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Aquatic animals are exposed to a variety of natural and

anthropogenic xenobiotics. Biotransformation of xenobiotics was

examined in three aquatic animals: a primitive mollusc (chiton);

a shellfish which is an important human food source (oyster);

and, a lower vertebrate model for toxicological studies (rainbow

trout). Since digestive glands of Cryptochiton stelleri

possessed cytochrome P-450 (P-450), various Phase II conjugation

and flavin-containing monooxygenase (FMO) activities, primitive

marine molluscs have the appropriate enzymes to cope with organic

xenobiotics. Studies examining gill and visceral mass microsomes

from the oyster (Crassostrea gigas) demonstrated strong evidence

for FMO and co-oxidation pathways and possible involvement of P-

450. FMO and one electron co-oxidation pathways appear to

catalyze N-oxidation of 2-aminofluorene in microsomes from oyster

visceral mass suggesting that, like the chiton, multiple enzymes

are involved in xenobiotic biotransformation in the oyster. In

rainbow trout, (Oncorhynchus mykiss), the in vitro and in vivo

biotransformation of aldicarb was examined to help determine the

role of FMO in this species. Since FMO was responsible for the

formation of the major metabolite, aldicarb sulfoxide, this enzyme

plays a significant role in the biotransformation of nitrogen and

sulfur-containing chemicals. FMO enzyme activity was used in

conjunction with immunochemical quantitation to observe the tissue

distribution, sexual and developmental differences of trout FMO.

Immunoquantitation of FMO was directly correlated with enzyme

activity. These data showed that trout FMO is structurally and

biochemically related the mammalian forms of FMO. Since the trout

has been used as an alternative vertebrate model to study

mammalian toxicology, the demonstration of FMO in this species

will provide a better understanding of the enzymatic basis for the

biotransformation of nitrogen and sulfur-containing chemicals.

Xenobiotic Biotransformation
in Aquatic Organisms

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XENOBIOTIC BIOTRANSFORMATION IN AQUATIC ORGANISMS

INTRODUCTION

Aquatic organisms are exposed to a host of toxic natural and anthropogenic chemicals (Faulkner, 1977; Mix, 1984). A better understanding of the mechanisms involved in foreign chemical or "xenobiotic" biotransformation is essential in assessing the toxicity of these chemicals to any organism.

Once a chemical enters an organism, the chemical can be either eliminated unchanged, spontaneously degraded, or undergo the most common fate, enzymatic biotransformation (Caldwell, 1980). Two general pathways are responsible for the biotransformation of xenobiotics, Phase I and Phase II reactions (Williams, 1967). Phase I reactions involve either oxidation, reduction, and hydrolysis, followed by the second phase (Phase II) in which the original compound or metabolite is linked with an endogenous molecule in a conjugation reaction. It is important to note that not all compounds undergo both processes; some products of Phase I reactions are eliminated unchanged. Likewise, some chemicals can bypass Phase I reactions and be acted upon solely by Phase II processes. Products of xenobiotic metabolism are generally less reactive, more water soluble, and, consequently readily eliminated. However, often in an attempt to make chemicals more water soluble, certain Phase I and Phase II reactions can occur that actually make the chemical more reactive and usually more toxic. Therefore, the ratio of detoxification pathways versus

bioactivation pathways is critical when examining the toxicity of any given chemical ingested by an organism (Buhler and Williams, 1988). Since the following dissertation primarily focused upon Phase I pathways, only the major Phase I enzymes will be discussed.

The most dominant enzyme involved in Phase I xenobiotic metabolism is the cytochrome P-450 system (Sato and Omura, 1978). Cytochrome P-450 activity has been observed throughout phylogeny from humans (Distlerath and Guengerich, 1987) to bacteria (Poulos, 1986). By far, the bulk of the studies involving cytochrome P-450 have focused upon mammalian systems (Guengerich, 1987a). Cytochrome P-450 was initially identified by G.R. Williams who found that when microsomes (experimentally fragmented endoplasmic reticula) were treated with a reducing agent and carbon monoxide, the preparation absorbed light at 450 nm (Sato and Omura, 1978). In metazoan organisms, the tissue possessing the highest levels of cytochrome P-450 is primarily the liver or organs with homologous function (i.e. digestive gland). However, at least in mammals, cytochrome P-450 has also been found at varying concentrations in kidney, lung, skin, testis, adrenal cortex and small intestine (Sato and Omura, 1978).

The reaction mechanism of cytochrome P-450 has yet to be completely elucidated (Ortiz de Montellano, 1986a). Substrates of cytochrome P-450 appear to either covalently bind or come into close proximity to an iron atom in a high-spin 3 + state residing within a heme moiety associated with a hydrophobic pocket within

the protein. As mentioned above, the protein is bound to membranes of the smooth endoplasmic reticulum. The mechanism of catalysis involves the reduction of iron to the ferrous form which is dependent upon reducing equivalents transferred from primarily NADPH via a flavoprotein (NADPH-cytochrome P-450 reductase) which is thought to be adjacent to the heme-containing protein serving several distinct cytochrome P-450 isozymes (Peterson and Prough, 1986). Once the iron has been reduced, dioxygen comes into contact with the iron and is split with one atom forming water and the other atom becoming bound covalently to the heme iron. Following a second reduction, possibly catalyzed via cytochrome b_5 transfer of an electron from NADH, the covalently bound oxygen is then transferred to the substrate leading to a monooxygenated product and ferric iron.

The major reactions catalyzed by cytochrome P-450 include carbon monooxygenation (aliphatic hydroxylation and aromatic hydroxylation, alkene and alkyne epoxidation) nitrogen dealkylation and oxidative deamination, oxygen dealkylations, nitrogen and sulfur oxidations, along with oxidative desulfuration, dehalogenation and denitrification reactions (Wislocki et al., 1980). The reductive reactions of cytochrome P-450 include nitro, azo, tertiary amine N-oxide, and arene oxide reductions, as well as reductive dehalogenation. Consequently, since cytochrome P-450 can perform so many functions, the substrate specificity of cytochrome P-450 is extremely broad (Yang and Lu, 1987). Cytochrome P-450 exists as several distinct forms

(Sato and Omura, 1978). Although some overlap has been observed, each form is characterized by its own substrate specificity and the ability of certain substrates to induce specific forms (Guengerich, 1987b). For example, in mammalian liver, administration of polyaromatic hydrocarbons induces primarily a single form of cytochrome P-450, while administration of phenobarbital induces a completely different isozyme (Bresnick, 1980).

Besides xenobiotic biotransformation, cytochrome P-450 is also responsible for steroid metabolism and fatty acid catabolism (Sato and Omura, 1978). Nebert and Gonzalez (1987) have postulated that steroid biosynthesis and degradation along with fatty acid metabolism were the primary roles for cytochrome P-450 in prokaryotic and early eukaryotic organisms. Later in evolution, as animals evolved digestive and excretory systems, cytochrome P-450 became an enzyme responsible for detoxification of numerous foreign substances which were probably plant metabolites.

Although most studies of cytochrome P-450 have focused on mammals (Guengerich, 1987a), there has been much interest regarding cytochrome P-450 in fish stimulated by the recent utilization of fish as alternative vertebrate models to study carcinogenesis (Hendricks, 1982). Cytochrome P-450 isozymes have been purified and well characterized in rainbow trout (Williams and Buhler, 1982; Williams et al., 1986; Miranda et al., 1989), the scup (Klotz et al, 1983) and the atlantic cod (Goksoyr, 1985).

Since marine invertebrates have been shown to bioaccumulate polyaromatic hydrocarbons (Mix, 1984), and these compounds induce certain cytochrome P-450 activities (Livingstone and Farrar, 1984), cytochrome P-450 and its related activities in marine invertebrates have been proposed as biomonitors for polyaromatic hydrocarbon pollution (Payne et al., 1987). There is good evidence for the occurrence of cytochrome P-450 in invertebrates (see Lee, 1981 and Livingstone, 1985 for reviews). Cytochrome P-450 has been partially purified from the digestive glands of Mytilus edulis (Kirchin et al., 1988) and of the spiny crab, Maja crispata (Batel et al., 1986). Cytochrome P-450 activity has been characterized in the spiny lobster, Panulirus argus (James, 1984), the mussel Mytilus galloprovinciallis (Gilewicz et al., 1984), the southern oyster drill Thais haemostoma (Livingstone et al., 1986), the clams, Mercenaria mercenaria (Anderson and Angel, 1986) and Macrocallista maculata, Arca zebra, (Stegeman, 1985), the oyster Crassostrea virginica (Anderson and Angel, 1986), the snail, Littorina littorea, (Livingstone et al., 1985), the blue crab Callinectes sapidus (Lee, 1981), the fiddler crab, Uca pugnax, (Lee, 1981), the polychaete worms, Nereis virens, Capitella capitata, Nereis succinea, (Lee, 1981), the lobster, Hommarus americanus, (Bend et al, 1977), the barnacle, Balanus erbuneus, (Lee, 1981), and the echinoderms, Asterias and Strongylocentrotus, (Payne and May, 1979). However, it is still unclear whether cytochrome P-450 plays a dominant role in foreign chemical metabolism in invertebrates (Stegeman, 1985). Very few xenobiotic

metabolism studies have been performed in marine invertebrates and those that have been carried out have not included characterization of the enzymatic pathways responsible for the observed metabolites (Meyer and Bakke, 1977; Landrum and Crosby, 1981; Knezovich and Crosby, 1985).

Although cytochrome P-450 has been, clearly, the most studied xenobiotic biotransformation enzyme system, another important Phase I enzyme is the flavin-containing monooxygenase (FMO). FMO has been isolated and purified from several mammalian species and tissues (Ziegler, 1988). FMO was purified and characterized from hog liver by Ziegler and Poulsen (1979). Since Ziegler's pioneering work, FMO has been purified from rat and mouse liver (Kimura et al., 1983; Sabourin, et al., 1984) as well as rabbit and mouse lung (Tynes and Hodgson, 1985; Williams et al., 1985).

Cytochrome P-450 and FMO share many common characteristics. They are both localized within the smooth endoplasmic reticulum (Ziegler, 1980), although cytosolic forms have been identified in unicellular organisms (Agosin and Ankley, 1987). Both enzymes require NADPH and oxygen for catalysis and even share common substrates, such as 2-aminofluorene (Frederick et al., 1982) and N,N-dimethylaniline (DMA) (Houdi and Damani, 1985). Because there are so many similarities with cytochrome P-450, it is often difficult to determine the relative contribution of each enzyme to the biotransformation of certain chemicals which exist as cosubstrates (Ziegler, 1988).

However, there are several differences between the enzymes

which can be used to study the separate contribution of each enzyme. Although both enzymes require similar cofactors, the reaction mechanisms are quite different. Since FMO possesses a flavin moiety, reducing equivalents directly reduce the enzyme rather than having to be transferred via a reductase as is the case for cytochrome P-450 (Ziegler, et al., 1980). This difference has been exploited to inhibit cytochrome P-450 involvement through the use of inhibitory polyclonal antibodies raised against NADPH-cytochrome P-450 reductase (Tynes and Hodgson, 1985). The enzymes also differ in the pH optimal for catalysis. The optimal pH for FMO appears to be between 8.4 and 9.2 depending on the species and tissue (Ziegler, 1988). This contrasts with cytochrome P-450 which possesses a pH optimum usually around 7.2-7.6 (Sato and Omura, 1978). Cytochrome P-450 can also be inhibited through the use of various suicide substrate inhibitors (Ortiz de Montellano and Reich, 1986). However, these inhibitors are usually specific for certain isozymes and may, in fact, be substrates for FMO (Ziegler, 1988). Consequently, cytochrome P-450 inhibitors should be used in conjunction with other methods to distinguish the involvement of FMO from that of cytochrome P-450 (Ziegler, 1988).

As mentioned above the reaction mechanism for FMO is very different from cytochrome P-450. After flavin reduction by NADPH, $NADP^+$ remains bound to the enzyme providing it with a certain amount of stabilization. Dioxygen is then bound at the 4-alpha position of the flavin creating a hydroperoxy moiety at this site.

Therefore, nucleophilic xenobiotics which are prone to oxidation via a peracid are extremely good substrates for the enzyme. Consequently, secondary and tertiary amines as well as many sulfur-containing chemicals have been shown to be substrates for the enzyme (Ziegler, 1980). However, there do appear to be some protein-mediated constraints influencing chemical access to the active site (Taylor and Ziegler, 1987). For example, chemicals which possess more than one charged atom, such as glutathione, are usually not substrates for the enzyme (Ziegler, 1988). Once substrate oxidation has occurred, breakdown of the remaining pseudobase follows and $NADP^+$ is released. The psuedobase breakdown and release of $NADP^+$ appear to be the rate limiting steps in the enzyme reaction (Ziegler, 1988). Due to the reaction sequence, FMO can not be irreversibly inhibited as is cytochrome P-450 (Ziegler, 1980). Consequently, FMO activity can be competitively inhibited only with cosubstrates which possess preferably similar or smaller Michaelis affinity constants (Ziegler, 1980). Methimazole has been the most commonly used in studies endeavouring to eliminate FMO contribution (Prough et al., 1981; Baba et al., 1987; Chiba et al., 1988; Levi and Hodgson, 1988).

Although without as broad a range as cytochrome P-450, FMO also does act on a wide variety of substrates, including secondary and most tertiary amines (Ziegler, 1985). The products of secondary amine oxidation are usually nitrones which can hydrolyze and yield dealkylated products. Such is the case with methamphetamine (Baba

et al., 1987). Tertiary amine oxidation by FMO always leads to the formation of the N-oxide (Houdi and Damani, 1985). Substrates such as DMA are used routinely to characterize FMO activity (Cashman, 1987; Ziegler, 1988). Hydroxylamines and hydrazines are also excellent substrates for the enzyme (Ziegler, 1985). FMO has also been shown to catalyze the N-oxidation of several primary arylamines and pyrrolizidine alkaloids to the corresponding hydroxylamines and N-oxides (Ziegler, 1988). These substrates, however, are thought to be "atypical" (Ziegler, 1988). There do appear to be tissue-related differences in the metabolism of primary amines by FMO. Although most primary alkyl amines are not substrates for the liver forms, certain lipophilic primary amines are substrates for the pulmonary forms (Tynes and Hodgson, 1985; Williams et al., 1985).

Since most organic sulfur compounds are susceptible to oxidation by hydroperoxides, FMO catalyzes oxidation of many sulfur-containing chemicals (Ziegler, 1985). Just as DMA represents the prototype FMO substrate for amines, methimazole has been shown to be an excellent substrate to characterize sulfur oxidation via FMO (Ziegler, 1988). Besides thiocarbamides, other sulfur-bearing substrates include thioamides, mercaptopurines and pyrimidines (Ziegler, 1988). Substrates that are significant from an environmental viewpoint include several thioether sulfide and disulfide pesticides (Hajjar and Hodgson, 1980). Thiols are also susceptible to oxidation via FMO leading to the formation of disulfides (Ziegler, 1980). In fact, since FMO oxidizes

cysteamine to the disulfide, Ziegler (1980) has suggested that disulfide formation during protein synthesis may be the endogenous function for FMO in mammals.

The endogenous function for FMO in fish and possibly some marine invertebrates appears to be the oxidation of trimethylamine (Goldstein and Dewitt-Harley, 1973; Agustsson and Strom, 1981; Daikoku et al., 1987; Daikoku et al., 1988). In addition, FMO activity has been observed in numerous marine invertebrates (Kurelec, 1985; Kurelec et al., 1986a) and has been suggested to play a dominant role in xenobiotic biotransformation (Kurelec and Krca, 1987). However, since there appears to be some discrepancy regarding the validity of these data (Livingstone, 1987a), it still remains uncertain as to the role of invertebrate FMO in xenobiotic biotransformation.

Besides cytochrome P-450 and FMO, other pathways responsible for the oxidation of xenobiotics involve co-oxidation through lipid peroxidation, cyclic redox processes, lipoxygenase, or peroxidase catalyzed reactions (O'Brien, 1982; Eling et al., 1983; Bruchhausen et al., 1988). One or more of these complex pathways may be involved in co-oxidation of a single chemical. For example, Tien and Aust (1982) have shown that redox cycling can stimulate lipid peroxidation which can then lead to xenobiotic co-oxidation. Furthermore, one electron reduction of dioxygen by NADPH-cytochrome P-450 reductase has been shown to stimulate redox cycling and subsequent lipid peroxidation (Tien and Aust, 1982). Thus, the cytochrome P-450 system may also be responsible for co-

oxidation pathways for certain chemicals.

Possessing both lipoxygenase and peroxidase functionalities, prostaglandin synthase has been shown to catalyze co-oxidation of numerous chemicals (Eling et al., 1983). In fact, in certain mammalian tissues such as the seminal vesicles, where cytochrome P-450 activity is low, it has been suggested that co-oxidation via prostaglandin synthase may be the primary route of xenobiotic biotransformation in these tissues (Eling et al., 1983). Other peroxidases that have been shown to play a role in xenobiotic oxidation include chloroperoxidase and lactoperoxidase (Doerge, 1986). Although there have been recent studies examining the role of redox cycling and lipid peroxidation in fish and marine invertebrates (Wenning et al., 1988; Wofford and Thomas, 1988; Washburn and Di Giulio; 1988; Livingstone et al., 1989), the major portion of the work in the area of co-oxidation pathways has focused on mammals. Consequently, little is known about xenobiotic co-oxidation mechanisms in non-mammalian systems.

Since information regarding xenobiotic biotransformation pathways is scarce, the following dissertation examines xenobiotic biotransformation in three aquatic organisms, the marine molluscs, Cryptochiton stelleri and Crassostrea gigas, as well as the freshwater fish, Oncorhynchus mykiss. Two marine molluscs, representing two diverse classes with distinctive natural histories and habitats were utilized to compare and contrast xenobiotic metabolism and the enzymes responsible for the observed biotransformation in marine invertebrates. In addition, an

attempt was made to determine the relative roles of Phase I

oxidation pathways in each species. In studies utilizing the

rainbow trout, the role of a lesser known xenobiotic

biotransformation enzyme, FMO, was established aiding future work

aimed at a better understanding of the mechanisms of

carcinogenesis in trout and other organisms.

Chapter 1

Cytochrome P-450 and Phase II Activities
in the Gumboot Chiton <u>Cryptochiton</u> <u>stelleri</u>.

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ABSTRACT

The digestive gland of <u>Cryptochiton</u> <u>stelleri</u> was shown to contain cytochrome P-450, benzo(a)pyrene hydroxylase (BPH), and NADPH-cytochrome P-450 reductase. Glutathione-S-transferase, UDP-glucuronosyltransferase, and UDP-glucosyltransferase activities also were observed. After treating chitons three weeks with a cytochrome P-450 inducer, β-naphthoflavone (BNF), a significant (p<0.05) increase in BPH was found in treated animals. In digestive gland microsomes from BNF-treated chitons, western blot analysis revealed an increase in staining intensity with antibodies raised against purified trout cytochrome P-450, LM_{4b}. In addition, a decrease in protein staining intensity subsequent to treatment with antibodies raised against trout cytochrome P-450 LM_2 was observed in digestive gland microsomes from BNF-treated chiton. The major metabolites extracted after incubation of BP with chiton digestive gland microsomes were quinones. However, a quantitative difference in quinone production was seen between controls (72.7%) and BNF-treated animals (42.9%). Neither ethoxycoumarin-O-deethylase (ECOD) or ethoxyresorufin-O-deethylase (EROD) was detected in controls or BNF-treated animals.

INTRODUCTION

How marine organisms cope with pollutants is an intriguing

question. Cytochrome P-450 has been suggested as the most

important xenobiotic metabolizing enzyme system in vertebrates,

invertebrates, and plants (Sato and Omura, 1978; Black and Coon,

1986). Furthermore, fish (Kurelec et al., 1977; Davies et al.,

1984; Collier et al., 1986) and certain invertebrates (Lee, 1981;

Lee et al., 1981; Livingstone, 1985) preexposed to organic

pollutants were shown to possess increased levels of cytochrome P-

450 catalyzed activities. Several researchers have examined the

possible utilization of marine invertebrate cytochrome P-450

activities as indicators of exposure to organic chemicals such as

polyaromatic hydrocarbons (Bayne et al., 1979; Mix, 1984). Other

investigators have studied the relationship between marine

pollutants and the occurrence of neoplasia in invertebrates

(Anderson and Doos, 1983; Al-Sabti and Kurelec, 1985; Mix, 1986).

