^{-1}$, 50 m*M* Tris pH 7.4, and about 3 mg microsomal protein $ml^{-1}$ were incubated for 30 min at 25 °C. Reactions were stopped with one volume cold acetone, and 6B-OH-testosterone was added as an internal standard. The metabolites were extracted twice with 1.5 volumes of ethyl acetate, the extracts pooled, dried under $N_2$ and dissolved in 100 $\mu$l methanol. Blank reactions with heat-inactivated enzyme were treated in the same fashion. Metabolites were resolved on a DuPont Zorbax C-18 column, using a modification of the gradient protocol of Morgenstern *et al.* (1982) with solvent A, water, and solvent B, methanol:ethanol (2:1). The gradient was 55 to 65% B over 15 min, then 65 to 75% B over 25 min, the flow rate was 1.5 ml $min^{-1}$ and the column oven temperature was 35 °C. Fractions eluting from the column were dried at 45 °C, redissolved in Scintaverse II and [$^3$H] was quantified by counting in a Beckman LS-100 C scintillation counter. Label eluting from equivalently treated blank reactions was subtracted and values that were three times the blank values or greater were considered detectable.

Metabolites were identified by coelution with authentic BP metabolite standards that had been coinjected with the microsomal metabolites, and by UV spectral analysis. Peaks eluting from injections without standards were analyzed and compared to results obtained for authentic standards that were similarly injected, collected and analyzed. Analyses were performed in a Cary 118-dual beam UV/visible spectrophotometer scanning from 600 to 200 nm. Appropriate regions of column eluent from a gradient run following injection of methanol only were used in the reference cuvette. All procedures were carried out under red light.

## Results

Both NADPH-cytochrome *c* reductase activity and BP metabolism were localized principally in the microsomal fractions prepared from most organs analyzed, exemplified by the distribution in gill and digestive gland depicted in Fig. 1. NADH-cytochrome *c* reductase was localized predominantly in the mitochondrial fraction of some organs (e.g. gill, Fig. 1), but was prominent in both mitochondrial and microsomal fractions of digestive gland (Fig. 1) and of mantle and foot. By contrast, succinate cytochrome *c* reductase activity was clearly localized in the mitochondrial fraction prepared from various organs. This activity was, however, very low being less than 1 nmol $min^{-1}$ $mg^{-1}$ protein in gill and mantle and less than 2 nmol

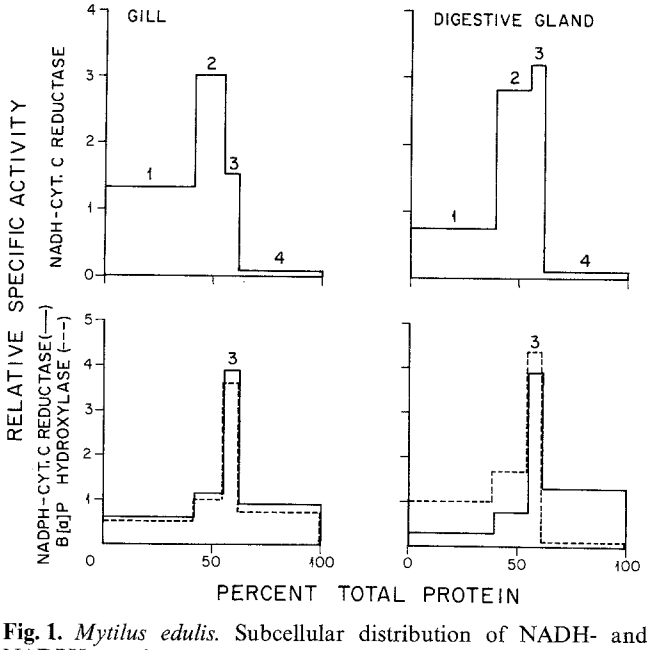


**Fig. 1.** *Mytilus edulis.* Subcellular distribution of NADH- and NADPH-cytochrome *c* reductase and benzo[a]pyrene hydroxylase activities in tissues. Values are means of determinations on two samples with tissues pooled from more than ten specimens in each. Data for benzo[a]pyrene hydroxylase activity were obtained both radiometrically and fluorometrically. 1 = nuclear fraction; 2 = mitochondrial fraction; 3 = microsomal fraction; 4 = cytosolic fraction. Relative specific activity calculated according to de Duve *et al.* (1962)

**Table 1.** *Mytilus edulis.* Microsomal enzyme activities in various organs

| Microsome[a] source | Microsomal yield (mg $g^{-1}$ organ) | NADH-cyt. *c* reductase (units $mg^{-1}$)[b] | NADPH-cyt. *c* reductase (units $mg^{-1}$)[b] | Benzo[a]pyrene metabolism (units $mg^{-1}$)[b] |
|---|---|---|---|---|
| Digestive gland | 5.6 | 46 | 7.6 | 0.035 |
| Gill | 3.1 | 16 | 3.2 | 0.013 |
| Mantle[c] | 2.6 | 28 | 2.6 | ND |
| Posterior adductor | 0.9 | 52 | 2.1 | ND |
| Foot[c] | 0.8 | 18 | 2.8 | 0.012 |
| ♂ gonad[d] | 1.2 | 5 | 5.5 | 0.003 |
| ♀ gonad[d] | 6.1 | 113 | 4.9 | – |

[a] Data obtained with microsomes from organs pooled from 14 individuals except for gonads, which were from 7 individuals. Mantle was from individuals that were not gonadally mature. Values are means of 4 replicates and variation in each assay was about 15%. ND, not detectable