Lee (1981) and Livingstone (1985) have reviewed the responses

of xenobiotic metabolizing enzyme systems of marine invertebrates.

Although cytochrome P-450 activity has been clearly observed in

crustaceans, evidence for the occurrence of this system in

molluscan species has been somewhat controversial. Recent

investigations of the mussels Mytilus edulis (Kurelec, 1985) and

Mytilus galloprovincialis (Kurelec et al., 1986; Britvic and

Kurelec, 1986) also have failed to show the presence of cytochrome

P-450 linked activity. By contrast, Livingstone and Farrar (1984),

Livingstone et al. (1985) and Stegeman (1986) found cytochrome P-450 activity in the gastropod Littorina littorea and the mussel Mytilus edulis. Furthermore, Ade et al. (1982) observed significant levels of benzo(a)pyrene hydroxylase in the mussel Mytilus galloprovincialis. Since there is substantial disagreement regarding the occurrence of cytochromes P-450 in molluscs, and their induction after exposure to organic chemicals, research on this question has continued (Livingstone et al., 1985; 1986; Stegeman, 1986).

Metabolism of xenobiotics by animals not only involves oxidative metabolism, usually catalyzed by cytochromes P-450, but conjugation processes also can occur. Two of the more important classes of conjugation enzymes are glutathione S-transferase and UDP-glucuronosyltransferase. UDP-glucuronosyltransferase activity has been observed in the postmitochondrial fraction of the digestive gland in the mussel Mytilus galloprovincialis (Kurelec et al., 1986) and cytosolic glutathione S-transferase activity has been demonstrated in Mytilus edulis (Bend et al., 1977). Glutathione S-transferase also was isolated from the terrestrial gastropod Achatina fulica (Balabaskaran et al., 1986). Although UDP-glucuronosyltransferase was not observed in another terrestrial gastropod Arion ater, a similar enzyme, UDP-glucosyltransferase was found (Leakey and Dutton, 1975).

Most studies of molluscan cytochrome P-450 activity have focused on marine bivalves because of the relative abundance, ease of maintenance, and sessility of these animals. Only two

gastropod molluscs (L.littorea and Thais haemastoma) (Livingstone et al., 1985; 1986) and one cephalopod (Illex illecebrosus) (Lee, 1981) have been examined previously for cytochrome P-450 activity. Up to the present time, no study has examined any species in the class Amphineura.

Along the coast in the Pacific Northwest, the gumboot chiton, Cryptochiton stelleri, is an abundant low intertidal-subtidal mobile organism. Its size can reach 18 inches with digestive glands that weigh approximately 20 g. Because of the large size of the digestive gland, single animal responses can be observed rather than sample pools. The purpose of this study was to examine cytochromes P-450 and associated mixed-function oxidase (mfo) activities and also search for Phase II conjugation enzymatic processes in the amphineuran Cryptochiton.

METHODS AND MATERIALS

Chemicals

β-Naphthoflavone (BNF) was purchased from Aldrich Chemical
Company (Milwaukee, WI) and [G-^{3}H]-Benzo(a)pyrene (BP) was
purchased from ICN Radiochemicals (Irvine, CA). [^{14}C-7,10]-BP was
obtained from Amersham (Arlington Heights, IL). All other
biochemicals were purchased from Sigma Chemical Company (St.
Louis, Mo).

Animal maintenance

Gumboot chitons, Cryptochiton stelleri, were obtained using
SCUBA from rock jetties at Newport, Oregon. These animals were
maintained in flowing seawater tanks at the Oregon State
University Hatfield Marine Science Center. Seawater was
unfiltered and the temperature varied with the season (10-15°C).
These animals were fed Ulva lactuca which was obtained year round
from nearby mudflats in Yaquina Bay at all times of the year.
Animals were maintained approximately one month before they were
killed. For induction studies, 10 animals (5 treated and 5
control) were not fed for 2 weeks prior to treatment. Treated
animals were dosed orally at 10 mg/kg with β-naphthoflavone (BNF)
in corn oil three times a week for three weeks. Control animals

received only corn oil.

Microsomal Preparations

Initial Cryptochiton microsomal preparations pooled tissues
(Digestive gland, Stomach, Intestine, Gonad and Gill) from 3
animals collected in July. Digestive gland microsomal enzyme
assays used tissue from varied numbers of (usually 2-3) individual
animals.

Animals were sacrificed by freezing at -20°C for approximately
20 min and dissected. Microsomes were prepared at 4°C by a
modified procedure as described in Livingstone and Farrar (1984).
Tissues were placed in a 1:4 w/v ratio of 20 mM Tris-HCl buffer,
pH 7.6, containing 0.5 M sucrose, 0.15 M KCl, 1 mM
ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT)
and 0.1 mM phenylmethylsulfonylfluoride (PMSF). Homogenizations
were carried out using a teflon Potter-Elvehjem homogenizer. The
homogenate was centrifuged at 12,000 x g for 20 min in a Sorvall
RC-2B refrigerated centrifuge. Following a second 12,000 x g spin
of the supernatant, a final centrifugation at 100,000 x g for 90
min was performed. The microsomal pellet was resuspended in a
storage buffer which contained 20 mM Tris-HCl pH 7.6, 20% w/v
glycerol, 1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF. Unused microsomes
were stored at -80°C until needed. The cytosol was retained and
frozen at -80°C for the glutathione S-transferase assay. For
comparison, approximately 100 juvenile female rainbow trout

(Oncorhynchus mykiss) were treated with BNF (80 mg/kg ip injection) and pooled liver microsomes were prepared by the method of Williams and Buhler (1984). Liver microsomes were prepared following the method of Guengerich (1982) by pooling the livers of three mature male Long-Evans rats.

<center>Enzyme Assays</center>

Cytochrome P-450 was quantitated from the carbon monoxide difference spectrum of sodium dithionite treated samples on a Cary 219 spectrophotometer using a extinction coefficient of 100 mM^{-1} cm^{-1} (Estabrook and Werringloer, 1978). The limits of detection for this assay were approximately 20 pmol/mg microsomal protein.

NADPH-cytochrome c reductase activity was measured spectrophotometrically by the increase in absorbance at 550 nm (extinction coefficient: 19.6 mM^{-1} cm^{-1}; Shimakata et al., 1972). The reaction mixture contained (in a final volume of 1.0 ml), 50 mM Tris-HCl pH 7.6, 1 mM KCN, 0.03 mM cytochrome c and 0.26 mM NADPH. Control incubations excluded NADPH. Pooled rat liver microsomal incubations with and without NADPH were used as a positive control. The limits of detection for this assay were approximately 10 pmol/min/mg microsomal protein.

Benzo(a)pyrene hydroxylase (BPH) activity was measured radiometrically following the procedure of Livingstone and Farrar (1984). The incubation mixture contained 50 mM Tris-HCl pH 7.6, 0.4 mM NADPH, 2 mg bovine serum albumin, 0.06 mM of $[1,3,6-^{3}H]$-BP

(110 mCi/mmol), and 1-2 mg microsomal protein in a final volume of 1.0 ml. The reaction was initiated by the addition of NADPH and allowed to run for 20 min at 25°C and stopped by the addition of 1.0 ml 0.15 M KOH in 85 % DMSO. Control incubations did not contain NADPH. The reaction mixture was extracted twice with 7.5 ml of hexane and 1.0 ml of the aqueous phase was added to 10 ml of scintillation fluid (ACS-Amersham) and counted after acidification in a Packard TriCarb scintillation counter. Activity was expressed as the difference between means of samples (+NADPH) and controls (-NADPH). Pooled BNF-treated trout liver microsomal incubations were used as positive controls. The limits of detection for this assay were approximately 1 pmol/min/mg microsomal protein.

BPH activity was assayed fluorometrically at 25°C by the method of Dehnen et al. (1973). In a final volume of 1 ml, incubations contained 50 mM triethanolamine-HCl pH 7.25 buffer, 0.2 mM NADPH and 1-2 mg of microsomal protein. The reaction was initiated by the addition of 0.06 mM BP in dimethylformamide and stopped after 10 min by the addition of 1 ml of ice-cold acetone. Controls omitted the addition of NADPH. The tubes were centrifuged (3000 x g) for 10 min and an aliquot of the supernatant mixed with 2.3 times its volume of 8% triethylamine and centrifuged as before. The fluorescence of the supernatant was measured using an excitation wavelength of 467 nm and an emission of 525 nm. The results were reported in terms of arbitrary units of fluorescence and represent the difference between samples and controls. Pooled

rat liver microsomes were incubated at 37°C as a positive control. The limits of detection for this assay were approximately 5-10 pmol/min/mg microsomal protein.

Glutathione S-transferase was determined by the method of Habig et al. (1974). The reaction mixture contained (in a final volume of 1.0 ml) 0.35 M potassium phosphate buffer, pH 8.0, 0.1 M reduced glutathione, and 0.3-0.5 mg of cytosolic protein. The reaction was initiated by the addition of 1 mM chlorodinitrobenzene, and incubations then carried out at 25°C for two min. Activity was measured as an increase in absorbance at 340 nm using a Cary 219 spectrophotometer. A background rate for control (without protein) was routinely determined and the resulting value subtracted from the absorbance before calculating activity using an extinction coefficient of 9.6 mM^{-1} cm^{-1}. Since rates in chiton were easily observable, no positive control was necessary. The limits of detection for the glutathione-S-transferase assays were approximately 1 nmol/min/mg cytosolic protein.

Dealkylation of 7-ethoxycoumarin (ECOD) and 7-ethoxyresorufin (EROD) was measured fluorometrically with a Perkin-Elmer 650-10S recording spectrofluorometer (Prough et al., 1978). The ECOD assay mixture contained (in a final volume of 1.0 ml) 0.1 mM Tris-HCl buffer (pH 7.6), 0.01 mM ethoxycoumarin, and 1.0 mg of microsomes in a fluorescence cuvette. The reaction was initiated by the addition of 0.1 mM NADPH and incubations carried out at 25°C for two min. Activity was measured as a change in

fluorescence (360 nm excitation; 460 nm emission) and quantitated

with 7-hydroxycoumarin as the standard. Also performed in a final

volume of 1.0 ml, the EROD assay contained 0.1 M Tris-HCl buffer

(pH 7.8), 1 μM 7-ethoxyresorufin, and 1.0 mg of microsomal

protein. After adjusting the excitation and emission wavelengths

to 530 and 585 nm, respectively, the rate of fluorescence change

versus time was recorded by adding 0.1 mM NADPH. Resorufin, 0.1

mM in 0.1 M Tris-HCl buffer, pH 7.8, was used as the standard.

Control runs were performed without the addition of NADPH and

subtracted from sample runs. Pooled liver microsomal incubations

from BNF-induced trout were used initially as a positive control

in both assays. Pooled rat liver microsomal preparations,

incubated at 37°C, were used in ECOD determinations as a positive

control. The limits of detection for EROD and ECOD were 10

pmol/min/mg microsomal protein.

The disappearance of p-nitrophenol (PNP) was used to measure

UDP-glucuronosyltransferase (Fowler et al., 1982). The incubation

medium contained 9 mM UDP-glucuronic acid (UDPGA), 0.5 mM MgCl$_2$,

0.8 mM PNP, and 40 mM Tris-HCl, pH 7.5, at 37°C (optimum

temperature for Cryptochiton), in a total volume of 1.4 ml.

Microsomal suspensions were activated prior to addition to the

incubation medium by adding 0.2 μl Triton X-100 per mg microsomal

protein and the reaction was started by the addition of these

microsomes. The reaction was stopped after 5 minutes with the

addition of 5.0 ml of 0.2 M glycine buffer containing 0.15 M NaCl,

pH 10.4. PNP concentration was measured spectrophotometrically on

a Cary 219 spectrophotometer at 405 nm. Blank values were obtained by omitting UDPGA from the reaction medium. Pooled rat microsomes incubated at 37°C served as a positive control. The limits of detection for this assay were approximately 0.1 nmol/min/mg microsomal protein. A modification of the method of Dutton (1966) was used to assay UDP-glucosyltransferase. The incubation mixture contained (in a total volume of 0.6 ml) 0.8 mM PNP, 0.75 mM UDP-glucose (UDPG) in 33 mM Tris-HCl, pH 7.8, and 3 mg of Triton X-100 activated microsomes as above at 37°C. The reaction was initiated by the addition of PNP and allowed to run for 60 min. Blanks omitting UDPG and pooled rat microsomes incubated at 37°C were used as negative and positive controls, respectively. Reactions were terminated with the addition of 0.6 ml of 0.2 N trichloroacetic acid and centrifuged at 500 x g for 5 min. After removing 0.9 ml of the supernatant, 0.6 ml of 2 N NaOH was added and samples were read spectrophotometrically at 405 nm. The limits of detection for UDP-glucosyltransferase activity were 0.02 nmol/hr/mg microsomal protein.

The number of animals used in each assay depended on two factors: the simplicity of the assay and the presence and/or absence of the particular activity examined. For example, since EROD or ECOD activities were not observed in initial experiments, all 15 animals collected in October were used in this assay. Cytochrome P-450 was determined in all but one animal collected in October. Spectral determination of this enzyme was relatively quick and supported the conclusion that lack of EROD and ECOD

activity was not due to the denaturation or absence of cytochrome P-450.

Protein concentrations were determined using the method of Lowry et al. (1951).

Benzo(a)pyrene Metabolite Analysis

Metabolites of [^{14}C]-BP (specific activity 2 mCi/mmole) were obtained via in vitro incubations of digestive gland microsomes from control and BNF-treated chitons as described above. The reaction was terminated after 60 min with the addition of 1.0 ml of ethyl acetate:acetone (2:1). Following extraction, the organic layer was evaporated with N$_2$. The residue was redissolved in 100 μl methanol, and an aliquot was injected into a Zorbax ODS (4.6 mm i.d. X 25 cm) column. Using a modification of procedure of Williams and Buhler (1984), the metabolites were eluted with a gradient of methanol (40-100 %) in water at 35°C with a flow rate of 1 ml/min. Total metabolites were monitored at 254 nm with a Spectra-Physics u.v. detector, and radioactivity was assayed by collecting 1.0 min fractions directly into vials and scintillation counting. Individual [^{14}C] metabolites were identified by coelution with unlabeled BP metabolite standards provided by Dr. Donald M. Jerina of NIH. All analyses were done in duplicate.

Electrophoresis, immunoblotting, and immunostaining

Electrophoresis of microsomal proteins was performed using 8.0 %
separating and 3.0 % stacking polyacrylamide gels in the presence
of sodium dodecyl sulfate (SDS)(Laemmli, 1970). All materials
were purchased from BioRad (Richmond, CA). Samples were combined
with buffer (60 mM Tris HCl, pH 6.8; 2 % SDS; 10 % glycerol; 5 %
β-mercaptoethanol; 0.0025 % pyronin Y) and boiled for 3 min prior
to electrophoresis. Electrophoresis was performed utilizing a
BioRad Protean^{TM} II system and power source. Gels were run for 4
hr at 30 mV. Following electrophoresis, the separated proteins
were transferred via electroblot to nitrocellulose sheets
following a modification of the method of Towbin et al. (1979) and
stained by a modification of the method of Burnette (1981). A
BioRad transblot cell was utilized to transfer the protein from
the polyacrylamide gel to the nitrocellulose paper. The transfer
buffer contained 20 mM Tris base, 0.15 M glycine in 25 % methanol.
The transfer was performed overnight at 30 mV. After transfer,
the nitrocellulose sheets were incubated 60 min with constant
shaking in phosphate buffered saline solution (PBS), (20 mM
potassium phosphate, pH 7.4, 0.8 % sodium chloride) containing 2 %
bovine serum albumin (BSA). Transfers were then incubated for 90
min with FMO antibody (20 μg/ml) in PBS containing 2 % BSA.
Following antibody treatment, the sheets were sequentially washed
with PBS, PBS containing 0.05 % Triton X-100, a second PBS wash
and then incubated with constant shaking for 60 min with a PBS

solution containing [^{125}I]-protein A at 2 x 10^5 cpm/ml. After a second sequential wash as above, the nitrocellulose sheets were wrapped in cellophane plastic and exposed to Kodak XAR-5 X-ray film for 7 days at - 80°C. Relative quantitation of autoradiograms was performed utilizing laser densitometry (Zeineh).

RESULTS

Tissue survey

Stomach, intestine, gonad, and digestive gland were surveyed

for cytochrome P-450 content, BPH, ECOD, EROD, and glutathione S-

transferase activities (Table 1). Because of the limited amount

of microsomal protein obtained from the gill, only glutathione S-

transferase activity was examined in that tissue. Only digestive

gland and intestine possessed detectable levels of cytochrome P-

450 (0.18 $\pm$ 0.02 nmol/mg and 0.05 $\pm$ 0.01 nmol/mg, respectively).

High glutathione S-transferase activities, however, were found in

most chiton tissues, with the intestine and digestive gland having

the highest levels. Except for BPH activity in the digestive

gland, cytochrome P-450 mfo activities were not detected in any of

the tissues. Similar levels of BPH were observed previously in

molluscan digestive gland microsomes using the same assay

conditions (Livingstone and Farrar, 1984).

Digestive gland experiments

Since most of the previous work with invertebrates observed the

highest cytochrome P-450 levels and associated mfo activity in

the digestive gland (Livingstone, 1985; Lee, 1981), additional

studies were performed with that tissue (Table 2). Comparatively

low BPH activities were detected in chiton digestive gland using fluorometric (8.5 $\pm$ 4.1 U/mg/min relative to 118 $\pm$ 19 U/mg/min for rat) and radiometric (0.026 $\pm$ .024 nmole/mg/min) procedures. No difference in BPH activity was observed between frozen and fresh microsomal samples. Chiton BPH activity in October was approximately one-half of the activity observed in animals collected in July (Table 1). However, these activities could not be compared statistically because pooled digestive glands were used in July and individual digestive glands were examined in October. No ECOD or EROD activity was observed after 4 replicate analyses of each sample. However, NADPH-cytochrome c reductase activity was observed (5.5 $\pm$ 0.7 nmol/mg/min) along with continued presence of cytochrome P-450 (0.11 $\pm$ .06 nmol/mg).

Chiton digestive gland possessed UDP-glucuronosyltransferase activities comparable to that found in rat liver (Table 2). Since the optimal temperature for chiton digestive gland and rat liver UDP-glucuronosyltransferase activity was found to be the same (37°C), both were incubated at this temperature. While UDP-glucosyltransferase activities were present in chiton digestive gland, these activities were substantially lower than those seen in the rat hepatic microsomes.

Induction Experiments

Following a three week oral treatment of chiton with BNF, a significant increase in BPH was discovered (Table 3) in the

treated animals using the radiometric assay of Livingstone and Farrar (1984). However, when the fluorometric assay of Dehnen et al. (1973) was employed, a decrease in BPH was observed in the treated chitons. Other detectable activities showed no significant difference between control and BNF treated animals. In addition, no ECOD, or EROD activity was detected in either the control or treated chitons.

Western blot analysis

Microsomes from chiton digestive gland reacted with antibodies against purified trout LM_2 and LM_{4b} (Figures 1 and 2). A single band was observed to react with LM_{4b} antibodies at 60,000 daltons in both control and BNF-treated samples (Figure 2). A band at 60,000 also was observed in control and BNF-treated samples stained with LM_2 antibodies along with a much darker band at 54,000 daltons (Figure 1). Digestive gland microsomes from control chitons possessed greater intensities of staining with LM_2 antibodies relative to BNF treated (Figure 3). Conversely, BNF-treated chitons possessed greater levels of reaction when stained with LM_{4b} antibodies relative to controls (Figure 3).