[b] Units are nmol product produced per minute

[c] NADPH-cytochrome *c* reductase was localized most prominently in the cytosolic fraction of foot (relative specific activity 4.1) and mantle (relative specific activity 1.45), but microsomal specific activities are indicated for comparison

[d] Contribution of gametes cannot be distinguished from non-gametic gonadal material

**Table 2.** Cytochrome *c* reductase activities and benzo[a]pyrene metabolism in *Mytilus edulis* and *Stenotomus chrysops*

| Sample | Succinate-cyt. *c* reductase (units $g^{-1}$ organ)[a] | NADH-cyt. *c* reductase (units $g^{-1}$ organ) | NADPH-cyt. *c* reductase (units $g^{-1}$ organ) | BP metabolism (units $g^{-1}$ organ)[a] |
|---|---|---|---|---|
| *M. edulis,* dig. gl.[b] | | | | |
| mitochondria | 2 | 454 | 9 | – |
| microsomes | ND | 266 | 54 | 0.203[d] |
| *M. edulis,* gill | | | | |
| mitochondria | 2 | 120 | 6 | – |
| microsomes | ND | 58 | 13 | 0.047 |
| *S. chrysops.* liver[c] | | | | |
| mitochondria | 1 021 | 793 | 291 | – |
| microsomes | 19 | 2 555 | 1 463 | 13.0 |

[a] Units are nmol cyt. *c* reduced or nmol BP metabolites produced per minute in mitochondrial or microsomal fractions normalized to a gram of organ weight

[b] Data for *M. edulis* are means of values obtained for two separate samples with tissues pooled from 10 and 14 mussels each. The range was 30% or less

[c] Data for the marine teleost scup (*Stenotomus chrysops*) were taken from Stegeman *et al.* (1982)

[d] Values for BP metabolism were obtained by analysis of $^3$H-BP-derivatives. The same preparations analyzed for fluorescent products yielded specific activities of 0.05 units $g^{-1}$ for mussel digestive gland and about 5.0 for scup liver

$min^{-1}$ $mg^{-1}$ protein in mitochondria of every other organ examined except ovary, which had about 15.0 nmol $min^{-1}$ $mg^{-1}$ mitochondrial protein. Nevertheless, the subcellular distributions are consistent with those seen in most other eucaryotic systems.

Rates of BP metabolism and NADPH- and NADH-cytochrome *c* reductase activities in microsomes prepared from several organs of *Mytilus edulis* (Table 1) were highest in digestive gland and were also prominent in gill, particularly in light of the greater yield of microsomal protein in these as compared to most other organs. BP metabolism was also detected in whole homogenates of blood cells ($0.032 \pm 0.013$ nmol $min^{-1}$ $ml^{-1}$; SD, $N=3$), consistent with the observations of Moore *et al.* (1980). The total activities in mitochondrial and microsomal fractions normalized per gram of organ in gill and digestive gland, as compared to teleost liver, (Table 2) further describe the subcellular distribution of these activities in *M. edulis* tissues. The data indicate an apparent distinction in mitochondrial function based on succinate-cytochrome *c* reductase activity, and give a measure of the total capacity for BP metabolism in these organs.

## Cytochromes P-450 and $b_5$

Given the suggested role of digestive gland and gill in aromatic hydrocarbon metabolism, the levels of microsomal electron transport components in several species,

**Table 3.** Microsomal heme protein and enzyme activities in bivalve mollusc species

| Microsome sample | Microsomal yield (mg $g^{-1}$ organ) | Cytochrome P-450 (nmol $mg^{-1}$)[a] | Cytochrome $b_5$ (nmol $mg^{-1}$) | NADPH-cyt *c* reductase (U $mg^{-1}$)[b] | NADH-cyt *c* reductase (U $mg^{-1}$)[b] |
|---|---|---|---|---|---|
| *Mytilus edulis* | | | | | |
| digestive gland | 5.0±2.5[c] | 0.101±0.025 | 0.037±0.014 | 8.1±0.5 | 41.6±3.2 |
| gill | 3.3±0.2 | 0.024±0.001 | 0.013±0.003 | 3.0±0.3 | 13.8±2.1 |
| *Macrocallista maculata* | | | | | |
| digestive gland | 4.9 | 0.079 | 0.081 | 4.0 | 148 |
| gill | 1.5 | –[d] | 0.016 | 2.0 | 26 |
| *Arca zebra* | | | | | |
| digestive gland | 2.9 | 0.106 | 0.076 | 8.3 | 65 |
| gill | 1.6 | 0.089 | 0.010 | 5.4 | 59 |

[a] These values are for native cytochrome P-450. Putative cytochrome P-420 values in the various microsome samples were in order, 0.039±0.019, 0.100, 0.077, 0.039 and 0.037 nmol $mg^{-1}$

[b] Units (U) are nmol cytochrome *c* reduced per minute per mg microsomal protein

[c] Values for *M. edulis* dig. gl. are means for 6 pooled samples of 5 to 10 individuals each, from Scorton Creek, MA, ± SD and for gill are from 2 to 4 pooled samples of 4 to 10 individuals in each case, ± SD. Values for other species are means of replicate assays on single pooled samples from 7 to 10 individuals