BP Metabolite Profile

The proposed pathway for BP biotransformation in the chiton can be observed in Figure 4. In control chitons, the dominant BP

metabolites were quinones (77.8%) (Table 4). Total conversion of BP to metabolites in controls was 1.8 % compared to 2.7 % for the BNF treated chitons (Table 5). Furthermore, the metabolite profile differed between treated and control animals. In BNF chiton, for example, only 42.9 % of the total metabolites were quinones while the 3-hydroxy BP accounted for 29.4 % of the total metabolites. Since these findings showed that a majority of the metabolites in both treated and control animals were quinones, 1 mM EDTA was added to the incubation mixture to examine the contribution of lipid peroxidation to BP metabolism in chiton digestive gland. There were negligible differences in quinone production in both control and BNF treated animals with and without EDTA. However, a decrease in BP-7,8 dihyrodiol and an increase in 3-hydroxy BP formation was observed in incubations containing EDTA.

DISCUSSION

Marine animals encounter a diverse array of natural and anthropogenic chemicals in their ecosystems. For example, a medium preference algal family upon which <u>Cryptochiton</u> feeds, Rhodomelaceae (Yates, personal communication), possesses brominated aromatic compounds which can constitute nearly 5 % of the plant's dry weight (Pedersen <u>et al</u>., 1974; Phillips and Towers, 1982). Allen (1977) showed that the plasma of <u>Cryptochiton</u> contained a number of fatty acids and sterols. Besides natural products, these animals can be exposed to numerous foreign organic compounds that occur as environmental pollutants. Such chemicals enter waterways from industrial and urban wastes, fallout of airborne particles, runoff from agricultural areas treated with herbicides or insecticides, accidental and intentional shipping spillages, and natural seepage of hydrocarbons from oil deposits (Bend <u>et al</u>., 1977; Mix, 1984). Since cytochrome P-450 has been shown to play an integral role in metabolism of natural and xenobiotic substrates (Ortiz de Montellano, 1986), the demonstration of cytochrome P-450 activity in the gumboot chiton was expected.

In the digestive gland of the gumboot chiton, at least one cytochrome P-450 activity (BPH) is inducible after three week treatment with BNF. This increase was observed with both the radiometric BPH assay and the HPLC BP metabolite profiles in BNF

treated chitons. In addition, since one band of staining was shown to be augmented in BNF-treated chiton after western blot analysis with antibodies raised to the BNF-inducible form of trout cytochrome P-450, LM_{4b}, it appears that this band at 60,000 daltons may be responsible for the enhanced BPH activities. The decrease in protein staining observed by digestive gland microsomes from BNF-treated chitons when samples were stained with antibodies to the constitutive form of trout cytochrome P-450, LM_2, would also explain the similarities observed in total cytochrome P-450 content in digestive gland microsomes from control and BNF-treated animals.

It is interesting that the fluorometric assay (Dehnen et al., 1973) used in our investigation showed a decrease in BPH activity while the other two methods employed in this study (radiometric and HPLC metabolite analysis) yielded activity increases. The radiometric assay (Livingstone and Farrar, 1984) for BPH determines total production of all water soluble metabolites while the HPLC assay measures the formation of each specific metabolite. The fluorometric assay, in turn, is based on total fluorescence and primarily measures formation of the highly fluorescent 3-hydroxy-BP metabolite. However, the 3-hydroxy-BP formed by microsomes from the BNF treated animals was twice that seen with the controls. This discrepancy can be explained either by the presence of fluorescence-quenching metabolite(s) in the BNF-treated chiton incubation mixtures or by the formation of less fluorescent metabolite(s) other than the 3-hydroxy-BP in the

control chiton incubation samples. It also is important to note that statistical significance between control and treated animals is not observed due to the large variation between individual animals ($p \leq 0.05$; student's t-test). This large animal-to-animal variation may also explain the great differences in activities seen in control animals in Table 2 and Table 3.

Demonstration of the inducibility of cytochrome P-450 activities in invertebrates is important if these easily maintained marine animals are to be used as bioindicators for environmental stress by organic pollutants. Earlier studies failed to see induction within marine invertebrates (Payne and May, 1979; Vandermeulen and Penrose, 1978). In later reports, however, Gilewicz et al. (1984) saw significant increases in cytochrome b_5 and cytochrome P-450 in the mussel *Mytilus galloprovincialis* when exposed (injection and water) to a variety of petroleum hydrocarbons. Lee et al. (1981) also observed significant increases in BPH and cytochrome P-450 in crabs and polychaete worms exposed to oil, benzo(a)pyrene, and PCBs. Furthermore, Dixon (1985) noted an increase of sister-chromatid-exchange by cyclophosphamide in mussels co-treated with the mammalian cytochrome P-450 inducer phenobarbital (PB).

Because of the large quantity of quinone metabolites which he isolated, Stegeman (1986) suggested the contribution of cooxidation via lipid peroxidation or prostaglandin synthase to bivalve benzo(a)pyrene metabolism. Iron has been shown to readily catalyze lipid peroxidation through a Fenton- type reaction producing lipid hydroperoxides from which oxygen can be

nonenzymatically or enzymatically transferred to BP leading

primarily to 6-hydroxy BP with subsequent rearrangement to a host

of quinones. Existing as an effective chelator of iron, EDTA can

be used to prevent iron catalyzed cooxidation reactions. Since

only a slight change in quinone formation was observed between

animals with and without EDTA added to the incubation mixture in

our study, it is probable that quinone formation does not occur

via an iron catalyzed reaction in chiton digestive gland.

However, control incubations containing EDTA did cause a

significant decrease in BP-7,8-dihydrodiol and increase in 3-

hydroxy BP production. Thus, cooxidation mediated through a

reaction with iron may play a role in BP-7,8-dihydrodiol and 3-

hydroxy-BP formation in chiton control digestive gland

incubations. Furthermore, the absence of BP-4,5-dihydrodiol and

the presence of BP-7,8-and -9,10-dihydrodiol metabolites in the

chiton microsomal incubation mixtures also is interesting. These

metabolites are thought to be formed after initial epoxidation

resulting in the arene oxide and the subsequent addition of water

via epoxide hydrolase. Since BP-7,8-dihydrodiol can be further

oxidized to BP-7,8-dihydrodiol-9,10 epoxide which is the most

chemically reactive BP metabolite, the possibility for

cytotoxicity and/or tumor development in the digestive glands of

these animals is apparent.

In green gland and stomach microsomes of the blue crab

Callinectes, Singer _et_ _al_.(1980) found minimal ECOD and EROD

activities (pmol/hr/mg protein). We were unable to detect ECOD or

EROD activities in either control or BNF-treated microsomes from the digestive gland of chitons. Since ECOD and EROD activities were apparently not inducible by BNF-treatment (nor were cytochrome P-450 levels and NADPH-cytochrome c reductase activities), a direct comparison to cytochrome P-450 activities in species of higher phylogeny cannot be suggested. Either cytochrome P-450 in Cryptochiton digestive gland microsomes lacks the ability to catalyze detectible conversion of ethoxycoumarin and ethoxyresorufin to 7-hydroxycoumarin and 7-hydroxyresorufin, respectively, or possibly these chemicals may be metabolized to metabolites undetected by our fluorescence assay. In a study with crab cytochrome P-450, James (1984) examined substrate specificities in the presence of specific isozyme inhibitors. She concluded that the invertebrate cytochrome P-450 system differed from the mammalian cytochrome P-450 with respect to substrate specificities. Lee et al. (1981) observed significant differences in K_s values after exposure of crabs and worms to organic pollutants and suggested that these data indicate the presence of different forms of cytochrome P-450 in these species. Consequently, marine invertebrates may have different forms of cytochrome P-450 that respond to different inducing agents or possibly to different mechanisms of induction than those seen in mammals.

Earlier studies have been unable to document cytochrome P-450 activity in molluscs (Payne and Penrose, 1975; Kurelec, 1985; Kurelec et al., 1986; Britvic and Kurelec, 1986). This lack of

activity may actually be artifactual, due to the omission of protease inhibitors from the homogenization media. Proteases or endogenous inhibitors to cytochrome P-450 activity have been demonstrated to be present in invertebrates (James et al., 1977) and may explain the apparent lack of activity seen in certain species.

After initial Phase I oxidative activation, a chemical has the potential to become toxic by binding to certain endogenous macromolecules, be detoxified by a Phase II conjugation step or be excreted unchanged. The presence of common Phase II enzymatic activities in invertebrates has been noted previously, such as glutathione S-transferase and epoxide hydrolase (Bend et al., 1977), amino acid conjugation (James, 1982), and UDP-glucuronosyltransferase (Kurelec et al., 1986). Further, through the use of β-glucosidase with naphthalene metabolites in crab, Corner et al. (1973) suggested the presence of UDP-glucosyltransferase which has also been found in terrestrial gastropods (Leaky and Dutton, 1975).

Glutathione S-transferase was detected in relatively large amounts in chiton digestive gland and intestine and in smaller amounts in the gonads, gills, and stomach (Table 1). UDP-gluronosyltransferase and UDP-glucosyl transferase were also found to be present in the chiton (Table 2). These data indicate that Cryptochiton possesses sufficient activity to detoxify oxygenated intermediates produced by Phase I processes.

The discovery of Phase II conjugative enzymatic activities in

marine molluscs is consistent with findings in previous invertebrate studies (Livingstone, 1985). Regarding Phase I oxidative reactions, the detection of cytochrome P-450 and BPH activity in Cryptochiton stelleri digestive gland tissue does suggest a possible role in xenobiotic metabolism. However, the absence of certain characteristic cytochrome P-450 activities such as EROD and ECOD may suggest a more prominent role for endogenous substrate metabolism rather than xenobiotic metabolism, since a mild seasonal variation in cytochrome P-450 and benzo(a)pyrene hydroxylase was observed. Seasonal variations have been observed in crab and mussel and are thought to be related to the production of endogenous molting and spawning hormones (James,1984; Livingstone, 1985). In the summer months, Pacific Northwest marine algal growth is at a maximum. Consequently, Cryptochiton has been shown to be a more active feeder at this time of the year (Yates, personal communication). Thus, the greater levels of BPH and cytochrome P450 may be attributed to an overall enhanced rate of metabolism. Further work regarding endogenous substrate interactions with cytochrome P-450 must be performed to establish the main function of cytochrome P-450 in marine invertebrates. Also, examination of other Phase I processes such as flavin-containing monooxygenase, prostaglandin synthetase, or lipid peroxidation could provide some insight as to how marine organisms cope with xenobiotics.

ACKNOWLEDGEMENTS

Figure 1.   Western blot analysis of microsomes from the
            digestive glands of oyster, control and BNF-treated
            chiton stained with anti-trout LM_2.
            Lane 1-3, control, 100 μg protein; Lane 4-6, BNF, 100
            μg protein; Lane 7-8, oyster, 100 μg protein;   Lane 9-
            11, purified trout LM_2, 0.2, 0.5, 1.0 pmol protein.

Figure 1
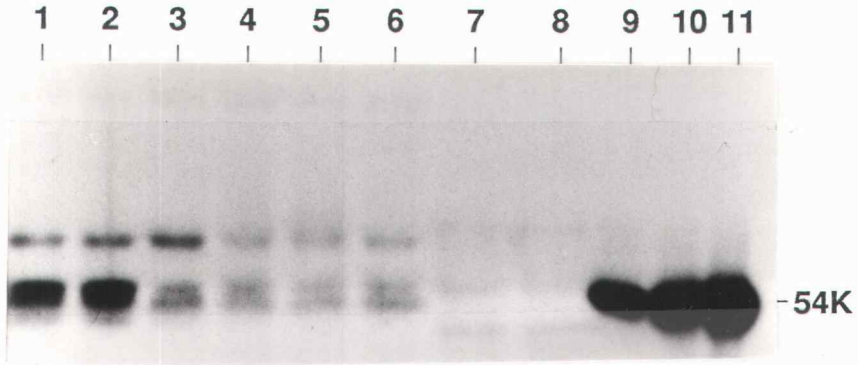
Figure 2.   Western blot analysis of microsomes from the digestive
            glands of control and BNF treated chiton stained with
            anti-trout LM_{4b}.
            Lane 1-3, control, 100 μg protein; Lane 4-6, BNF, 100
            μg protein; Lane 7-10, purified trout LM_{4b}, 2.0, 1.0,
            0.5, 0.2 pmol protein.

Figure 2
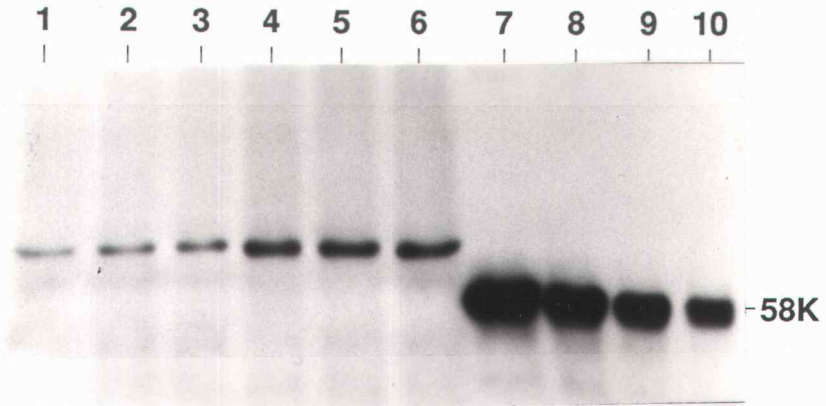
Figure 3.   Relative staining intensities of microsomes from the
            digestive glands of control and BNF-treated chitons
            stained with anti-trout LM_2 and LM_{4b}.
            Each value represents the mean of three sample
            replications from the same preparation $\pm$ S.D.

Figure 3
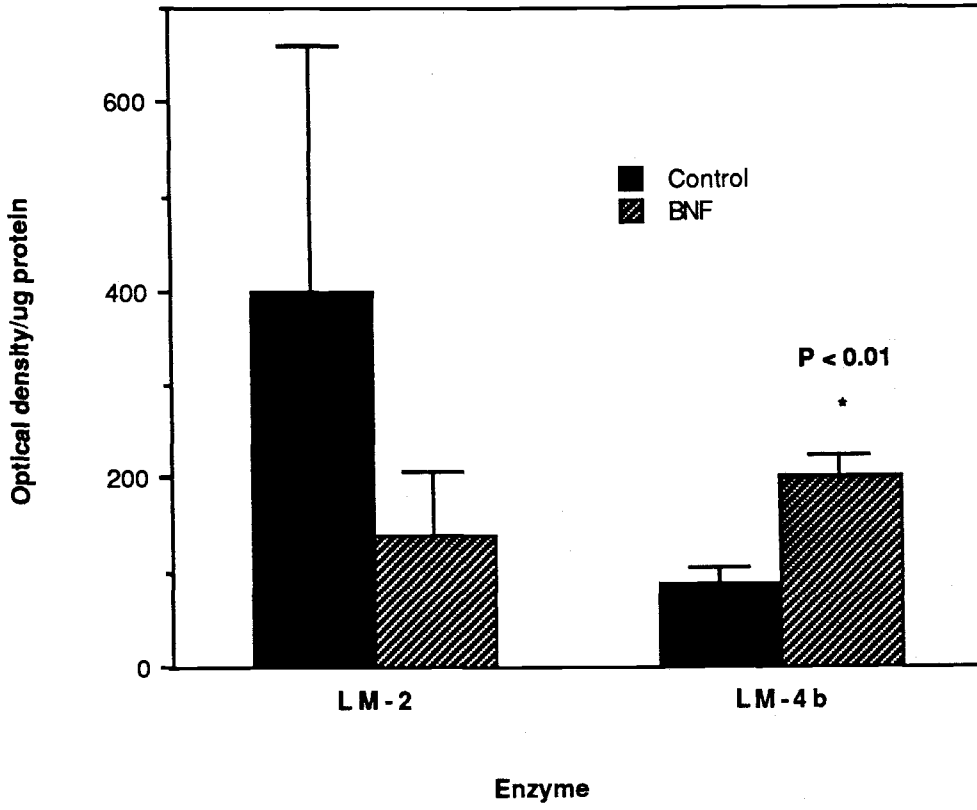
Figure 4.   Benzo(a)pyrene biotransformation pathway in digestive
            gland microsomes from chiton.

Figure 4
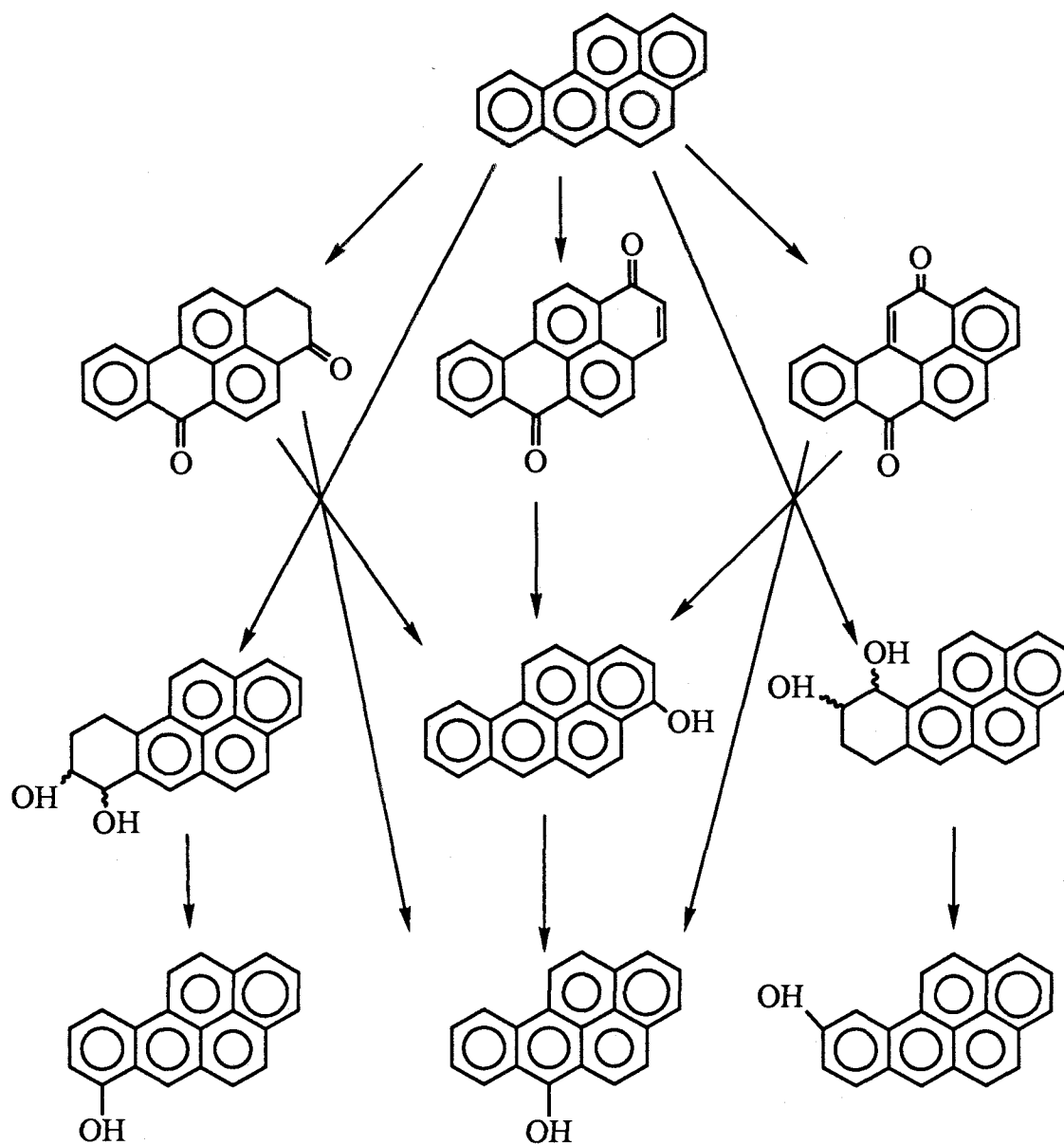
Table 1.   Xenobiotic metabolizing enzyme activity in chiton
            microsomes and cytosol from a single pooled sample of
            three animals[a].
            [a] Assay precision was measured as mean values $\pm$ SD of
            triplicate determinations on each pooled sample.
            Chitons collected in July.
            [b] nmol/mg.
            [c] nmol/mg/min.
            [d] μmol/mg/min.
            N.D. Not detected.