[d] Not analyzed

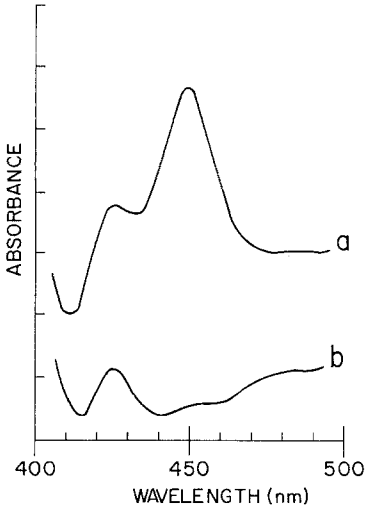


**Fig. 2.** *Mytilus edulis.* Difference spectra of digestive gland microsomal cytochrome P-450 and cytochrome $b_5$. Spectra were obtained as outlined in "Materials and methods" on a sample that contained 0.63 mg $ml^{-1}$ of microsomal protein. Absorption maxima were 450 and 424 nm on the P-450 spectrum. Maxima and minima were 425 and 415 nm on the $b_5$ spectrum

the blue mussel, the Bermuda mussel and the Calico clam were compared (Table 3). These data, which include the first values for cytochrome P-450 in gill of bivalves, reveal a strong similarity between species. The spectra for NADH-reduced cytochrome $b_5$ were typical and unambiguous, and the values presumably reflect content. The levels of cytochrome P-450 indicated in Table 3 might not, however, represent the total P-450 present, as a chromophore representing or including cytochrome P-420 was evident in each preparation (Fig. 2). Other investigators have reported such a chromophore in bivalve digestive gland microsomes (Gilewicz *et al.*, 1984; Livingstone and Farrar, 1984), and it might reflect a stability of P-450 different from that in vertebrate membrane preparations. However, we found that the putative P-420 content was variable, and in some preparations was only 5% of the P-450 content, much less than that seen by others. We also noted a time-dependence in the reduction of P-450 by dithionite, reaching maximal spectral signal at 20 min. There was a concomitant disappearance of the peak at 420, suggesting that some estimates of either P-450 or P-420 could reflect incomplete reduction of P-450, and a shift in a balance of some conversion.

## Requirements for activity

The typical suite of flavoprotein reductase activity and hemoproteins in mussel organ microsomes complements other characteristics of BP metabolism including dependence on native protein, NADPH and molecular $O_2$, and an inhibition by CO (Table 4), in indicating a hemoprotein monooxygenase could catalyze this activity in several organs. But whereas a dependence on native protein was always observed, dependence on NADPH was ephemeral, with some preparations having activity without NADPH that was about twice that obtained when NADPH was added (Table 4). Further, incubations with NADPH and cytochrome *c*, which efficiently competes for electrons from NADPH, also had activity that was about twice the activity with NADPH alone. No effect of CO was observed in some of these same samples. The higher activity in the absence of NADPH could have several possible origins, including an endogenous source of reducing power, or perhaps stimulation of some competitive pathway by NADPH. A physical influence of NADPH on partitioning was considered, but studies to assess an influence of NADPH on partitioning of $^3$H-BP were also inconclusive. The possibility of a seasonal distinction in these features (Table 4) is intriguing.

**Table 4.** *Mytilus edulis.* Requirements for benzo[a]pyrene metabolism

| Conditions | Digestive gland microsomes-A[a] | Blood cell homogenate-A[a] | Digestive gland microsomes-B[b] |
|---|---|---|---|
| Complete | 100±10% | 100± 9 | 100 |
| Heated enzyme | 35± 9 | 22± 4 | 46±20 |
| Minus NADPH | 52± 7 | – | 231±75 |
| $N_2$[c] | 53± 2 | 46±11 | – |
| $CO_2:O_2$, 80:20[c] | 24± 2 | 19± 6 | – |

[a] Blood and digestive gland samples were separately pooled from the same 10 mussels obtained in July and prepared and analyzed fresh. Values represent means of 4 replicate assays ± SD of the assay, normalized to complete reaction micture as 100%. Activity in digestive gland was 5 pmol $min^{-1}$ $mg^{-1}$ and in blood cells was 19 pmol $min^{-1}$ $ml^{-1}$ whole homogenate

[b] Samples were obtained in Feb–March and prepared and analyzed fresh. Values are means of determinations on 3 separate pools, ± SD, with more than 5 individuals in each pool

[c] $N_2$ or CO and $O_2$ were added to the atmosphere of sealed reaction vessels by repeated evacuation and replacement with defined gas mixtures

**Table 5.** Benzo[a]pyrene metabolism and ANF inhibition in bivalve mollusc microsomes

| Microsome sample | B[a]P Metabolism (nmol $min^{-1}$ $mg^{-1}$) | % Activity with 100 $\mu$m ANF |
|---|---|---|
| *Mytilus edulis* | | |
| digestive gland | 0.011±0.020[a] | 52±11[b] |
| gill | 0.004±0.002 | – |
| *Macrocallista maculata* | | |
| digestive gland | 0.009±0.002 | 62± 3 |
| gill | – | – |
| *Arca zebra* | | |
| digestive gland | 0.010±0.001 | 109±13 |
| gill | 0.020±0.001 | – |

[a] Average of values on 7 separate preparations, ranging in activity from ND to 0.035 nmol $min^{-1}$ $mg^{-1}$, ± SD, and gill was the average of 3 values ± SD. Values for other species are means of replicate assays on single preparations, ± SD of the assay