Table 1

Tissue	P-450[b]	BPH[c]	ECOD[c]	EROD[c]	GST[d]
Gill	- - - - - -	- - - -	- - - -	- - - -	0.60 ± 0.01
Gonad	N.D.	N.D.	N.D.	N.D.	0.05 ± 0.01
Stomach	N.D.	N.D.	N.D.	N.D.	0.59 ± 0.02
Intestine	0.05 ± 0.01	N.D.	N.D.	N.D.	1.51 ± 0.01
Digestive gland	0.18 ± 0.02	0.06 ± 0.03	N.D.	N.D.	1.22 ± 0.05

Table 2.   Xenobiotic metabolizing enzyme activity in microsomal
            preparations from chiton digestive glands and livers
            of rats and rainbow trout[a].
            [a] Mean values ± SD of triplicate determinations on
            n(#) animals. Chitons collected in October.
            [b] Enzyme activities or levels per mg microsomal
            protein.
            [c] nmol/mg.
            [d] μmol/mg/min.
            [e] fluorescence units/mg/min.
            [f] nmol/mg/h.
            N.D., Not detected.
            ---- Not determined.

Table 2

Enzyme activity[b]	Chiton	Rat	Trout
Cytochrome P-450[c]	0.110 ± 0.058 (14)	-----	-----
NADPH-cytochrome P-450 reductase[d]	5.5 ± 0.7 (3)	42.5 ± 4.7	-----
BPH[d]	0.026 ± 0.024 (5)	-----	0.48 ± 0.08
BPH[e]	8.5 ± 4.1 (6)	118 ± 19	-----
ECOD[d]	N.D. (15)	0.18 ± 0.03	0.20 ± 0.05
EROD[d]	N.D. (15)	-----	3.18 ± 0.04
UDP-glucuronosyl transferase[d]	38.0 ± 2.5 (3)	42.8 ± 22.5	-----
UDP-glucosyl transferase[f]	0.11 ± 0.04 (3)	2.5 ± 1.9	-----

Table 3.   Effects of BNF treatment on xenobiotic metabolizing
            enzyme activity in microsomes from chiton digestive
            glands[a].
            [a] Mean values ± SD of triplicate determinations on
            three animals collected in November.
            [b] Enzyme activities or levels per mg microsomal
            protein.
            [c] nmol/mg.
            [d] nmol/mg/min.
            [e] fluorescence units/mg/min.
            N.D., Not detected.
            * $p \leq 0.05$; Student's t-test

Table 3

Enzyme activity/level[b]	Control	BNF treated
Cytochrome P-450[c]	0.11 ± 0.04	0.094 ± 0.046
NADPH-cytochrome P-450 reductase[d]	10.9 ± 1.40	10.2 ± 1.21
BPH[d]	0.012 ± 0.003	0.075 ± 0.038*
BPH[e]	1.69 ± 1.20	0.44 ± 0.19
ECOD[d]	N.D.	N.D.
EROD[d]	N.D.	N.D.

Table 4. [^{14}C]-Benzo[a]pyrene (BP) metabolism with digestive gland microsomes from untreated chiton.
^{a} Percentage of each metabolite is based on total radioactivity in identified metabolite fractions.
^{b} Percentage of total radioactivity above blank which emerges from the column prior to BP.
^{c} Percentage of radioactivity emerging before BP in defined metabolite peaks.

Table 4

Incubation	Metabolite (%)				Total conversion[b]	Recovery (%)[c]
	7,8-diol[a]	9,10-diol[a]	Quinones[a]	3-OH[a]		
Control	6.1	4.4	77.8	8.4	1.8	75.5
Control + EDTA	2.0	5.0	72.7	16.6	1.4	86.3

Table 5.   [^{14}C]-BP metabolism in digestive gland microsomes from chiton pretreated with BNF.
[a] Percentage of each metabolite is based on total radioactivity in identified metabolite fractions.
[b] A phenol other than 3-OH BP or 9-OH BP.
[c] Percentage of total radioactivity above blank which emerges from the column prior to BP.
[d] Percentage of radioactivity emerging before BP in defined metabolite peaks.

Table 5

Incubation	Metabolite (%)						Total conversion[c]	Recovery (%)[d]
	7,8-diol[a]	9,10-diol[a]	Quinones[a]	9-OH[a]	3-OH[a]	Phenol[ab]		
BNF	13.3	4.9	42.9	4.1	29.4	4.0	2.7	89.2
BNF + EDTA	10.8	6.0	38.6	8.4	36.4	N.D.	3.0	85.3

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Chapter 2

Flavin-containing Monooxygenase Activity in

the Gumboot Chiton (Cryptochiton stelleri).

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ABSTRACT

Flavin-containing monooxygenase (FMO) activity was observed in
microsomes from the digestive gland of the gumboot chiton,
Cryptochiton stelleri. Digestive gland microsomal oxygen uptake
was initiated after the addition of 2-aminofluorene (2-AF), N,N-
dimethylaniline (DMA), methimazole, phenylhydrazine (PH), or 1,1-
dimethylhydrazine (DMH) to the incubations. 2-AF-dependent oxygen
uptake was optimal at pH 8.4 and required NADPH. The formation of
DMA N-oxide and the oxidation of methimazole was measured and
confirmed the presence of FMO activity in this tissue.

INTRODUCTION

The presence of Phase I xenobiotic metabolizing enzyme systems

has been demonstrated in various marine molluscs (Lee, 1981;

Livingstone, 1985; Britvic and Kurelec, 1986). While most

studies have focused on cytochrome P-450-dependent

biotransformation in marine bivalves (Livingstone et al., 1985;

Stegeman, 1985; Livingstone, 1987a; 1988), there has not been

general agreement concerning which enzyme systems are responsible

for the biotransformation of foreign compounds. Thus, while

Kurelec (1985) failed to find cytochrome P-450 activity in Mytilus

edulis, the same species of mussel that Livingstone and Farrar

(1984) utilized to demonstrate cytochrome P-450 dependent-

oxidations, he did observe significant flavin-containing

monooxygenase (FMO) activity in this species.

FMO has been isolated from several mammalian species (Ziegler,

1988). In addition to M. edulis, FMO activity has been observed

in various other invertebrates (Kurelec, 1985; Britvic and

Kurelec, 1986; Kurelec et al., 1986a; 1986b; Agosin and Ankley,

1987). In all of these organisms, the observed pH optimum for FMO

was between 8.3 and 8.4. Like cytochromes P-450, FMO exhibits a

broad substrate specificity. Substrates for the later enzyme

include thiocarbamides, thioamides, sulfides, thiols, secondary

and tertiary amines, hydrazines, N-hydroxylamines, and some

primary aryl amines (Ziegler, 1988).

Due to their ubiquitous usage in industrial processes and as

pesticides, many of these FMO substrates also pollute the marine environment. Consequently, the presence of FMO in marine animals may allow these animals to detoxify or bioactivate certain aromatic amines, or sulfur and nitrogen-containing pesticides.

In contrast to the number of comparative studies that have been undertaken on cytochromes P-450, very little work has been done concerning comparative aspects of FMO. In the following study, the _in vitro_ metabolism of several substrates which have been shown in mammalian systems to be substrates for FMO has been examined using tissue preparations from the marine chiton _Cryptochiton stelleri_. This animal has been shown to possess cytochrome P-450 along with various conjugation enzymatic systems (Schlenk and Buhler, 1988).

METHODS AND MATERIALS

Chemicals

N,N-Dimethylaniline (DMA) was purchased from Aldrich

Chemical Company (Milwaukee, WI) and was recrystallized as the

hydrochloride salt by dissolving 15 ml DMA in 50 ml of anhydrous

ether and bubbling in HCl gas generated by the dropwise addition

of concentrated sulfuric acid to solid sodium chloride. All other

chemicals were purchased from Sigma Chemical Company (St. Louis,

MO).

Animal maintenance

Gumboot chitons, <u>Cryptochiton</u> <u>stelleri</u>, were obtained from rock

jetties at Newport, Oregon using SCUBA. These animals were

maintained in flowing seawater (15°C) tanks at Oregon State

University's Hatfield Marine Science Center and fed assorted

marine algae.

Tissue preparations and enzyme assays

Three animals were killed by freezing at -20°C for

approximately 20 min and then dissected. Digestive gland

microsomes were prepared as described in earlier work (Schlenk and

Buhler, 1988). Briefly, each tissue was dissected, homogenized in
buffer A [20 mM Tris-HCl (pH 7.6), containing 0.5 M sucrose, 0.15
M KCl, 1.0 mM ethylenediaminetetraacetic acid (EDTA), 1.0 mM
dithiothreitol (DTT) and 0.1 mM phenylmethylsulfonylfluoride
(PMSF)] and centrifuged at 10,000 x g for 10 min. Following a
second centrifugation at 12,000 x g for 20 min, a final
centrifugation at 100,000 x g for 90 min was performed. The
resulting microsomal pellet was resuspended in buffer B [20 mM
Tris-HCl (pH 7.6), 20% (w/v) glycerol, 1.0 mM EDTA, 1.0 mM DTT,
and 0.1 mM PMSF] and frozen at -80°C until used.

Oxygen uptake was measured with a Clark-type electrode at 20°C.
In a total volume of 0.6 ml, reaction mixtures at pH 8.4 or 9.2
containing 0.05 M potassium phosphate, 0.05 M Tricine HCl, 0.25 mM
NADPH, and 1.0 mM EDTA were preincubated for 3-5 min. After the
addition of 1.0 mg microsomal protein, endogenous oxygen uptake
was recorded for about 1 min or until the rate was linear for at
least 30 sec. Substrate (1.0 mM) was added and oxygen uptake was
recorded for another 1-2 min. At pH 6.8 and 7.6, 0.1 M Tris-HCl
replaced Tricine.

Methimazole oxidation catalyzed by FMO was measured by the
spectrophotometric assay of Dixit and Roche (1984). Assay
mixtures contained 0.1 M Tricine HCl (pH 8.4), 1.0 mM EDTA, 0.06
mM 5,5'-dithiobis(2-nitrobenzoate) (DTNB), 6.0 mM potassium
phosphate (pH 8.0), 0.025 mM dithiothreitol (DTT), 0.10 mM NADPH,
1.0 mg microsomal protein, and 1.0 mM methimazole in a final
volume of 1.0 ml. Enzyme activity was initiated by addition of

methimazole to one cuvette, and reaction rates were measured with

a Cary 219 spectrophotometer as the rate of decrease in

absorbance at 412 nm between identical assay mixtures with and

without methimazole. Rat microsomes prepared in previous studies

(Schlenk and Buhler, 1988) served as positive controls.

N-oxide formation from DMA was determined

spectrophotometrically by the method of Ziegler and Pettit (1964).

The 1.0 ml incubation mixture contained 0.25 M potassium

phosphate, pH 8.4, 0.5 mM NADPH, and 1.0 mg microsomal protein.

The reaction was started by the addition of 4 mM DMA, and then

incubated at 20°C for 30 min. After extraction with ether to

remove unreacted substrate, DMA N-oxide is reduced by nitrous acid

back to DMA which then forms the nitroso compound that was

colorimetrically quantitated at 420 nm (extinction coefficient 8.2

mM^{-1}). Blanks consisted of boiled microsomes.

Protein concentrations were determined using the method of

Lowry et al. (1951).

RESULTS

All substrates examined showed measurable oxygen uptake, with 2-AF possessing the highest rate (0.5 nmol O_2/mg/min) (Figure 5). DMA had the second highest activity followed by methimazole, PH, and DMH, respectively. The mean endogenous rate of oxygen consumption was 0.05 ± .02 nmol O_2/min/mg. In view of its high rate of oxidation, 2-AF was utilized in determining the pH optimum for oxygen uptake.

The optimum pH for oxygen uptake when chiton digestive gland microsomal mixtures were incubated with 2-AF, was 8.4 (Figure 6). Uptake was dependent upon NADPH, since assays omitting NADPH demonstrated no oxygen uptake upon substrate addition. At pH 7.6, only a 20% decrease in activity was observed while a 50% reduction occurred at pH 7.2.

Figure 7 shows the comparison of methimazole oxidation rates determined by the Dixit and Roche (1984) method between rat liver and chiton digestive gland microsomal FMO activity. Rat liver microsomal activity was approximately five times greater than that of chiton digestive gland microsomes. Optimum temperatures were 37 and 20°C for rat and chiton microsomes, respectively (data not shown). Contrasting the rate of oxygen consumption when methimazole was the substrate (Figure 5) with the Dixit and Roche method, the latter assay demonstrates twice the activity seen with the former.

The effect of NADPH and temperature inactivation of the enzyme on the rate of DMA N-oxide formation in chiton digestive gland microsomes as determined spectrophotometrically is shown in Figure 8. Enzyme activities determined in this manner were roughly one-seventh of those observed in the oxygen uptake experiments. DMA N-oxide formation is dependent upon NADPH and is inactivated upon boiling.

DISCUSSION

The presence of FMO activity in marine molluscs is not uncommon (Kurelec, 1985; Britvic and Kurelec, 1986; Kurelec et al., 1986b). As in our study, FMO has been shown to possess a pH optimum at 8.4 and require NADPH as a cofactor. In addition, this study has shown that chiton digestive gland microsomes catalyze the formation of the N-oxide from DMA and the oxidation of methimazole, two reactions indicative of FMO activity. Moreover, other known FMO substrates elicited NADPH-dependent oxygen uptake upon incubation with chiton digestive gland microsomes. Marine animals have to cope with increasing quantities of organic nitrogen and sulfur-containing pollutants (i.e. pesticides, herbicides, industrial and petroleum wastes) entering marine habitats from many sources (Singer et al., 1980). Thus, FMO-catalyzed oxidations may play a part in activation or detoxication of these chemicals encountered by marine organisms.

There appears to be much controversy surrounding the rates of FMO in molluscs (Livingstone, 1987b; Kurelec, 1987). Kurelec (1985) reported rates of FMO-catalysis of approximately 1 μm/min/mg protein. Livingstone (1987b) has argued that the rates of FMO activity in molluscs are 2-3 orders of magnitude less than that observed by Kurelec (1985). FMO activity in Cryptochiton digestive gland microsomes was approximately four orders of magnitude less than that observed in bivalve digestive gland

microsomes (Kurelec, 1985). Consequently, our data appear to support the viewpoint of Livingstone (1987b).

The FMO activity in microsomes from Cryptochiton digestive gland was significantly less than mammalian liver microsomal levels. The relative rates of oxygen uptake by various nitrogen and sulfur-containing substrates in Cryptochiton differed from rates of substrate-dependent NADPH oxidation in a previous study using liver microsomes from rat, mouse, rabbit, and pig (Tynes and Hodgson, 1984). Whereas methimazole and DMA showed three times greater activity than PH and DMH in rat, mouse, rabbit and pig liver microsomes, DMA and methimazole-dependent oxygen uptake was only slightly greater than with PH and DMH in Cryptochiton digestive gland incubations. The mammalian liver microsomal rates were approximately five times greater than chiton digestive gland microsomes.

Rates of DMA-dependent oxygen uptake were approximately seven times greater than the rates of DMA N-oxide formation. Conversely, methimazole oxidation was more than twice the rate of methimazole-dependent oxygen uptake. Dixit and Roche (1984) state that the utilization of oxygen uptake as measurement of FMO activity is limited by interfering reactions in incubation mixtures and is, consequently, not very selective. This would explain the higher levels observed in the DTNB assay relative to the oxygen uptake experiments. However, regarding the differences observed with DMA, activation of other oxidative pathways which require molecular oxygen may be responsible for the inconsistency.

After examining benzo(a)pyrene metabolism in several bivalve molluscs, Stegeman (1985) suggested a possible contribution by co-oxidation pathways such as lipid peroxidation and prostaglandin synthase to xenobiotic metabolism which would also require oxygen. In fact, hydroxyl radicals, hydrogen peroxide, and superoxide have been reported to be present in _Mytilus_ digestive gland incubations containing xenobiotics (Livingstone et al., 1988). Cytochrome P-450 has also been observed in _Cryptochiton_ and oxidation of FMO substrates by P-450 may be responsible for additional oxygen uptake (Schlenk and Buhler, 1988).

The endogenous function and overall contribution of FMO to xenobiotic biotransformation in marine molluscs are still unknown. Kurelec has suggested that this activity in marine animals probably serves more of a physiological rather than a "detoxicating" function, metabolizing yet unknown endogenous and/or exogenous xenobiotics and excreting them _in vivo_ (Kurelec et al., 1986a). Ziegler has stated that only cysteamine has been identified as an endogenous substrate for FMO in mammals (Ziegler, 1980; 1988). One possible endogenous substrate in molluscs might be trimethylamine which is found in molluscan cytosol as the N-oxide, functioning as an osmotic effector (Burton, 1983). However, since digestive gland microsomal nitrogen and sulfur-containing substrate biotransformation by FMO was ten times that of cytochrome P-450-dependent benzo(a)pyrene hydroxylase in _Cryptochiton_ (Schlenk and Buhler, 1988), contribution to xenobiotic metabolism cannot be excluded. In addition, other

pathways may play a role in molluscan xenobiotic metabolism. The process of biotransformation of foreign compounds in these animals may be mediated by a complex of enzymes each perhaps contributing a small amount that enables the animal to survive in a complex environment.

ACKNOWLEDGEMENTS

Figure 5.   Oxygen uptake after the addition of various nitrogen
             and sulfur-containing chemicals to chiton digestive
             gland microsomes.
             Each value represents the mean ± S.D. of three
             individual animals. (DMA = N,N-dimethylaniline; PH =
             phenylhydrazine; 2-AF = 2 aminofluorene; DMH = 1,1-
             dimethylhydrazine).
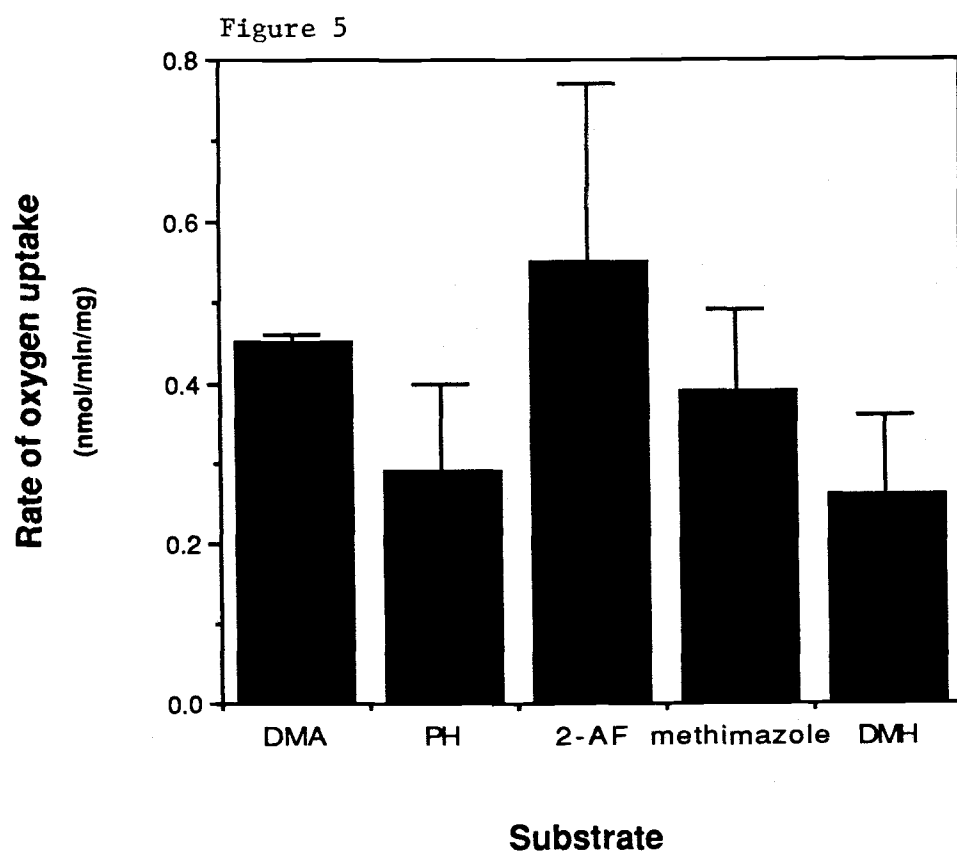
Figure 5

Figure 6.   pH profile of oxygen uptake rates from chiton
             digestive gland microsomes utilizing 2-AF as
             substrate.
             Each value represents the mean of two replications on
             an individual animal.