[b] Data obtained with 4 replicate assays on a single preparation

## Population and seasonal Variation

Uncertainties concerning mechanism notwithstanding, the metabolism of BP was clearly associated with the microsomal fraction. The characterization of this fraction could thus indicate the nature of or capacity for xenobiotic metabolism in comparative studies. Metabolism of BP and the effect of ANF *in vitro* were similar in the three species of bivalves (Table 5) with low BP metabolism rates, were highly variable in *Mytilus edulis,* and had modest or no ANF inhibition. EROD activity was below the limits of detection in all cases but one, in which digestive gland microsomes had activity at about 5 pmol $min^{-1}$ $mg^{-1}$. The rates of BP metabolism in *M. edulis* sampled from different regions around Cape Cod revealed little variation that could be ascribed to environmental characteristics. Thus, the rates of BP metabolism in digestive gland were similar, about 1–5 pmol $min^{-1}$ $mg^{-1}$, in animals sampled at the same time of year from creosote-treated pilings near abundant ship traffic in Woods Hole, at Scorton Creek (a putative "clean" site on the north of Cape Cod), and from areas near Nantucket Island where hydrocarbon residues were proven low[1]. Likewise, the levels of digestive gland cytochromes P-450 and $b_5$ were similar at these sites, e.g. at Woods Hole, 0.066 and 0.025 nmol $mg^{-1}$ and at Scorton Creek 0.075 and 0.044 nmol $mg^{-1}$ for cytochromes P-450 and $b_5$ respectively. By comparison, fish sampled near Nantucket and Woods Hole showed marked differences in rates of BP metabolism, with those near Woods Hole exhibiting apparent strong induction ascribed to environmental chemicals (Stegeman, unpublished observations). The small differences in mussels could just reflect the low BP metabolism rates. Nevertheless, the data suggest that if environmental factors do influence microsomal systems in *M. edulis,* this does not necessarily result in obvious differences in activity between populations.

There was a suggestion that BP metabolism within a single population could vary with season. Levels of activity were highest during the periods of August–September, were barely detectable during December and January, and began to increase in February, reflecting reproductive cycles (Bayne *et al.,* 1976). The lowest levels of activity were apparently not the result of any endogenous monooxygenase inhibitor in molluscan microsomal preparations. Addition of 75 $\mu$g of *Mytilus edulis* digestive gland microsomal protein having ca 0.5 pmol $min^{-1}$ $mg^{-1}$ BP metabolism, to 200 $\mu$g hepatic microsomal protein from dogfish did not alter BP hydroxylase activity in the latter. Addition of the protease inhibitor phenylmethane sulfonyl fluoride just prior to homogenization did not influence the activity in resulting microsomes, although some inactivation might result from release of lysosomal hydrolases during homogenization and membrane isolation.

[1] Polychlorinated biphenyl residues in mussels from Nantucket determined by Farrington *et al.* (1982b) were 20 to $80 \times 10^{-9}$ g $g^{-1}$ dry wt, the lowest seen in Massachusetts, and petroleum hydrocarbons were undetectable, ($< 3 \times 10^{-6}$ g $g^{-1}$ dry wt; Farrington, personal communication)

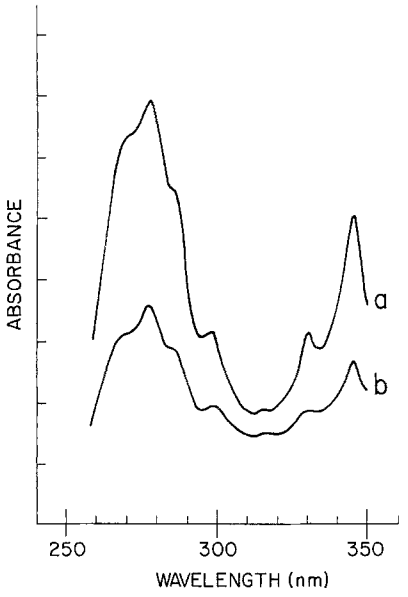


**Fig. 3.** *Mytilus edulis.* UV spectra of benzo[a]pyrene metabolites. (a) digestive gland metabolite. (b) authentic 9,10-dihydrodiol. Compounds were obtained from HPLC as described in "Materials and methods". Compare also to spectrum 38 in McCaustland *et al.* (1976)

**Table 6.** *Mytilus edulis.* Metabolites of benzo[a]pyrene formed *in vitro* by *M. edulis* digestive gland microsomes

| Metabolite[a] | % of total[b] |
|---|---|
| 9,10-Dihydrodiol | 0.8± 0.7[b] |
| 4,5-Dihydrodiol | 1.2± 0.5 |
| 7,8-Dihydrodiol | 1.0± 0.9 |
| 1,6-Quinone | 28.6± 0.3 |
| 3,6-Quinone | 13.1± 7.1 |
| 6,12-Quinone | 23.0± 6.0 |
| 9-hydroxy | 8.5± 0.5 |
| 7-hydroxy[c] | 21.0± 17 |

[a] Metabolites were obtained in 30-min incubations carried out as described in "Materials and methods"

[b] Values are means of 2 separate analyses, ± range. Total activity was like that in Table 6; less than 2% of substrate was metabolized during the incubation