Figure 6
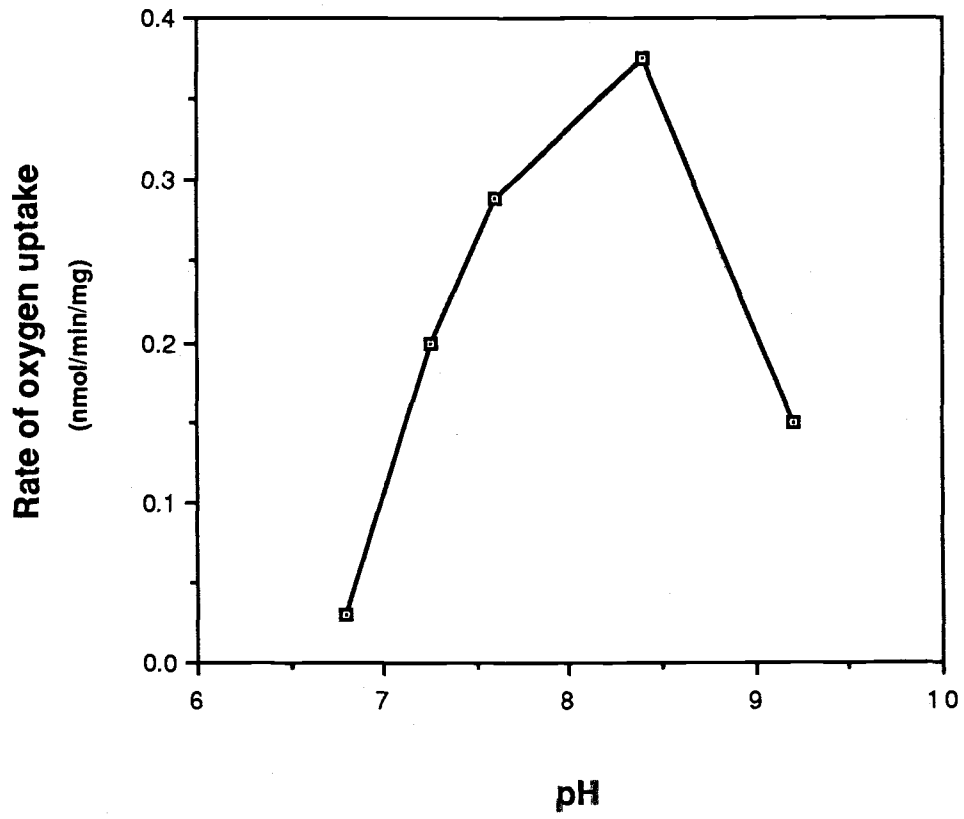
Figure 7.   Methimazole-enhanced TNB-oxidation catalyzed by rat
            liver and chiton digestive gland microsomes.
            Rat liver microsomal methimazole oxidation values are
            represented as the mean of three replications $\pm$ S.D.
            at 37°C.   Chiton digestive gland activity was
            represented as the mean of three individual animals $\pm$
            S.D. at 20°C.
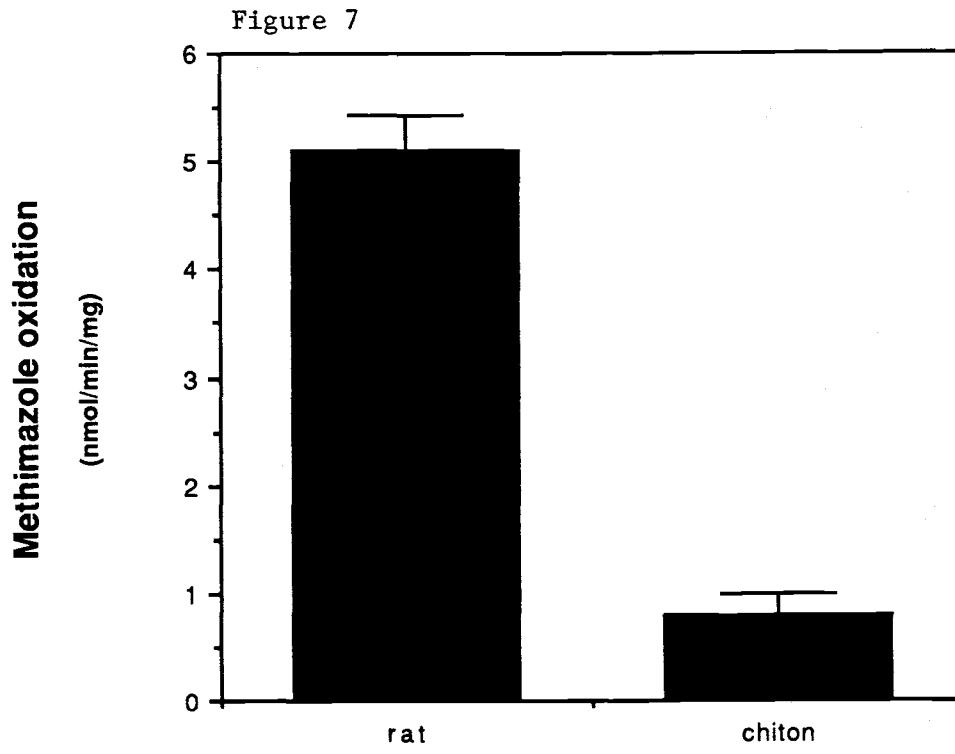
Figure 7

Figure 8.   Effect of NADPH and boiling on DMA N-oxide formation
             by chiton digestive gland microsomes.
             Each value represents the mean ± S.D. of three
             individual animals.

Figure 8
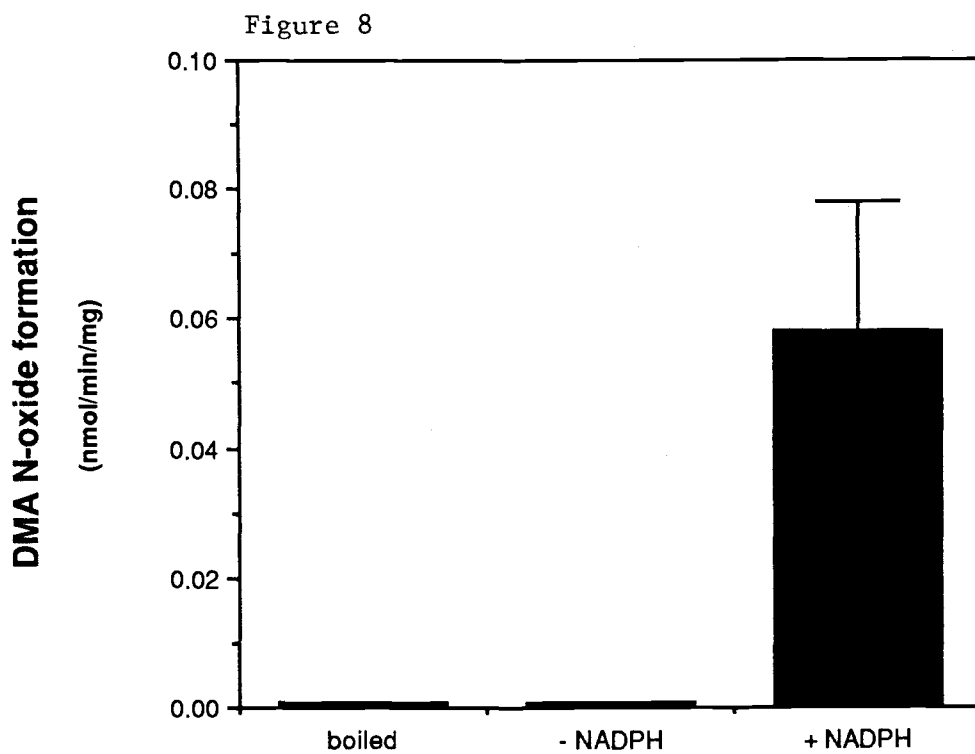
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Chapter 3

Xenobiotic Biotransformation

in the Pacific Oyster

(Crassostrea gigas).

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ABSTRACT

Oyster visceral mass and gill tissues possessed measurable
flavin-containing monooxygenase (FMO) activity. Although lacking
spectral evidence for cytochrome P-450, a peak at 418 nm was
observed along with NADPH-cytochrome c reductase activity in
visceral mass and gill microsomes. FMO activity was confirmed in
visceral mass microsomes by oxygen uptake experiments utilizing
various nitrogen and sulfur-containing chemicals along with
measurement of N,N-dimethylaniline (DMA) N-oxidase and methimazole
oxidation activities. DMA N-oxidase and methimazole oxidation
activities also were present in gill microsomes. NADPH-
independent benzo(a)pyrene hydroxylase (BPH) activity was observed
in both oyster visceral mass and gill microsomes suggesting a co-
oxidation pathway. However, additions of
ethylenediaminetetraacetic acid (EDTA), superoxide dismutase
(SOD)/catalase, indomethacin, or induction of lipid peroxidation
caused no significant change in BPH. Additions of tertiary butyl
hydroperoxide and glutathione led to respective increases and
decreases in visceral mass BPH. Additions of antibody raised
against trout cytochrome P-450 reductase did not affect NADPH-
cytochrome c reductase activity in visceral mass microsomes.
Excluding oyster gill methimazole oxidation, there were no
consistent seasonal differences in FMO activity in oyster gill or
visceral mass microsomes.

INTRODUCTION

The oyster is a major seafood resource in many countries, especially along the east coast of the United States where these and other marine animals must tolerate increased levels of pollution in their ecosystems. Consequently, a better understanding of the biochemical processes that allow these organisms to survive is necessary.

Oysters have been shown to possess very low levels of xenobiotic biotransformation activity. Working with the eastern species of oyster, Crassostrea virginica, Anderson and Angel (1986) observed visceral mass microsomal benzo(a)pyrene hydroxylase (BPH) activities in the pmol/min/mg protein range and they stated that this activity was mediated through cytochrome P-450. In the pacific oyster Crassostrea gigas, Knezovich and Crosby (1985) demonstrated low levels of in vivo metabolism of o-toluidine which was indicative of monooxygenase involvement.

Two dominant xenobiotic metabolizing enzyme systems are cytochrome P-450 and the flavin-containing monooxygenase. Recent studies have shown the presence of these systems in various marine molluscs (Kurelec, 1985; Livingstone et al., 1985; Livingstone et al., 1986; Livingstone, 1988; Schlenk and Buhler, 1988; Schlenk and Buhler, 1989). While both enzyme systems require molecular oxygen and NADPH, cytochrome P-450 differs from FMO in substrate specificity and pH optimum. For example, FMO generally catalyzes the oxidation of basic nucleophilic nitrogen and sulfur-containing

chemicals while cytochrome P-450, in addition to oxidizing various other functional groups, acts on acidic nitrogen and sulfur-containing compounds. The pH optima for cytochrome P-450 and FMO are 7.6 and 8.4, respectively.

The purpose of the research reported in this paper was to examine the pacific oyster, <u>Crassostrea</u> <u>gigas</u>, for the presence of cytochrome P-450 and FMO activities and to determine their relative contributions to xenobiotic metabolism in this important human food source.

METHODS AND MATERIALS

Chemicals

N,N-Dimethylaniline (DMA) was purchased from Aldrich Chemical Company (Milwaukee, WI), and recrystallized as the hydrochloride salt by dissolving 15 ml DMA in 50 ml of anhydrous ether and bubbling in HCl gas generated by the dropwise addition of concentrated sulfuric acid to solid sodium chloride. $[G-^3H]$-Benzo(a)pyrene (BP) was purchased from ICN Radiochemicals (Irvine, CA). All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO).

Animal maintenance

Animals were collected from the American Oyster Company in Toledo, OR and held until use in either flowing seawater tanks at the Oregon State University Hatfield Marine Science Center (Newport, OR) at ambient temperature (11-14°C) or at Oregon State University (Corvallis, OR) in an aquarium containing synthetic seawater at 13°C. Animals were collected in February, April, May and June.

Tissue preparations and enzyme assays

Visceral mass and gill were dissected from 10 oysters and
microsomes were prepared as previously described (Schlenk and
Buhler, 1988). Briefly, each tissue was dissected, homogenized in
buffer A [20 mM Tris-HCl, pH 7.6, containing 0.5 M sucrose; 0.15
M KCl; 1.0 mM ethylenediaminetetraacetic acid (EDTA); 1.0 mM
dithiothreitol (DTT); and 0.1 mM phenylmethylsulfonylfluoride
(PMSF)] and centrifuged at 10,000 x g for 10 min. Following a
second centrifugation at 12,000 x g for 20 min, a final
centrifugation at 100,000 x g for 90 min was performed. The
microsomal pellet was resuspended in buffer B (20 mM Tris-HCl, pH
7.6, 20% w/v glycerol; 1.0 mM EDTA; 1.0 mM DTT; and 0.1 mM PMSF).
All assays were performed on fresh unfrozen samples.

The quantitation of cytochrome P-450 was determined from the
carbon monoxide difference spectrum of sodium dithionite treated
samples on a Cary 219 spectrophotometer using an extinction
coefficient of 100 mM^{-1} (Estabrook and Werringloer, 1978). NADPH-
cytochrome c reductase activity was measured
spectrophotometrically by the increase in absorbance at 550 nm-
extinction coefficient: 19.6 mM^{-1} (Shimakata et al., 1972). The
reaction mixture contained (in a final volume of 1.0 ml), 50 mM
Tris-HCl, pH 7.6, 1.0 mM KCN, 0.03 mM cytochrome c and 0.26 mM
NADPH. Benzo(a)pyrene hydroxylase (BPH) activity was measured
radiometrically (Livingstone and Farrar, 1984). The incubation
mixture contained 50 mM Tris-HCl (pH 7.6), 0.4 mM NADPH, 0.06 mM

of [G-^{3}H] Benzo(a)pyrene (BP) (110 mCi/mmol), and 1.0 mg

microsomal protein in a final volume of 1.0 ml. Induction of

lipid peroxidation was achieved utilizing the method of Dix and

Marnett (1983). Lipid peroxidation-induced BPH incubations were

identical to the typical BPH samples above with the addition of 4

mM adenosine diphosphate (ADP) 0.015 mM ferric chloride, and 1.0

mM ascorbic acid. Microsomal preparations excluded EDTA and DTT

during homogenization and resuspension. Total water soluble BP

metabolites were extracted and counted via scintillation.

Incubations with boiled microsomal protein served as controls.

Antibodies to purified trout NADPH-cytochrome P-450 reductase were

raised in rabbits (Williams et al., 1983) and rabbit serum

controls were generously provided by Dr. David E. Williams. The

dealkylation of 7-ethoxyresorufin (EROD) was measured

fluorometrically as described previously (Prough et al., 1978).

Oxygen uptake was measured with a Clark-type electrode at 15,

20, 25, and 37°C. In a total volume of 0.6 ml, reaction mixtures

containing 0.05 M potassium phosphate, 0.05 M Tricine HCl, each at

a varied pHs, 0.25 mM NADPH, and 1.0 mM EDTA were preincubated for

3-5 min. After the addition of 1.0 mg microsomal protein,

endogenous oxygen uptake was recorded for about 1 min or until the

rate was linear for at least 30 sec. Substrate (1.0 mM) was added

and oxygen uptake was recorded for another 1-2 min. FMO

substrates consisted of 2-aminofluorene (2-AF), N,N-

dimethylhydrazine (DMH), phenylhydrazine (PH), methimazole, and

DMA.

FMO catalyzed methimazole oxidation was measured by the spectrophotometric assay of Dixit and Roche (1984). Assay mixtures contained 0.10 M Tricine HCl, pH 8.4, 1.0 mM EDTA, 0.06 mM 5,5'-dithiobis(2-nitrobenzoate) (DTNB), 6.0 mM potassium phosphate, pH 8.0, 0.025 mM DTT, 0.10 mM NADPH, 1.0 mg microsomal protein, and 1.0 mM methimazole in a final volume of 1.0 ml. Enzyme activity was initiated by addition of methimazole to one cuvette, and reaction rates were measured with a Cary 219 spectrophotometer as the rate of decrease in absorbance at 412 nm between identical assay mixtures with and without methimazole.

DMA N-oxidation was determined by the method of Ziegler and Pettit (1964). A 1.0 ml incubation mixture contained 0.25 M potassium phosphate, pH 8.4, 0.5 mM NADPH and 1.0 mg microsomal protein. The reaction was started by the addition of 4 mM DMA, and then incubated at 20°C for 30 min. After extraction with ether to remove unreacted substrate, DMA N-oxide was reduced by nitrous acid back to DMA, converted to the nitroso compound and was colorimetrically quantitated at 420 nm (extinction coefficient 8.2 mM^{-1}). Blanks consisted of incubations containing boiled microsomes.

Experimental Design

Animals collected in February were used exclusively for FMO assays (oxygen uptake and methimazole oxidation). Due to a limited yield of gill microsomes, oxygen consumption studies,

including temperature and pH optima, were performed exclusively on visceral mass microsomes. Gill and visceral mass microsomes from animals collected in April were used primarily for cytochrome P-450 assays (CO-difference spectra, BPH, NADPH-cytochrome c reductase and EROD activities). One FMO assay (DMA-oxidase) was performed on gill and visceral mass microsomes from April animals. Due to a lack of gill microsomes, antibody inhibition studies were performed only with visceral mass microsomes. To assess seasonal differences in oxygen uptake, experiments using visceral mass microsomes also were done in animals collected in late May. Gill and visceral mass microsomes prepared from May animals were also used in methimazole oxidation assays. Due to differences observed in DMA N-oxide formation and DMA-dependent oxygen uptake, oxygen uptake experiments were repeated using DMA N-oxide and DMA in samples collected in June. Visceral mass microsomes from animals taken in June were also used for tertiary butyl hydroperoxide, glutathione and lipid peroxidation BPH incubations.

Statistical analyses (Student's t-test) were performed using the statistical software, Statview, from Brainpower Inc. (Calabasas, CA).

Protein concentrations were determined using the method of Lowry et al. (1951).

RESULTS

There was no spectral evidence for the presence of cytochrome
P-450 in oyster visceral mass or gill microsomal preparations.
The limits of detection using this method were approximately 20
pmol/mg microsomal protein. Oysters collected at different times
of the day and different locations were examined. Cytochrome P-
450 determinations were attempted in Corvallis and Newport
utilizing varied buffer and microsome concentrations,
temperatures, and pH. However, a consistent peak at 418 nm and no
peak at 450 nm was observed in the CO difference spectra in
microsomes from both tissues (Figure 9). NADPH-cytochrome c
reductase activity was present in gill (6.0 $\pm$ 0.01 nmol/min/mg; N
= 4 replications) and visceral mass (3.9 $\pm$ 0.003 nmol/min/mg; N =
4 replications) microsomes but EROD activity was not observed in
either tissue after multiple assay attempts (data not shown).
However, low levels of NADPH-catalyzed BPH activity was observed
in oyster gill and visceral mass along with measurable levels of
NADPH-independent BPH in visceral mass (Table 6). After
discovering that NADPH-independent biotransformation was possible,
boiled microsomes were utilized as controls.

Since cytochrome P-450 was not observed spectrally in
oyster microsomes, the possible occurrence of NADPH-independent
cooxidation pathways for BPH was examined. No significant change
in BPH or NADPH-cytochrome c reductase was observed when antibody

to trout NADPH-cytochrome P-450 reductase was added to visceral
mass microsomal incubations (Figure 10). In addition, neither
EDTA, SOD/catalase nor indomethacin had a significant effect on
BPH when added to gill or visceral mass microsomal incubation
mixtures (Table 6). Although induction of lipid peroxidation
failed to alter BPH activity, the addition of tertiary butyl
hydroperoxide (1.0 mM) led to significant increases in BPH in
visceral mass microsomes taken from oysters collected in June
(Figure 11). In these same incubations, a decrease in BPH was
observed when glutathione (1 mM) was included in the microsomal
mixtures (Figure 11).

In experiments utilizing the Clark oxygen electrode, the pH
optimum for oxygen uptake was 8.4 when 2-AF and NADPH were added
to visceral mass microsomal mixtures (Figure 12) and the optimum
temperature for oxygen uptake was 20°C (data not shown). Of the
various nitrogen and sulfur-containing chemicals tested, 2-AF gave
the highest rate of oxygen uptake when incubated with visceral
mass microsomes from animals collected in early February (Figure
13). 2-AF also had the greatest activity in visceral mass and
gill microsomal mixtures from animals killed in late May. DMH and
PH activities in May visceral mass microsomal mixtures were
enhanced while other substrates showed a decrease (methimazole and
2-AF), or no change (DMA).

Gill and visceral mass microsomes both possessed similar
methimazole oxidation rates in February animals (Figure 14).
However, gill microsomes from May oysters had one-half the

activity of the gill microsomes from animals collected in
February. Visceral mass microsomal methimazole oxidation rates
were unchanged.

Three replications of DMA oxidase assays were carried out only
on a set of animals collected in April. Rates of DMA N-oxide
formation from visceral mass microsomes (0.024 ± 0.01 nmol/min/mg)
were more than two-fold higher than the activity observed with
microsomes from gill tissue (0.007 ± 0.028 nmol/min/mg). Due to
the large differences between DMA-dependent oxygen uptake and DMA
N-oxide formation, DMA-dependent oxygen uptake was assayed a
second time and compared with the combination of DMA N-oxide-
dependent oxygen uptake and DMA N-oxide formation. The rate of
DMA-dependent uptake was still one-third higher than the combined
rates of DMA N-oxide-dependent oxygen uptake and DMA N-oxide
formation (Figure 15).

DISCUSSION

Xenobiotic biotransformation in marine bivalves is relatively
unexplored. However, there is convincing evidence for the
presence of cytochrome P-450 in these species (Stegeman, 1985;
Livingstone et al., 1985; Livingstone et al., 1986). Likewise,
FMO activity has been shown to be present in Mytilus edulis and
Mytilus galloprovincialis (Kurelec, 1985; Britivic and Kurelec,
1986). The lack of spectral evidence for cytochrome P-450 in
Crassostrea gigas and the ready detection of FMO activity were
somewhat unexpected.

Initially, rates of oxygen uptake were used as a general
measure of FMO activity. This relatively quick assay used
compounds containing nitrogen and sulfur atoms previously observed
as substrates for mammalian FMO enzymes (Ziegler, 1980; 1988).
The pH optimum of 8.4 and the requirement for both NADPH and
oxygen uptake upon addition of these nitrogen and sulfur-
containing substrates provide strong evidence for the presence of
FMO in oyster visceral mass microsomes. Furthermore, the
demonstration of methimazole and DMA oxidations support this claim
in both visceral mass and gill microsomes. However, rates of
oxygen uptake were considerably lower than those seen by Kurelec
(1985).

The differences in oxygen uptake when compared to the specific
assays for methimazole and DMA oxidations are interesting. Oxygen

uptake using DMA was more than ten times greater than N-oxide formation. In mammalian systems, DMA N-oxide is rapidly reduced back to DMA, thus underestimating N-oxide formation _in_ _vivo_ (Ziegler, 1988). In experiments utilizing the oxygen electrode, DMA N-oxide was added to microsomes and oxygen consumption was again observed indicative of the cycling effect noted by Ziegler (1988). Reduction of DMA N-oxide to DMA could occur via cytochrome P-450 or another unknown two electron microsomal reductase. Although the DMA-dependent oxygen uptake and levels of N-oxide formation/reuptake still differed by a third, they were not statistically different ($p \geq 0.4$; Student's t-test). The remaining differences between the two assays may be due to a lack of sensitivity of the assay for N-oxide formation (Ziegler, 1988). The ratio of oxygen uptake using methimazole as substrate to methimazole oxidation via the spectrophotometric assay of Dixit and Roche (1984) was observed to be roughly 1:2. In contrast to the differing DMA oxidation assay values, methimazole oxidation assay discrepancies are most likely explained by the enhanced sensitivity of the Dixit and Roche method compared to methimazole-dependent oxygen uptake (Ziegler, 1988).

Since cytochrome P-450 and BPH levels have been shown to vary with season in molluscs (Livingstone, 1985), seasonal differences in FMO were examined. However, due to large variability in visceral mass oxygen uptake rates among the nitrogen and sulfur-containing chemicals examined in this study, it was difficult to determine whether a seasonal difference in FMO activity exists in

the oyster. In visceral mass microsomes, one substrate showed no difference (DMA), two showed an increase in May (DMH and PH), and two showed a decrease in May (2-AF and methimazole). However, when methimazole oxidation alone was examined utilizing a more sensitive assay, visceral mass microsomal activity was found to be similar in both February and May animals. Conversely, gill microsomal activity in May animals was only one-half that observed in February animals. With the exception of methimazole oxidation in the gill, a seasonal difference in FMO activity does not seem to be present. Oysters become more metabolically active in the summer due to increased food availability from Pacific Northwest summer upwelling and subsequent consumption (Langdon, personal communication). Also, since Crassostrea spawns in the summer, the animals possess greater quantities of gonadal material. Hence, greater levels of enzyme activities associated with feeding or reproduction would be expected. However, it is possible that these enzymes may not play an integral role in digestion or hormone function.

The lack of BPH inhibition by antibody to NADPH-cytochrome P-450 reductase may be due to a failure of the antibodies to trout NADPH-cytochrome P-450 reductase to react with the oyster reductase due to epitope differences between fish and oyster reductases. Nevertheless, the absence of detectible spectral evidence for cytochrome P-450 and the existence of NADPH-independent BPH, coupled with the absence of EROD activity, is consistent with the absence of cytochrome P-450 catalyzed

oxidation of BP in the oyster and lends further support to the presence of a cooxidation pathway in Crassostrea visceral mass microsomes.

Stegeman (1985) has suggested the possible involvement of lipid peroxidation and/or prostaglandin synthase in BP metabolism. However, when SOD and catalase, indomethacin or EDTA, were added to BPH incubations, no significant decrease was observed. Furthermore, when lipid peroxidation was initiated in visceral mass microsomal incubations, there were no differences observed between lipid peroxidation incubations and controls (+ NADPH). However, the addition of tertiary butyl hydroperoxide and glutathione led to significant enhancement and supression of BPH, respectively. These data suggest the presence of a co-oxidation mechanism which does not involve an iron, cyclooxygenase, hydrogen peroxide or superoxide anion catalyzed reaction, but which is inactivated by protein denaturation (i.e. enzymatic), stimulated by an alkyl hydroperoxide and inhibited by a free radical scavenger. Livingstone et al., (1988) has observed the presence of hydroxyl radical, hydrogen peroxide, and superoxide anion in Mytilus digestive gland. Each of these activated forms of oxygen may be a likely intermediate in a cooxidation pathway which might involve lipid and xenobiotic substrates (see Figure 16a). Perhaps, a microsomal "lipoxygenase-like" enzyme may be responsible for the formation of lipid hydroperoxides which then transfer a molecule of oxygen via a nonenzymatic hydroxyl radical mechanism or a second "peroxidase-like enzyme" to the xenobiotic

leading to oxygenated product and a lipid alcohol. An alternative hypothetical process (see Figure 16b) may involve a microsomal enzyme which reduces oxygen directly to the hydroxyl radical which could then rapidly react with a chemical within close proximity of the radical. Livingstone et al. (1989) have suggested the occurrence of a microsomal component capable of storing reducing equivalents and causing NADPH-independent reactions. A third possiblity may involve a combination of these two mechanisms where a microsomal component/reductase could reduce oxygen, forming a lipid hydroperoxide which could then transfer oxygen to substrate via a second peroxidase enzyme (Figure 16c). Obviously, much more work needs to be performed to confirm or disprove these suggestions.

Since tissue preparation and difference spectra analysis were identical to that of previous molluscan work (Livingstone and Farrar, 1984; Schlenk and Buhler, 1988), the lack of spectral data for cytochrome P-450 was definitely unexpected. However, this result is consistent with western blot analysis (see Figure 1) where only a faint reaction occured against antibodies raised to purified trout cytochrome P-450 LM_2. The presence of NADPH-cytochrome-c reductase activity, indicative of NADPH-cytochrome P-450 reductase, further complicates the issue. One possible explanation for the failure to detect cytochrome P-450 linked activity could be the presence of endogenous proteases which were not neutralized by PMSF in the tissue homogenization procedure. The occurrence of a "418" peak is consistent with previous studies

involving molluscs (Livingstone, 1988) and suggests possible denaturation of cytochrome P-450 to P-420. Perhaps the cytochrome P-450 system in Crassostrea gigas is more unstable than that of other molluscs.

The presence of FMO and the evidence for cooxidation pathways warrant further investigation of Crassostrea microsomal xenobiotic biotransformation enzymes in order to better understand how bivalves cope with foreign chemicals.

ACKNOWLEDGEMENTS

Figure 9.   Typical CO-difference spectrum of oyster visceral mass
and gill microsomes.

Figure 9

"418" peak

450 nm
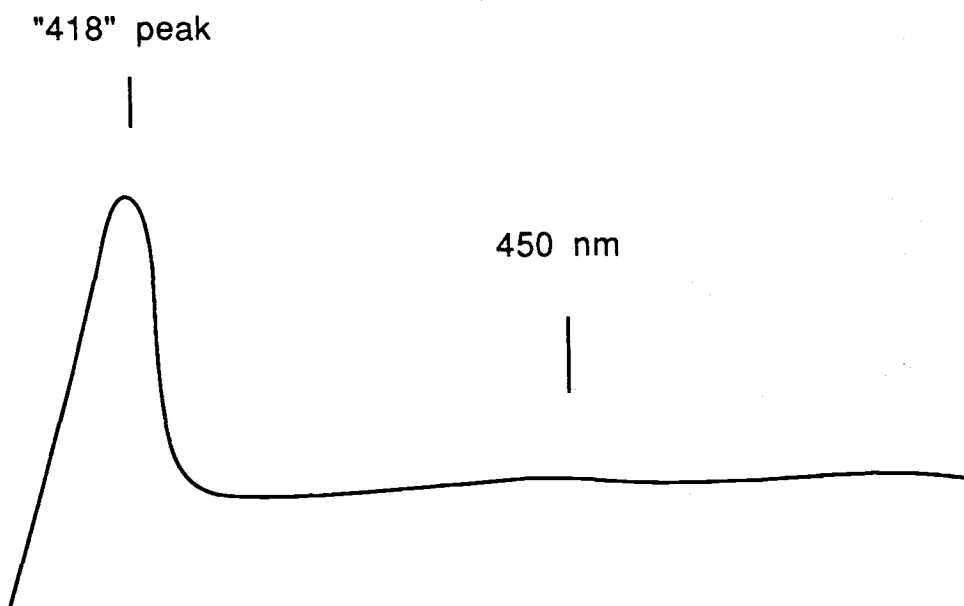
Figure 10.   The effect of anti-trout NADPH-cytochrome P-450
             reductase antibodies on NADPH-cytochrome-c-reductase
             in microsomes from oyster visceral mass.
             All values represent the mean of three replications
             from the same preparation $\pm$ S.D.. Animals were
             collected in April.
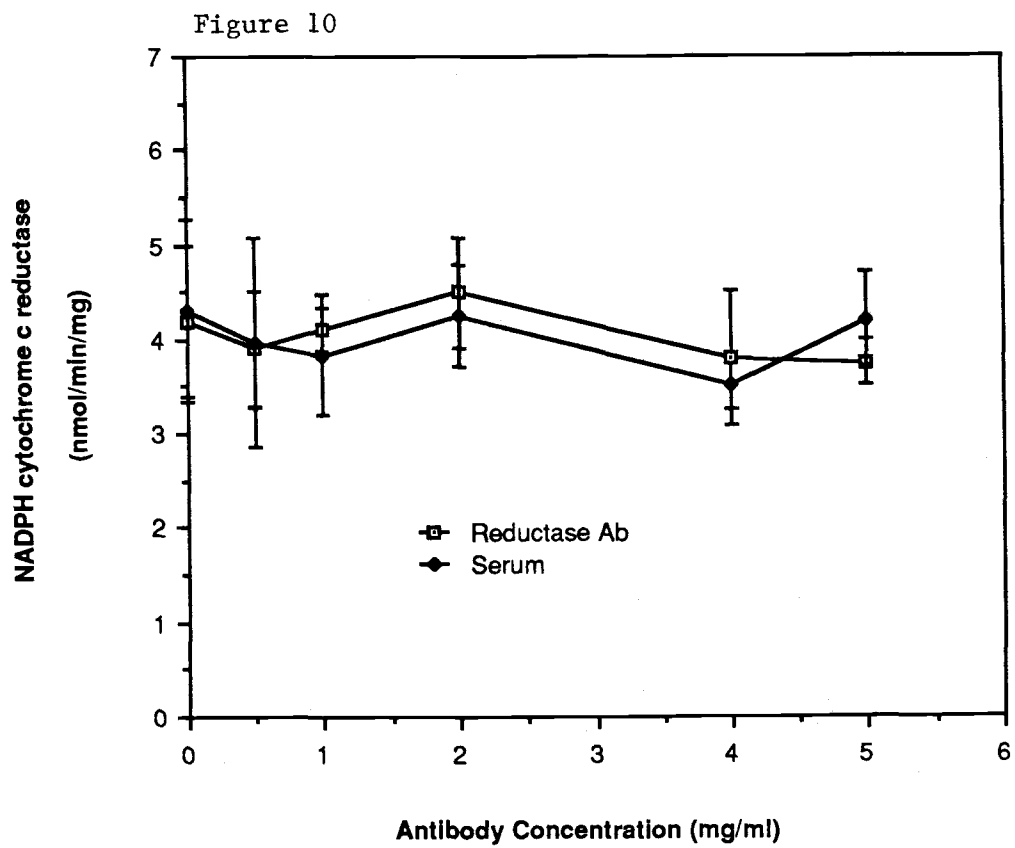
Figure 10

Figure 11. The effect of lipid peroxidation (LP), glutathione
(GSH), and tertiary butyl hydroperoxide (TerBuOOH) on
benzo(a)pyrene hydroxylase in microsomes from oyster
visceral mass.
Each value represents the mean of four replications
from the same preparation ± S.D.. Animals were
collected in June.
a 1.0 mM tertiary butyl hydroperoxide.
b 1.0 mM glutathione.
c 4.0 mM ADP, 15 μM FeCl$_3$, 1.0 mM ascorbate.
* p $\leq$ 0.01; Student's t-test.
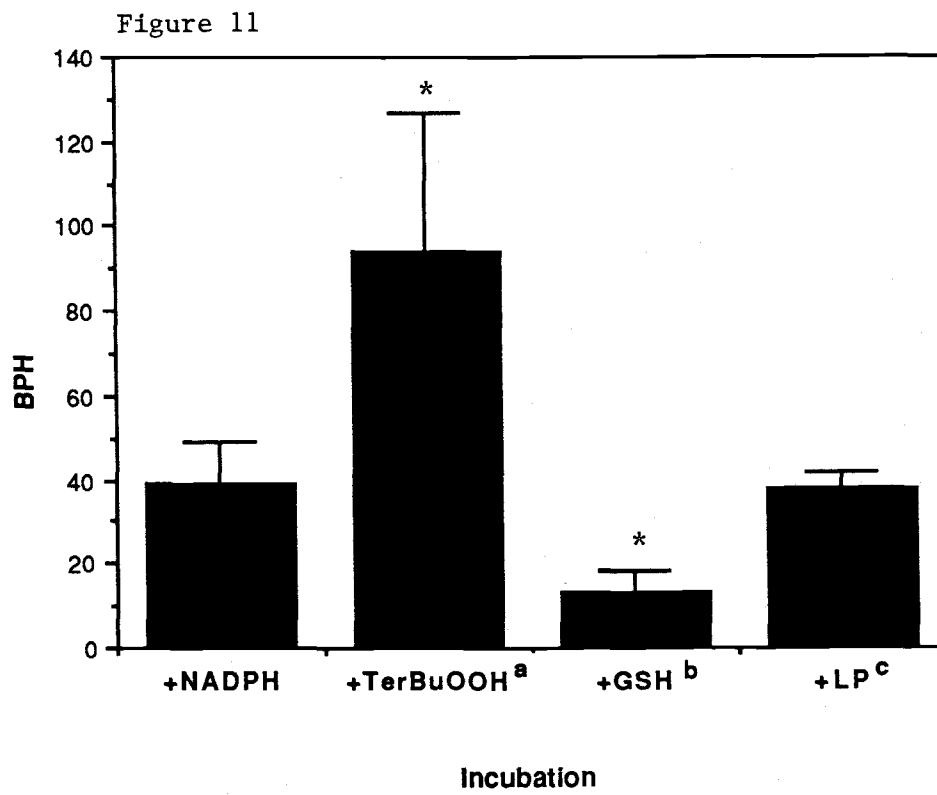
Figure 11

Figure 12. pH profile of oxygen uptake rates from oyster
visceral mass microsomes utilizing 2-AF as substrate.
Each value represents the mean of two replications
from the same preparation. Animals were collected in
February.
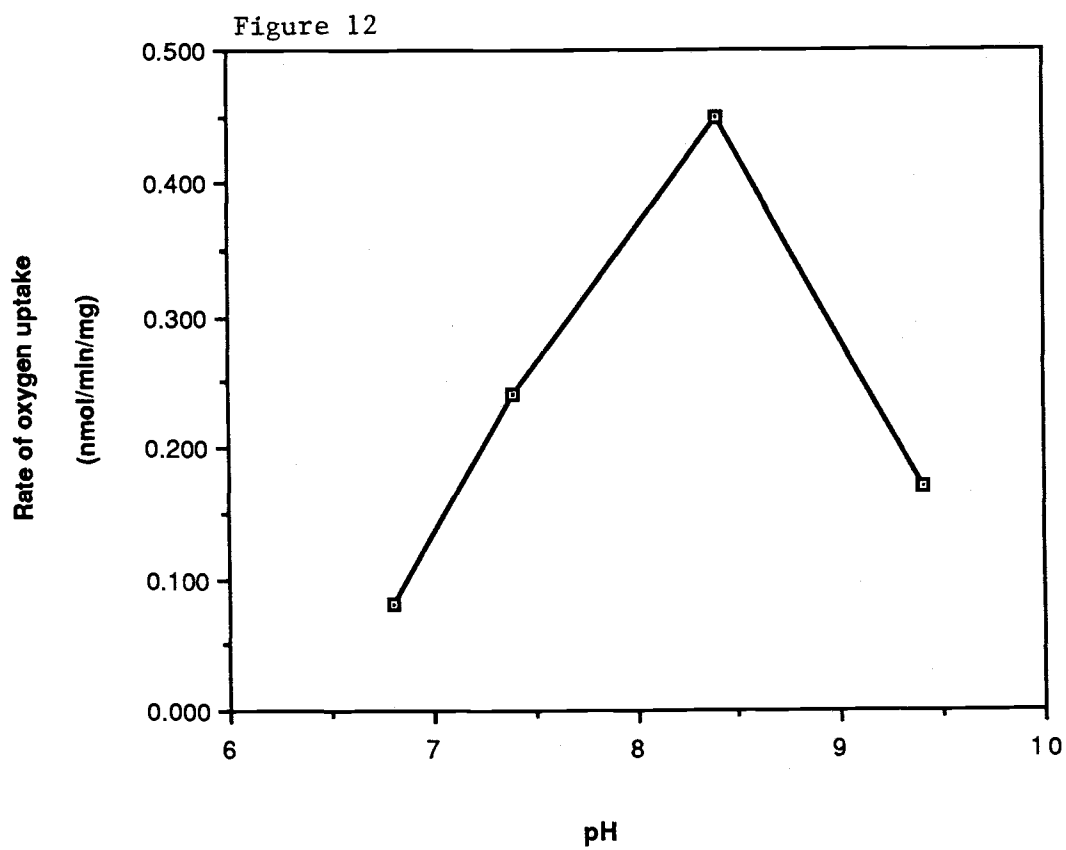
Figure 12