[c] 1-OH, 3-OH and 7-OH-BP coelute in this fraction

### Benzo(a)pyrene metabolites

Metabolites of BP formed *in vitro* by *Mytilus edulis* digestive gland microsomes included those precisely coeluting with 9,10-, 4,5- and 7,8-dihydrodiols, 1,6-, 3,6- and 6,12-quinones, and 9-OH and 7-OH-BP. The identity of these metabolites was further indicated by the close similarity between the UV spectra obtained for *M. edulis* metabolites, with those obtained for authentic derivatives similarly resolved, collected and analyzed. Figure 3 provides the spectral analysis for the first of these, the 9,10-dihydrodiol, but similar data were obtained for the quinones and for 9-OH-BP. The data for 4,5- and 7,8-dihydrodiol and the 7-OH-BP peaks were complicated by other chromophores, although the indicated identities were suggested. Three additional peaks having strong absorbance between 250 to 370 nm did not coelute with any standards. Tritium counts were also present at low levels in these peaks. The three quinone derivatives were the predominant metabolites, accounting for 60 to 70% of the total ethyl acetate-extractable metabolites formed (Table 6), while the benzo-ring dihydrodiols comprised less than 2% of the total metabolites and phenolic derivatives amounted to about 30% of the total.

## Discussion

The complement and levels of microsomal enzymes, including a capacity for BP metabolism, seen in different organs of bivalve species from both temperate and subtropical waters indicate a similarity in these systems in bivalves. The tissue distribution seen here and by Livingstone and Farrar (1984) and the rates of BP metabolism seen in other bivalve genera (Anderson, 1978; Stegeman, 1980) are sufficiently similar so that one can conclude not only that BP metabolism occurs broadly in the bivalvia, but that the digestive gland also possesses the major activity, and that *in-vitro* rates are generally similar. The data here also establish a strong similarity in microsomal enzymes between *Mytilus edulis* from both sides of the Atlantic. Rates of BP metabolism, normalized to protein or organ weight, and the levels of microsomal cytochrome *c* (P-450 and $b_5$) reductase activities and cytochromes P-450 and $b_5$ in *M. edulis* from Massachusetts were approximately the same as those (averaging values for males and females) observed in this species from British waters (near Plymouth) by Livingstone and Farrar (1984), suggesting that the levels are characteristic for this species.

A typical suite of microsomal components, the characteristics and requirements of BP metabolism seen here and by others (Livingstone and Farrar, 1984), and the spectrum of reactions reportedly catalyzed by bivalve microsomes, including epoxidation, demethylation and hydroxylation (e.g. Trautman *et al.*, 1979) indicate a role for cytochrome P-450 in oxidative metabolism of xenobiotics in this group. Certain BP metabolites identified here, particularly BP-9,10-dihydrodiol and the 9-OH-BP, indicate further that cytochrome P-450 is likely involved as a catalyst and, coincidentally, are consistent with the presence of epoxide hydrolase, previously reported in bivalves (James *et al.*, 1979). However, the fact that dependence on NADPH and inhibition by CO were not consistently evident suggests the possibility that catalysts additional to cytochrome P-450 could be involved in hydrocarbon metabolism in bivalves. This is further considered below.

There have been suggestions that BP metabolism or the levels of cytochrome P-450 can be induced in bivalves

by exposure to hydrocarbon compounds. Anderson (1978) reported 2- to 3-fold increased rates of BP metabolism in *Crassostrea virginica* exposed to 3-methylcholanthrene (3-MC) or BP, but the response was not consistently seen. Gilewicz *et al.* (1984) reported increased levels of cytochrome P-450 in *Mytilus galloprovincialis* treated with a variety of hydrocarbon compounds, but known inducers, including 3-MC, had little effect, and both the variability and the mechanism of response, important to interpretation, were not apparent. Furthermore, one cannot infer the nature of any catalytic function merely from the aggregate levels of cytochrome P-450. The catalytic evidence of some ANF inhibition of BP metabolism seen here and by Livingstone and Farrar (1984) might indicate activity of a cytochrome P-450 similar to those MC-induced forms in vertebrates, but EROD activity, catalyzed by those same forms, was virtually undetectable. The possibility that metabolism of foreign compounds might be induced in bivalves by exposure to environmental chemicals requires substantiation. At present, the lack of conclusive evidence for induction of metabolism like that in other systems, the low levels of xenobiotic metabolism often difficult to measure, and the uncertain mechanism of reported elevation of cytochrome P-450 levels indicate that these systems in bivalves are not yet suitably understood for their analysis to indicate effects of exposure to xenobiotics in the environment.

The seasonal pattern seen here, though not strong, and the sex differences in *Mytilus edulis* BP metabolism reported by Livingstone and Farrar (1984), suggest that a biological variable, presumably hormonal, might regulate the catalysts for BP metabolism in bivalves. Hormonal regulation of monooxygenase systems has been documented in aquatic vertebrates (Stegeman *et al.*, 1982), has been implied in some invertebrate species (Singer and Lee, 1979) and could be involved in bivalves. That seasonal variation might also be related to organic contaminants is suggested by a seasonal pattern in residues of PAH in other *M. edulis* (Mix and Schaffer, 1979) that is inverse to the changes in metabolism. Variation in residue levels could stem either from seasonal variation in the input of contaminants or to a seasonal release of contaminants, and it is conceivable that rates of hydrocarbon metabolism could be a factor in determining the residue levels of compounds like BP.

Whether or not the rates of hydrocarbon metabolism might be regulated in part by environmental chemicals or by hormonal factors, the rates observed in various studies, could be sufficient to have an effect *in vivo* on levels of those contaminants subject to metabolism. Considering contribution by all organs, we might calculate a capacity of 200 ng BP transformed $d^{-1}$ $g^{-1}$ animal. There have been speculations that changes in amount and/or composition of hydrocarbon residues in bivalves, including those species studied here, could be influenced by metabolism (Stegeman and Teal, 1973; Farrington *et al.*, 1982a; Solbakken *et al.*, 1982). However, real relationships between *in vitro* activity and *in-vivo* disposition are not known, and there is no evidence demonstrating that biotransformation processes are quantitatively significant to the bulk of hydrocarbon residues either present in animals in the field or experimentally introduced to animals in the laboratory. This leaves uncertain the mechanism(s) for disposition, and although partitioning is still indicated, further studies on this question are needed.

The predominant *in-vitro* formation of BP-dione derivatives seen here is similar to the pattern of metabolism reported for another bivalve, the oyster *Crassostrea virginica* (Anderson, 1978), although in that study multiple quinone derivatives were not resolved. The present study provides the first indication of metabolite identity based on properties other than HPLC retention. The origin of the 1,6- and 3,6-quinone derivatives could include the autoxidation of 1-hydroxy- and 3-hydroxy-BP. This has been suggested in teleost systems, but only prominently under conditions of low protein not pertaining here, and even then no 6,12-Q was observed (Stegeman *et al.*, 1984). The occurrence of all three quinones here suggests that they may be also arising by metabolism at the 6-carbon of BP, which can result in the formation of all three dione derivatives (Lesko *et al.*, 1975). If cytochrome P-450 is responsible for this metabolism, it would appear to be highly specific for oxidation at C-6, or involve peroxidatic function of cytochrome P-450. Oxidation at the 6-carbon can proceed by various pathways involving peroxidative mechanisms, including co-oxygenation by prostaglandin synthetase (Marnett *et al.*, 1980), lipid peroxidation (possibly involving NADPH cytochrome *c* reductase) (Morgenstern *et al.*, 1981) or other endoperoxidase mechanisms (Sloan, 1981). Given that some data allow for a catalyst in addition to cytochrome P-450, it is attractive to speculate that some, such as lipid peroxidative mechanisms or prostaglandin synthetase, which apparently does occur with seasonal variation in molluscs (Ono *et al.*, 1982), may be responsible. Preliminary studies in our hands have indicated a stimulation of BP metabolism by arachidonic acid but not linoleic acid added to digestive gland microsomes. However, metabolite identity is not known here, and endogenous substrate, e.g. arachidonic acid or $PGG_2$, would still be required to fit the observations that some activity proceeds without added cofactors.

The significance of PAH metabolism to the appearance of neoplastic disease in bivalve molluscs is not clear, although in light of the prevalence of leukemia-like diseases, the appearance of BP metabolism in blood cells is intriguing. In *Mytilus edulis,* neoplasms were not correlated with BP residues in any case but one (Mix *et al.*, 1981), and the slight metabolism on the benzo-ring argues against much formation of highly mutagenic diol-epoxides, particularly in light of uncertainties about *in-vivo* metabolism. However, BP-quinones could exert some mutagenic activity (Chesis *et al.*, 1984), and Anderson and Döös (1983) observed that the clam (*Mercenaria mercenaria*) digestive gland had a slight capacity to activate BP to bacterial mutagens. More importantly, they observed a substantial activation of several aromatic amines (e.g. 2-

acetylaminofluorene) to mutagens, suggesting a possible role for such compounds in environmental neoplasms in molluscs, a role deserving of exploration. It is also interesting that this activation, which generally proceeds by N-hydroxylation, was not inhibited by CO (Anderson and Döös, 1983), indicating again that some catalyst other than cytochrome P-450 could be involved. Those results are consistent with flavoprotein (amine) monooxygenase activity (Ziegler and Mitchell, 1972), which is not inhibited by CO but does catalyze N-oxidations, and we suggest that this activity will be found to be prominent in bivalves.

Regardless of the influence of xenobiotic metabolism on residue levels or tumorigenesis in bivalves, the presence of such activity holds substantial interest from the standpoint of comparative biochemistry and phylogeny of mechanisms for xenobiotic metabolism. Of further interest are questions of the major biological role(s) of that cytochrome P-450 apparently present, and the function of oxygenases during periods of anaerobic metabolism common to many bivalves.

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## Literature cited

Ade, P., M. G. Banchelli Soldaini, M. G. Castelli, E. Chiersara, F. Clementi, R. Fanelli, E. Funari, G. Ignesti, A. Marabini, M. Oronesu, S. Palmero, R. Pirisino, A. Ramundo Orlando, V. Silano, A. Viarengo and L. Vittozzi: Comparative biochemical and morphological characterization of microsomal preparations from rat, quail, trout, mussel and *Daphnia magna*. *In:* Cytochrome P-450. Biochemistry, biophysics and environmental implications, pp 387–390. Ed. by E. Hietanen, M. Laitinen and O. Hanninen. Amsterdam: Elsevier Biomedical 1982

Anderson, R. S.: Benzo[a]pyrene metabolism in the American oyster *Crassostrea virginica*. EPA Ecol. Res. Ser. Monogr. (EPA-600/3-78-009) 1978

Anderson, R. S. and J. E. Döös: Activation of mammalian carcinogens to bacterial mutagens by microsomal enzymes from a pelecypod mollusk, *Mercenaria mercenaria*. Mutat. Res. *116*, 247–256 (1983)

Bayne, B. L., R. J. Thompson and J. Widdows. *In:* Marine mussels: their ecology and physiology, Ch. 5. Ed. by B. L. Bayne. Cambridge: Cambridge, Univ. Press 1976