Figure 13. Oxygen uptake after the addition of various nitrogen
and sulfur-containing substrates to oyster visceral
mass microsomes from animals collected in February and
May.
Each value represents the mean of two replications
from the same preparation at pH 8.4 and 20°C.
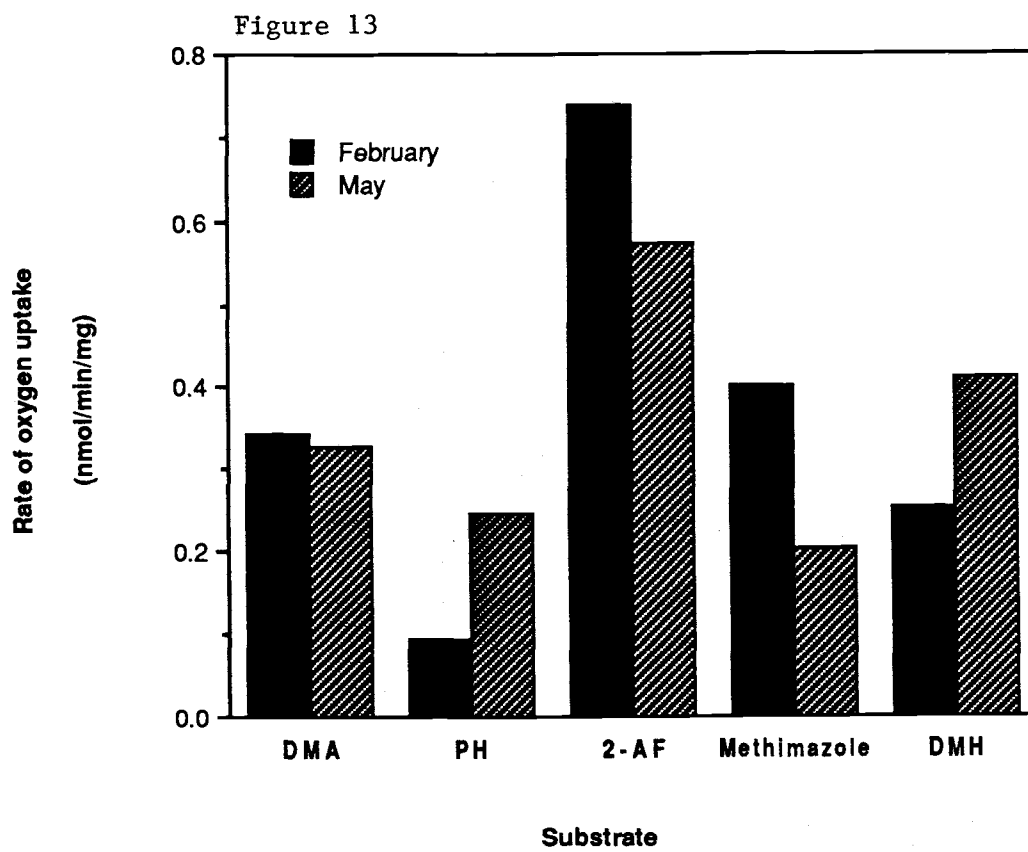
Figure 13

Figure 14.   Methimazole-enhanced TNB-oxidation catalyzed by
             oyster visceral mass and gill microsomes from animals
             collected in February and May.
             Each values represent the mean of three replications
             from the same preparation ± S.D..
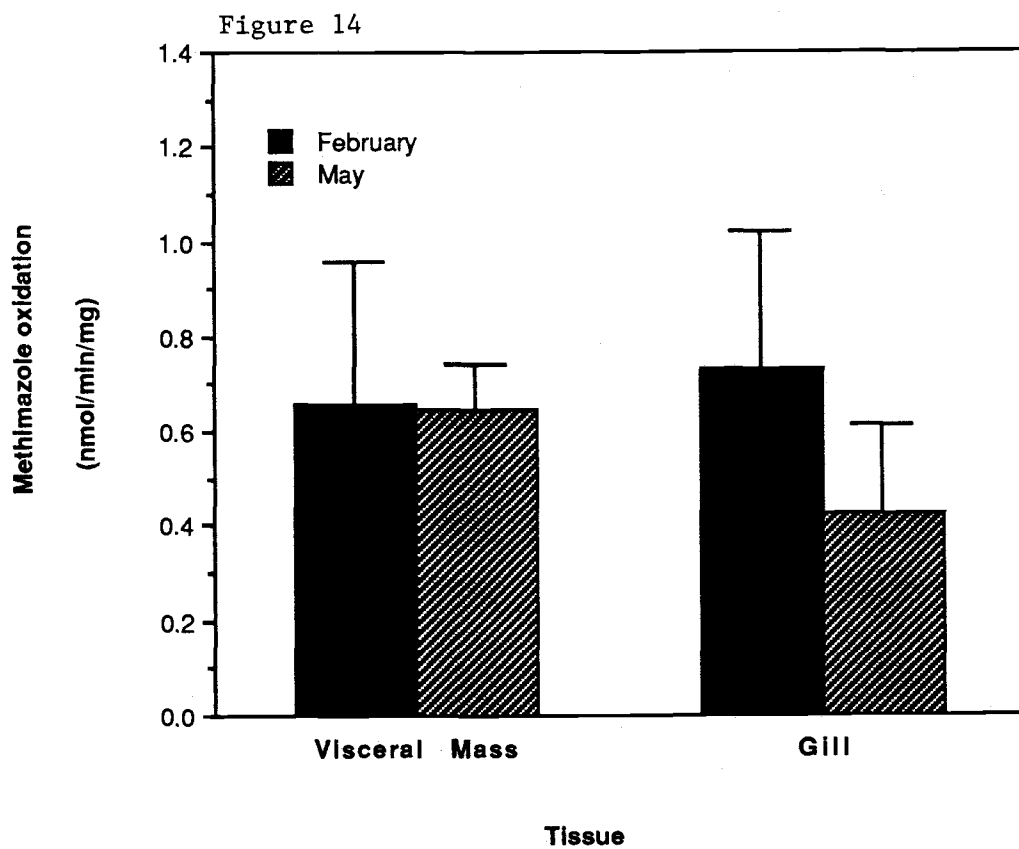
Figure 14

Figure 15. Comparison of DMA-dependent oxygen uptake and the
            combination of DMA N-oxide-dependent oxygen uptake
            and DMA N-oxide formation.
            Each value is the mean of three assay replications
            from the same preparation ± S.D.
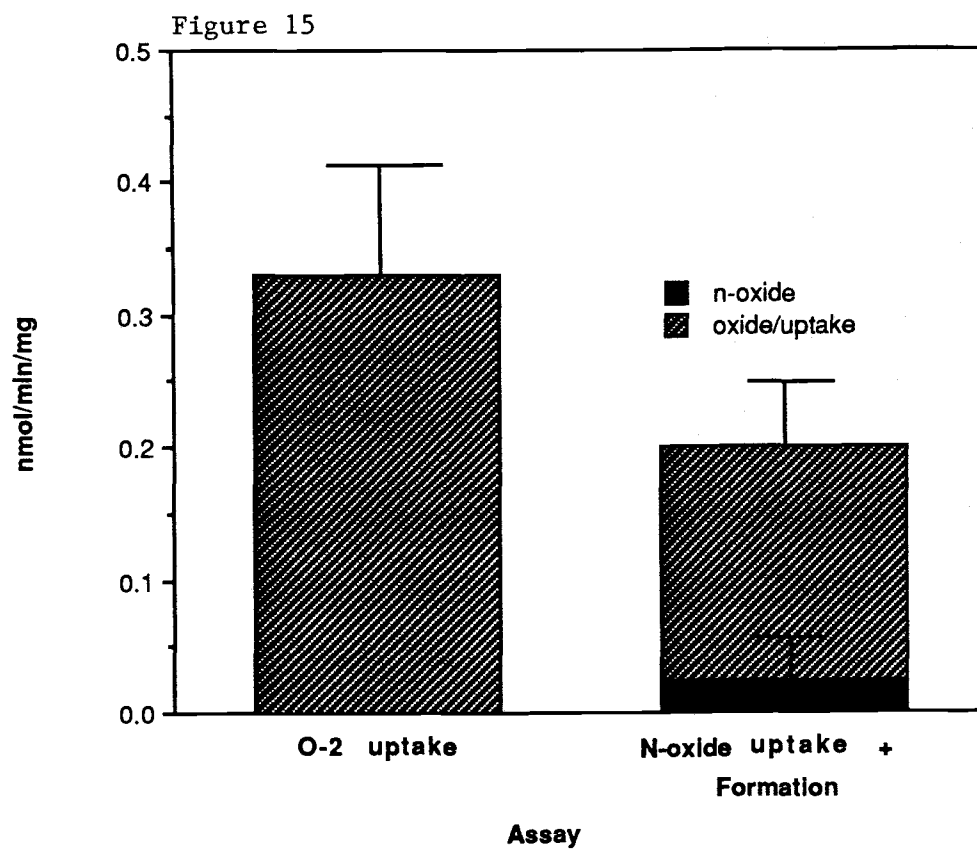
Figure 15

Figure 16. Proposed mechanisms of co-oxidation: a) via lipid
            hydroperoxide intermediate b) via microsomal
            reductase c) combination of a and b.

Figure 16

(a)

(b)

(c)

I
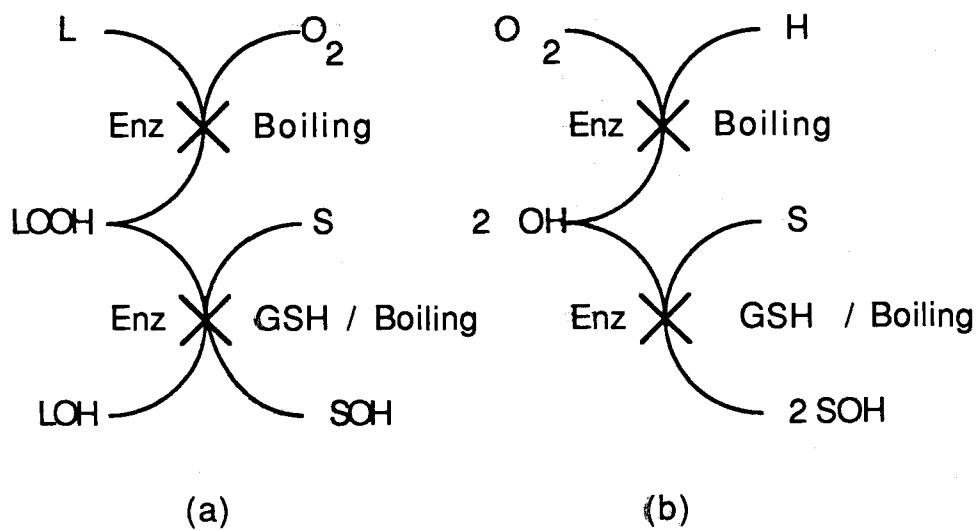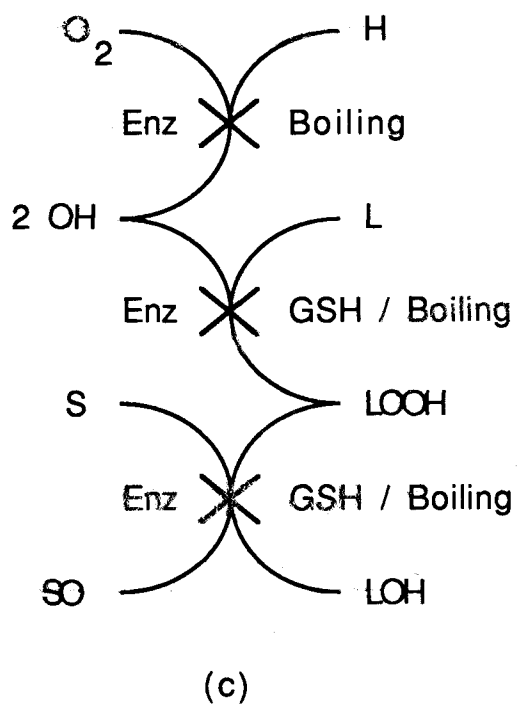
Table 6. The effect of NADPH and various inhibitors on
          benzo(a)pyrene hydroxylase activity in microsomes from
          oyster gill and visceral mass[a].
          a Values represent the means of three assay
          replications from the same preparation $\pm$ S.D.
          Animals were collected in April.
          b pmol/min/mg microsomal protein.
          c 1 mM EDTA.
          d 1.0 mg/ml SOD; 0.1 mg/ml Catalase.
          e 1.0 mM Indomethacin.

Table 6

Tissue	Condition	BPH[b]
Visceral mass	-NADPH[b]	19.4 ± 2.4
	+NADPH[b]	22.0 ± 1.5
	+EDTA[c]	23.5 ± 0.9
	+SOD/Cat[d]	21.0 ± 0.8
	+Indometh[e]	21.6 ± 5.1
Gill	-NADPH[b]	23.0 ± 1.7
	+NADPH[b]	25.4 ± 2.2
	+ETDA[c]	20.9 ± 0.6
	+SOD/Cat[d]	27.0 ± 1.1
	+Indometh[e]	24.2 ± 6.0

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Chapter 4

The In Vitro

Biotransformation of 2-aminofluorene in

the Pacific Oyster

Crassostrea gigas.

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ABSTRACT

The in vitro biotransformation of 2-aminofluorene (2-AF) was
examined in visceral mass microsomes from the pacific oyster
Crassostrea gigas. The major metabolite was N-formyl-2-
aminofluorene with smaller quantities of N-hydroxy-2-
aminofluorene and nitroso-2-aminofluorene or 2-nitrofluorene.
The mechanism of N-oxidation was explored using various
inhibitors, stimulators, cofactors, and pH modifications within
incubations. N-oxidation appears to occur via a one electron
peroxidative mechanism which involves a transition metal and/or
lipid peroxidation. Flavin-containing monooxygenase and possibly
prostaglandin synthase also seem to play a role in N-oxidation.

INTRODUCTION

The pathways and mechanisms of xenobiotic biotransformation in marine molluscs are relatively unknown (Livingstone, 1985). The balance between bioactivation and detoxication by Phase I pathways and the roles each system plays in biotransformation are important factors which allow marine molluscs to survive in chemically-hostile environments. However, there is much debate surrounding the enzymatic mechanism(s) responsible for the formation of bioactivated or detoxified organic compounds (Livingstone, 1987; Kurelec, 1987). Certain Phase I enzymes that have been shown to play a role in biotransformation processes in marine molluscs include the cytochrome(s) P-450 system (Schlenk and Buhler, 1988; 1989b; Livingstone 1988), flavin-containing monooxygenase (Schlenk and Buhler, 1989a; 1989b; Kurelec 1985) and cooxidation via lipid peroxidation or peroxidase (Schlenk and Buhler, 1989b).

In mammalian systems, the in vitro N-oxidation of the aromatic amine, 2-aminofluorene (2-AF) to the more toxic compound, N-hydroxy-2-AF, has also been shown to be mediated through cytochrome(s) P-450 (Vanderslice et al. 1987), flavin-containing monooxygenase (Hammons et al, 1985) and cooxidation via the peroxidase, prostaglandin synthase (Robertson et al., 1983). In molluscan systems, Kurelec et al. (1986) have suggested that FMO is exclusively responsible for the N-oxidation and subsequent bioactivation of 2-AF to mutagenic metabolites.

A major class of xenobiotics, aromatic amines, can enter the marine environment as constituents of fuel oils (Later et al., 1982) and synthetic dyes (Ellis et al., 1982). Knezovich et al. (1988) examined the in vivo biotransformation of 2-AF in the bivalve Mytilus, and found measurable levels of the mutagen, N-hydroxy-2-AF. Consequently, a potential hazard may exist to marine animals which are capable of bioactivating compounds of this nature.

Most of the previous work in molluscan xenobiotic biotransformation studies have utilized the mussel, Mytilus (Kurelec et al., 1986; Knezovich et al., 1988; Livingstone 1988). However, xenobiotic biotransformation in the pacific oyster Crassostrea gigas, an important commercial fishery resource, has also been receiving attention (Knezovich and Crosby, 1985; Schlenk and Buhler, 1989b). The purpose of this research was to determine the in vitro biotransformation pathway of 2-AF in visceral mass microsomes from Crassostrea gigas and determine the mechanism(s) responsible for N-oxidation in this species.

METHODS AND MATERIALS

Chemicals

2-Aminofluorene (2-AF) and 2-nitrofluorene were purchased from Aldrich Chemical Company, Inc.(Milwaukee, WI). 2-Acetylaminofluorene was purchased from Sigma Chemical Co.(St Louis, MO). N-Methyl-2-aminofluorene and 7-hydroxy-2-acetylaminofluorene were obtained from the National Cancer Institute's chemical repository (Midwest Research Institute, Kansas City, MO). 2-Amino[ring-G-^{3}H]fluorene (1.85 Ci/mmole), N-hydroxy-2-aminofluorene (N-hydroxy-2-AF) and N-nitroso-2-aminofluorene (N-nitroso-2-AF) were purchased from Chemsyn Science Laboratories (Lenexa, KA). N-Formyl-2-aminofluorene (N-formyl-2-AF) was synthesized according to the method of Sheehan and Yang (1958) and structurally verified by 400 MHz ^{1}H-NMR (1H, s, δ 8.92 ppm, 1H, s, δ 11.88 ppm). 7-Hydroxy-2-aminofluorene was obtained via acidic deacetylation of 7-hydroxy-2-acetylaminofluorene by the method of Miller and McQueen (1986).

Animal maintenance

Western oysters (<u>Crassostrea</u> <u>gigas</u>) were obtained from The American Oyster Co. in Toledo, OR. The average shell lengths were 15.2 $\pm$ 5.7 cm. Four groups of ten oysters were collected in June,

1987. Each group was placed on ice and transported to Corvallis and maintained in an 18.9 liter (5 gal) aquarium containing Instant Ocean (Aquarium Systems, Inc., East Lake, OH) at a temperature of 15 $\pm$ 1 °C, salinity of 28 $\pm$ 1 o/oo and a pH of 7.8. Oysters were allowed to equilibrate in these surroundings for 2 days and then dissected for microsomal preparation.

Tissue preparations and enzyme assays

Visceral mass was dissected from 10 oysters and microsomes were prepared as previously described (Schlenk and Buhler, 1988). Briefly, each tissue was dissected, homogenized in buffer A [20 mM Tris-HCl (pH 7.6),containing 0.5 M sucrose, 0.15 M KCl, 1.0 mM ethylenediaminetetraacetic acid (EDTA), 1.0 mM dithiothreitol (DTT) and 0.1 mM phenylmethylsulfonylfluoride (PMSF)] and centrifuged at 10,000 x g for 10 min. Following a second centrifugation at 12,000 x g for 20 min, a final centrifugation at 100,000 x g for 90 min was performed. The microsomal pellet was resuspended in buffer B [20 mM Tris-HCl (pH 7.6), 20% w/v glycerol, 1.0 mM EDTA, 1.0 mM DTT, and 0.1 mM PMSF]. Microsomal preparations utilized to determine the contribution of lipid peroxidation did not contain EDTA or DTT in the homogenization and resuspension buffers. All assays were performed on fresh unfrozen samples. Lipid peroxidation was confirmed in assigned incubations by the measurement of malondialdehyde production (Beuge and Aust, 1978) (data not shown). Microsomal glutathione-S-transferase

activities were examined by the method of Habig et al. (1974).

Protein concentration was determined by the method of Lowry et

al. (1951).

Experimental Design

Six groups of ten oysters were labeled groups A through G.

Group A was used to determine optimum assay conditions and the

standard 2-AF biotransformation profile. To compare the formation

of 2-AF metabolites at pH 7.6, 8.4 and +/- NADPH, a complete block

design was employed utilizing groups B, C and D. Data were

statistically evaluated via a one-way analysis of variance

performed using statistical software (Statview) from Brainpower

Inc., (Calabasas, CA). Groups E, F and G were used to perform

three replications of each incubation mixture comparing the

effects of various inhibitors and stimulators on 2-AF metabolite

formation. In this last set of experiments, a randomized

incomplete block design was used. The data were analyzed using a

Least Squares Mean model with 95 % confidence bounds. This model

provides estimates of the treatment effects which are not

confounded by the variable of different sample blocks.