Bend, J. R. and M. O. James: Xenobiotic metabolism in marine and freshwater species. *In:* Biochemical and biophysical perspectives in marine biology, Vol. 4, 128 pp. Ed. by D. C. Malins and J. R. Sargent, New York: Academic Press 1978

Binder, R. L. and J. J. Stegeman: Induction of aryl hydrocarbon hydroxylase activity in embryos of an estuarine fish. Biochem. Pharmacol. *29*, 949–951 (1980)

Chesis, P. L., D. E. Levin, M. T. Smith, L. Ernster and B. N. Ames: Mutagenicity of quinones: pathways of metabolic activation and detoxification. Proc. natl Acad. Sci. *81*, 1696–1700 (1984)

de Duve, C., R. Wattiaux and P. Baudhin: Distribution of enzymes between subcellular fractions in animal tissues. Adv. Enzymol. *24*, 291–358 (1962)

Farrington, J. W., A. C. Davis, N. M. Frew and K. S. Rabin: No. 2 fuel oil components in *Mytilus edulis:* retention and release after an oil spill. Mar. Biol. *66*, 15–26 (1982 a)

Farrington, J. W., R. W. Risebrough, P. L. Parker, A. C. Davis, B. de Lappe, J. K. Winters, D. Boatwright and N. M. Frew: Hydrocarbons, polychlorinated biphenyls, and DDE in mussels and oysters from the U.S. coast, 1976–1978 – the mussel watch. Woods Hole Oceanog. Inst. Tech. Rept. WHOI-82-42 (1982 b)

Gilewicz, M., J. R. Guillaume, D. Carles, M. Leveau and J. C. Bertrand: Effects of petroleum hydrocarbons on the cytochrome $P_{450}$ content of the mollusc bivalve *Mytilus galloprovincialis*. Mar. Biol. *80*, 155–159 (1984)

Goldberg, E. D., V. T. Bowen, J. W. Farrington, G. Harvey, J. H. Martin, P. L. Parker, R. W. Risebrough, W. Robertson, E. Schneider and E. Gamble: The mussel watch. Environ. Conserv. *5*, 2 (1979)

Hanzel, M. E. and G. P. Carlson: Azoreductase activity in the hard clam, *Mercenaria mercenaria* (L.). J. exp. mar. Biol. Ecol. *14*, 225–229 (1974)

James, M. O., E. R. Bowen, P. M. Dansette and J. R. Bend: Epoxide hydrase and glutathione S-transferase activities with selected alkene and arene oxides in several marine species. Chem. Biol. Interact. *25*, 321 (1979)

Klotz, A. V., J. J. Stegeman and C. Walsh: An alternative 7-ethoxyresorufin O-deethylase activity assay: a continuous visible spectrophotometric method for measurement of cytochrome P-450 monooxygenase activity. Analyt. Biochem. *140*, 138–145 (1984)

Krieger, R. I., S. J. Gee, L. O. Lim, J. H. Ross and A. Wilson: Disposition of toxic substances in mussels (*Mytilus californianus*): preliminary metabolic and histologic studies. *In:* Pesticide and xenobiotic metabolism in aquatic organisms, pp 259–277. Ed. by M. A. Q. Khan, J. J. Lech and J. J. Menn. Am. Chem. Soc. Symp. Ser. *99*, 1979

Lee, R. F., R. Sauerheber and A. A. Benson: Petroleum hydrocarbons: uptake and discharge by the marine mussel *Mytilus edulis*. Science, N.Y. *177*, 344–346 (1972)

Lesko, S., W. Caspary, R. Lorentzen and P. O. P. Ts'o: Enzymatic formation of 6-oxobenzo[a]pyrene radical in rat liver homogenates from carcinogenic benzo[a]pyrene. Biochemistry *14*, 3978–3984 (1975)

Livingstone, D. R. and S. V. Farrar: Tissue and subcellular distribution of enzyme activities of mixed-function oxygenase and benzo[a]pyrene metabolism in the common mussel *Mytilus edulis* L. Sci. tot. Environ. *39*, 209–235 (1984)

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall: Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193*, 265–275 (1951)

Marnett, L. J., M. J. Bienkowski, M. Leithauser, W. R. Pagels, A. Panthananickal and G. A. Reed: Prostaglandin synthetase-dependent cooxygenation. Prostaglandins and cancer: First Int. Conf., pp 97–111. New York: A. R. Liss, Inc. 1982

McCaustland, D. J., D. L. Fischer, K. C. Kolwyck, W. P. Duncan, J. C. Wiley, Jr., C. S. Menon, J. F. Engel, J. K. Selkirk and P. P. Roller: Polycyclic aromatic hydrocarbon derivatives: synthesis and physicochemical characterization. *In:* Carcinogenesis, vol. 1, Polynuclear aromatic hydrocarbons: Chemistry, metabolism, and carcinogenesis, pp 349–411. Ed. by R. I. Freudenthal and P. W. Jones. New York: Raven Press 1976

Mix, M. C.: Haemic neoplasms of bay mussels, *Mytilus edulis* L., from Oregon: occurrence, prevalence, seasonality and histopathological progression. J. Fish Dis. *6,* 239–248 (1983)

Mix, M. C. and R. L. Schaffer: Benzo[a]pyrene concentrations in mussels (*Mytilus edulis*) from Yaquina Bay, Oregon during June 1976–May 1978. Bull. environ. Contam. Toxicol. *23,* 677–684 (1979)

Mix, M. C., R. L. Schaffer and S. J. Hemingway: Polynuclear aromatic hydrocarbons in bay mussels (*Mytilus edulis*) from Oregon. *In:* Phyletic approaches to cancer, pp 167–177. Ed. by C. J. Dawe, J. C. Harshbarger, S. Kondo, T. Sugimura and S. Takayama. Tokyo: Japan Scientific Societies Press 1981

Moore, M. N., D. R. Livingstone, P. Donkin, B. L. Bayne, J. Widdows and D. M. Lowe: Mixed function oxygenases and xenobiotic detoxication/toxication systems in bivalve molluscs. Helgoländer wiss. Meeresunters. *33,* 278–291 (1980)

Morgenstern, R., J. W. DePierre, C. Lind, C. Guthernberg, B. Mannervik and L. Ernster: Benzo[a]pyrene quinones can be generated by lipid peroxidation and are conjugated with glutathione by glutathione S-transferase B from rat liver. Biochem. Biophys. Res. Commun. *99,* 682–690 (1981)

Morgenstern, R., C. Guthernberg, B. Mannervik, J. W. DePierre and L. Ernster: Benzo[a]pyrene metabolism by rat liver microsomes: effects of adding purified glutathione S-transferase A, B, and $C_1$. Cancer Res. *42,* 4215–4221 (1982)

Nebert, D. W. and H. V. Gelboin: Substrate inducible microsomal aryl hydrocarbon hydroxylase in mammalian cell culture. I. Assay and properties of induced enzyme. J. Biol. Chem. *234,* 6242–6249 (1968)

Ono, K., M. Osada, T. Matsutani, K. Mori and T. Nomura: Gonadal prostaglandin $F_2$ profile during sexual maturation in the oyster, *Crassostrea gigas* Thunberg, Mar. Biol. Lett. *3,* 223–230 (1982)

Payne, J. F.: Mixed function oxidases in marine organisms in relation to petroleum hydrocarbon metabolism and detection. Mar. Pollut. Bull. *9,* 112–116 (1977)

Phillips, A. H. and R. G. Langdon: Hepatic triphosphopyridine nucleotide-cytochrome *c* reductase: isolation, characterization and kinetic studies. J. Biol. Chem. *237,* 2652–2660 (1962)

Singer, S. C. and R. F. Lee: Mixed-function oxygenase activity in the blue crab *Callinectes sapidus:* tissue distribution and correlation with changes during molting and development. Biol. Bull. mar. biol. Lab., Woods Hole *153,* 377–386 (1977)

Sloan, N.: Enzymic hydroxylation of benzo[a]pyrene at the 6-position by vitamin $K_1$-hydroperoxide and rat lung hydroperoxidase. Xenobiotica *11,* 267–274 (1981)

Solbakken, J. E., F. M. H. Jeffrey, A. H. Knap and K. H. Palmork: Accumulation and elimination of [9-$^{14}$C]phenanthrene in the Calico clam (*Macrocallista maculata*). Bull. environ. Contam. Toxicol. *28,* 530–534 (1982)

Stegeman, J. J.: Mixed-function oxygenase studies in monitoring for effects of organic pollution. Rapp. P.-v. Reun. Cons. int. Explor. Mer *179,* 33–38 (1980)

Stegeman, J. J.: Polynuclear aromatic hydrocarbons and their metabolism in the marine environment. *In:* Polycyclic hydrocarbons and cancer, Vol. 3, pp 1–60. Ed. by H. V. Gelboin and P.O.P. Ts'o. New York: Academic Press 1981

Stegeman, J. J., R. L. Binder and A. Orren: Hepatic and extrahepatic microsomal electron transport components and mixed-function oxygenases in the marine fish *Stenotomus versicolor.* Biochem. Pharmacol *28,* 3431–3439 (1979)

Stegeman, J. J., A. M. Pajor and P. Thomas: Influence of estradiol and testosterone on cytochrome P-450 and monooxygenase activity in immature brook trout, *Salvelinus fontinalis.* Biochem. Pharmacol. *31,* 3979–3989 (1982)

Stegeman, J. J. and J. M. Teal: Uptake, release and retention of petroleum hydrocarbons by the oyster *Crassostrea virginica.* Mar. Biol. *22,* 37–44 (1973)

Stegeman, J. J., B. R. Woodin and R. L. Binder: Patterns of benzo[a]pyrene metabolism by varied species, organs and developmental stages of fish. J. nat. Cancer Inst. Monogr. *65,* 371–377 (1984)

Stegeman, J. J., B. R. Woodin, A. V. Klotz, R. W. Wolke and N. R. Orme-Johnson: Cytochrome P-450 and monooxygenase activity in cardiac microsomes from the fish *Stenotomus chrysops.* Mol. Pharmacol. *21,* 517–526 (1982)

Trautman, T. D., S. J. Gee, R. I. Krieger and T. Thongsinthusak: Sensitive radioassay of microsomal O-demethylation of $^{14}CH_3O$- or $C^3H_3O$-p-nitroanisole for comparative studies. Comp. Biochem. Physiol. *63C,* 333–339 (1979)

Vandermeulen, J. H. and W. R. Penrose: Absence of aryl hydrocarbon hydroxylase (AHH) in three marine bivalves. J. Fish. Res. Bd Can. *35,* 643–647 (1978)

Ziegler, D. M. and C. H. Mitchell: Microsomal oxidase IV: properties of a mixed-function amine oxidase isolated from pig liver microsomes. Arch. Biochem. Biophy. *150,* 116–125 (1972)

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