Assay conditions

In a total volume of 1.0 ml, standard assay incubations

contained buffer (50 mM Tris-HCl for pH 7.6; 50 mM Tricene for pH

8.4), 0.5 mM NADPH, 0.06 mM [^{3}H]-2-AF and 2.0 mg microsomal

protein. Substrate concentration and protein content was based on

previous studies with visceral mass microsomes from oyster

(Schlenk and Buhler, 1989a). Reactions were started by the

addition of [^{3}H]-2-AF. After incubation at 18°C for 1.0 hr, the

reactions were terminated by extraction with 2.0 ml (95:5, v/v)

ethyl acetate:methanol. The organic extracts were combined,

evaporated to dryness by gaseous nitrogen, and resuspended in 0.5

ml methanol. To minimize auto-oxidation 0.05 ml samples were

immediately injected on a Whatman partisil C-8 HPLC column (4.6 mm

i.d. x 25 cm). Analyses were performed using a modification of

the method of Miller and McQueen (1986). The initial solvent

conditions were 73% 0.02 M acetic acid + 0.01% desferal

mesylate:27% isopropanol. The isopropanol was increased to 100%

over a 45 min concave gradient. The flow rate was 1.0 ml/min.

Quantitation of radioactive metabolites was obtained by the

collection of 1.0 min fractions of column effluent and subsequent

scintillation counting. These values were compared to the

retention times of known standards observed at 254 nm. To confirm

HPLC analyses, TLC was also performed on organic extracts using a

benzene:methanol:acetic acid (45:8:4, v/v) solvent system.

Plastic-backed (20 x 20 cm) Silica Gel F TLC sheets (E. Merck,

Darmstadt, F.R.G.) were used for all TLC analyses. Boiled

microsomes served as control since no metabolites were observed in

these incubations (data not shown).

RESULTS

Separation of 2-AF (20 min), 7-hydroxy-2-AF (5 min), N-methyl-2-AF (29 min) and N-hydroxy-2-AF (16 min) was achieved using a mixture of 0.02 M acetic acid and isopropanol (Figure 17). Although clearly separated from 2-AF, 2-AAF could not be separated from N-formyl-2-AF (26 min). Likewise, N-nitroso-2-AF and 2-nitrofluorene possessed identical retention times, but were distinguishable from 2-AF and N-formyl-2-AF having a retention time of 35 min. N-Formyl-2-AF and 2-AAF could be differentiated utilizing TLC (Figure 18). Since N-nitroso-2-AF is spontaneously converted to 2-nitrofluorene (Lindeke, 1982), no attempt was made to separate these compounds by TLC. Initial HPLC analyses (+NADPH at pH 7.6) (Figure 19), demonstrated radioactivity which coeluted with the standards: N-hydroxy-2-AF, N-formyl-2-AF/2-AAF and N-nitroso-AF/2-nitrofluorene. Subsequent TLC analysis revealed the presence of N-formyl-2-AF rather than 2-AAF. To verify this result, incubations containing acetylcoenzyme A and the N-acetyltransferase inhibitor, NaF, respectively, failed to alter metabolite formation profiles (data not shown). The average percent recovery for 51 sample injections was 95 ± 4 %.

The percent conversion of 2-AF to metabolites including the formation of N-oxidation and N-formyl metabolites did not vary significantly with the addition of NADPH or adjustment of pH (Table 7) (One-way ANOVA; $p \geq 0.25$). A slight increase and

decrease in percent conversion was observed in incubations

omitting NADPH and containing methimazole plus NADPH,

respectively. Likewise, an increase in N-oxidation was observed in

incubations without NADPH at both pH 7.6 and 8.4. Addition of

methimazole at pH 8.4 caused a reduction in N-oxidation compared

to incubations omitting NADPH at pH 8.4. N-Oxidation was

generally more prominent in incubations at pH 8.4 rather than pH

7.6. Conversely, N-formylation was slightly decreased in

incubations at pH 8.4 compared to those at pH 7.6. Similar to

percent conversion data, incubations containing methimazole

possessed lower rates of N-formyl-2-AF formation compared to the

remaining samples. Furthermore, the inclusion of NADPH failed to

cause a change in N-formylation at either pH 7.6 or pH 8.4.

Although marked differences appear to be present in percent

conversion, rates of N-oxidation and N-formylation in incubation

mixtures treated with various inhibitors and stimulators of lipid

peroxidation, statistically significant differences were not

observed (Figures 20 and 21). However, incubations possessing

glutathione (GSH), at both pH 7.6 and 8.4, drastically reduced

levels of N-oxidation compared with those of the standard (+

NADPH). The addition of EDTA also caused a reduction of percent

conversion, decreased rates of N-formylation at pH 7.6 and 8.4,

and N-oxidation at pH 7.6, but only a slight decrease in N-

oxidation was observed at pH 8.4. Incubations containing SOD/CAT

had little effect on N-oxidation at pH 8.4 and an unexpected

increase in activity was observed at pH 7.6. However, a decrease

in 2-AF percent conversion and N-formylation was observed in incubation at pH 7.6 and 8.4.

With the exception of the lipid peroxidation incubations at pH 7.6, the percent conversion of 2-AF was reduced in all stimulator-containing incubations relative to levels observed with NADPH (Figures 22 and 23). Similarly, all incubations possessing stimulators had reduced rates of N-formylation. Stimulation of N-oxidation generally occurred in incubations at pH 7.6 and 8.4 with the addition of arachidonate and Fe^{3+}-ADP. A slight increase in N-oxidation was also observed in incubations at pH 7.6 containing hydrogen peroxide while a decrease was seen at pH 8.4.

DISCUSSION

Since N-hydroxy-2-AF is formed by microsomes from oyster
visceral mass, the oyster possesses the enzymatic potential to
bioactivate 2-AF to mutagenic metabolites capable of binding to
DNA. Kurelec (1988) has observed DNA-adduct formation in vitro by
2-AF bioactivated via homogenates from Mytilus digestive gland.
Knezovich et al. (1988) has also demonstrated that N-hydroxy-2-AF
is formed in vivo by Mytilus. Thus, it appears that shellfish DNA
may be vulnerable to bioactivated compounds in vivo which could
cause genotoxic damage. However, the percent conversion of 2-AF
to reactive metabolites by microsomes from oyster visceral mass
was less than 0.05 %. Furthermore, the small amount of
bioactivated chemical may be made even more readily excretable via
Phase II conjugation reactions. UDP-Glucuronosyltransferase
activity has been observed in bivalves (Kurelec et al., 1986).

In microsomes from oyster visceral mass, N-formyl-2-AF
constituted more than 80 % of the total observed metabolites. The
occurrence of the N-formyl metabolite is consistent with in vivo
studies examining the biotransformation of o-toluidine in the
western oyster (Knezovich and Crosby, 1985). Since bioactivation
of aromatic amines is usually initiated by oxidation at the
nitrogen of the amine, the formation of N-formyl metabolites has
been suggested to be a pathway of detoxication (Santti and Hopsu-
Havu, 1968, Knezovich and Crosby, 1985). Earlier studies have

shown that N-formyl-2-AF possesses much less carcinogenic activity in rodents than N-hydroxy-2-AF (Miller et al., 1962). The N-formylation of amines in mammalian systems has been shown to be mediated through a transfer of formate from N-formylkynurenine via the cytosolic enzyme, kynurenine formamidase (Tatsumi and Amano, 1987). However, it is unknown whether microsomal N-formylation is catalyzed through this mechanism in molluscs.

One well studied mechanism of activation of 2-AF involves N-oxidation to N-hydroxy-2-AF (Miller and Miller, 1981). Upon the loss of water, the hydroxylamine is rapidly transformed to the electrophillic nitrenium ion. N-Hydroxy-2-AF may also be further oxidized, spontaneously or enzymatically, to N-nitroso-2-AF or 2-nitrofluorene (Lindeke, 1982; Gorrod and Manson, 1986). Reduction of 2-nitrofluorene back to the free amine is also a possible pathway of transformation which can be catalyzed by certain strains of bacterial and microsomal reductases (Lindeke, 1982; Rickert, 1987).

In order to elucidate the enzymatic mechanism(s) responsible for N-oxidation, various inhibitors or incubation modifications were employed to evaluate the contributions of individual enzyme systems. Cytochrome(s) P-450 and FMO activity in microsomes from oyster visceral mass can be differentiated by their differences in optimum pH, 7.6 for the former and 8.4 for the latter (Schlenk and Buhler, 1989b). Incubations at pH 7.6 and pH 8.4 containing NADPH are indicative of the involvement of cytochrome(s) P-450 and FMO, respectively. It is important to note that previous studies in

our laboratory examining benzo(a)pyrene biotransformation in microsomes from oyster visceral mass and gill suggested little cytochrome P-450 activity possibly due to denaturation during microsomal preparation (Schlenk and Buhler, 1989b). An alternative N-oxidation reaction which occurs at pH 7.6 and is NADPH-dependent was postulated (Schlenk and Buhler, 1989b). After observing NADPH-dependent redox cycling and enhanced lipid peroxidation in bivalve and snail digestive gland microsomes, Livingstone et al. (1989) have suggested that reactive oxygen transfer may be responsible for xenobiotic co-oxidation in molluscs.

Kurelec and Krca (1987) have shown that methimazole inhibited the presumed FMO-mediated bioactivation of 2-AF to mutagenic metabolites in bivalve homogenates. Consequently, this inhibitor was used in our study to reduce microsomal FMO-catalyzed N-oxidation. The reduction of N-oxidation upon the addition of methimazole and the greater levels of activity at pH 8.4 suggest that FMO does play a role in the mechanism of N-oxidation. FMO activity, dependent upon NADPH and optimal at pH 8.4, has been previously demonstrated in the oyster (Schlenk and Buhler, 1989b). However, the formation of N-oxidized metabolites in incubations omitting NADPH is not consistent with an exclusive FMO-mediated pathway because NADPH is a necessary cofactor in FMO reactions (Ziegler, 1988). Kurelec et al. (1986) have stated that FMO is the predominant enzyme responsible for aromatic amine N-oxidation in marine bivalves. However, NADPH-independent oxidations have been

observed previously in bivalve microsomal preparations (Kirchin et al., 1988; Schlenk and Buhler, 1989b). NADPH has also been shown to even cause inhibition of certain microsomal oxidation reactions (Kirchin et al. 1988). Therefore, since NADPH-independent N-oxidation was observed in digestive gland microsomes from oyster, this reaction must have occurred via another mechanism which is able to catalyze oxidation reactions without the requirement of exogenous reducing equivalents.

Stimulation of N-oxidation by the addition of lipid (arachidonate), hydrogen peroxide and Fe^{3+}-ADP-generated lipid peroxidation were not consistent with results observed previously with benzo(a)pyrene oxidation as these incubations showed no enhancement of benzo(a)pyrene hydroxylase activity (Schlenk and Buhler, 1989b). In order to explain NADPH-independent oxidation in previous xenobiotic biotransformation studies in molluscs, we suggested a peroxidative enzymatic mechanism of substrate oxidation which is lipid hydroperoxide-dependent (Schlenk and Buhler, 1989b). An alternative mechanism may involve a microsomal reductase (possibly NADPH-cytochrome P-450 reductase) initiating reduction of dioxygen to hydroxyl radical from endogenous stores of reducing equivalents (Schlenk and Buhler, 1989b). Integrating the two hypotheses, the production of hydroxyl radical may then act to initiate lipid peroxidation leading to lipid hydroperoxide formation and subsequent peroxidase-catalyzed xenobiotic co-oxidation. Livingstone et al.(1989) have postulated the existence of a microsomal component capable of two-electron-acceptance and

one-electron-transfer reactions. According to our reductase model, dioxygen must bypass superoxide anion and hydrogen peroxide since SOD/catalase had no effect on N-oxidation or benzo(a)pyrene oxidation (Schlenk and Buhler, 1989b). However, microsomal lipid peroxidation and redox cycling has been shown to occur in the presence of SOD and catalase (Morehouse and Aust, 1988). Two hypotheses have been invoked to account for the failure of SOD and catalase to inhibit microsomal lipid peroxidation. The first proposes that SOD or catalase cannot gain access to the site(s) of microsomal oxygen reduction (Mimnaugh and Trush, 1983). The second argues that oxidative damage, dependent upon bound transition metal catalysts, is produced in a localized environment where its direct involvement with endogenous macromolecules cannot be prevented by the added enzymes (Samuni et al., 1981).

Inhibition of N-oxidation by GSH is consistent with a one electron peroxidative mechanism. Inhibition could have been a result of the radical scavenging properties of GSH and/or mediated through the enzyme GSH-S-transferase. However, since enzyme activities were not observed nor expected in oyster microsomes (data not shown), it is assumed that inhibition of lipid peroxidation in microsomes was due to the free radical scavenging properties of GSH.

A decrease of N-oxidation activity was also observed with the addition of EDTA to incubations. These data suggest perhaps the involvement of a transition metal (most likely iron or copper) which may exist either as a contaminant in the microsomal

preparations or as the electron donor of a metalloenzyme complex.
In either case, hydroxyl radical potentially can be produced from
a lipid hydroperoxide via the Fenton reaction and transferred as a
free radical or as a ferryl radical intermediate to a xenobiotic
substrate (Bruchhausen et al., 1988). Since incubations
containing boiled microsomes failed to demonstrate a metabolite
profile, N-oxidation observed in all incubations is certainly
enzyme-mediated. Consequently, it appears a metalloenzyme
peroxidase and/or reductase could be responsible for the N-
oxidation observed in microsomes from oyster visceral mass.

Although arachidonate stimulation of N-oxidation at pH 7.6 is
compatible with the lipid hydroperoxide-dependent peroxidase
model, the specific enzyme capable of substrate co-oxidation may
be prostaglandin synthase. Various prostaglandins have been shown
to be biosynthesized from arachidonate in bivalves (Srivastava and
Mustafa, 1985). However, it is not known to what extent this
particular enzyme occurs in bivalves nor the role it plays in
molluscan biochemical pathways.

Although cytochrome(s) P-450 and FMO are important enzymes
regulating xenobiotic biotransformation in microsomes from _Mytilus_
digestive gland, it appears enzymes such as prostaglandin synthase
and Phase II pathways may play a greater role in microsomes from
oyster visceral mass. Various biochemical pathways in oysters
have been shown to differ from those in mussels (Livingstone et
al., 1983). Consequently, xenobiotic biotransformation may be
unique in oysters. In any case, as in "advanced" organisms, the

mechanism of N-oxidation in oysters appears to be mediatated
through several enzymatic pathways. Future research should
involve further characterization of the microsomal peroxidase and
reductase activities to gain a better understanding of the
processes of xenobiotic biotransformation in marine molluscs.

ACKNOWLEDGEMENTS

Figure 17. HPLC trace of 2-AF standards and the _in vitro_
biotransformation pathway of 2-AF in digestive gland
microsomes from the western oyster, _Crassostrea gigas_.

Figure 17
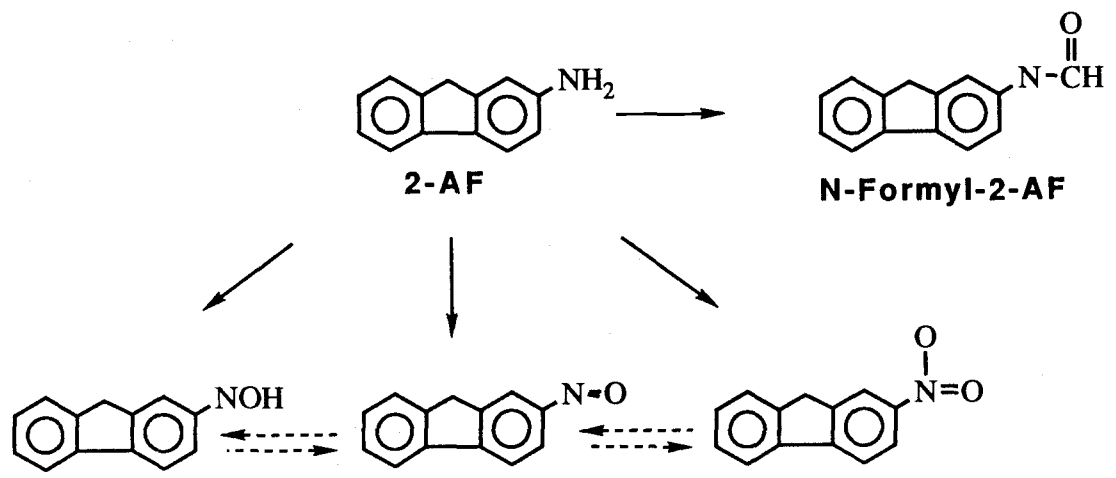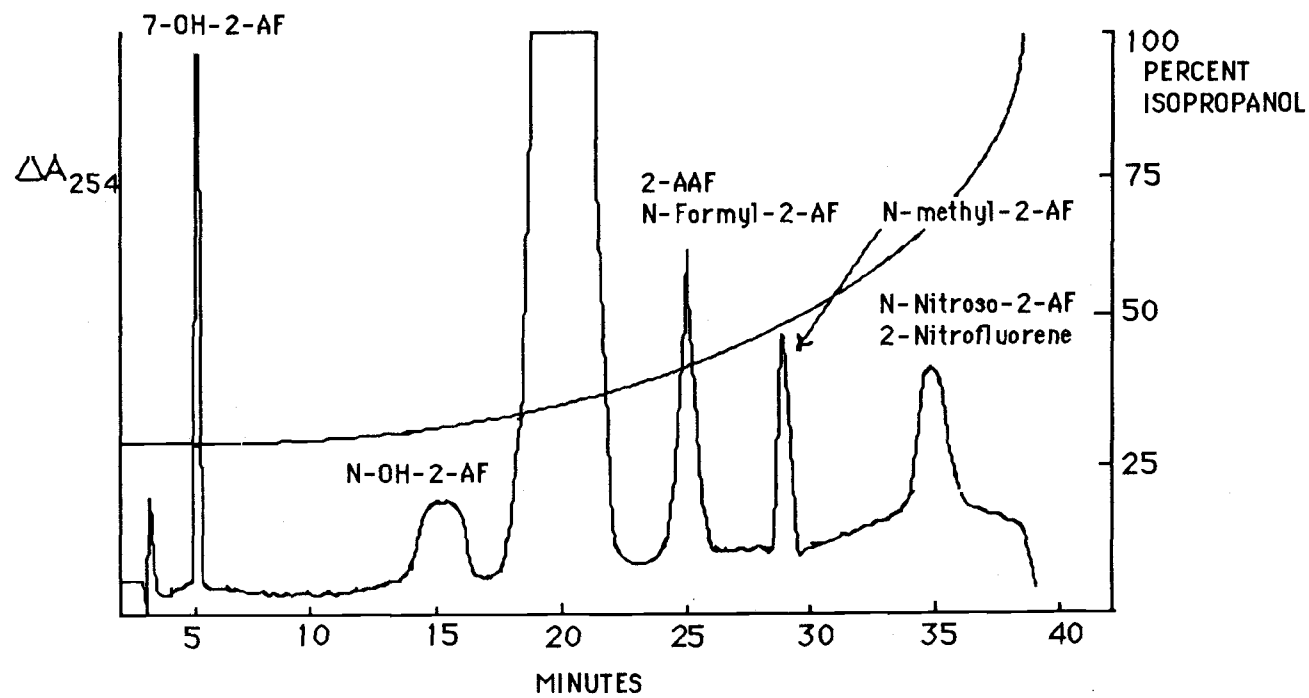
Figure 18.　TLC trace of 2-AF standards.
　　　　　　Lane 1, Combination of standards; Lane 2, 7-hydroxy-2-
　　　　　　AF; Lane 3, N-hydroxy-2-AF; Lane 4, 2-AF; Lane 5, 2-
　　　　　　acetylAF; Lane 6, N-formyl-2-AF; Lane 7, N-nitroso-2-
　　　　　　AF/ 2-nitrofluorene; Lane 8, N-methyl-2-AF.

Figure 18
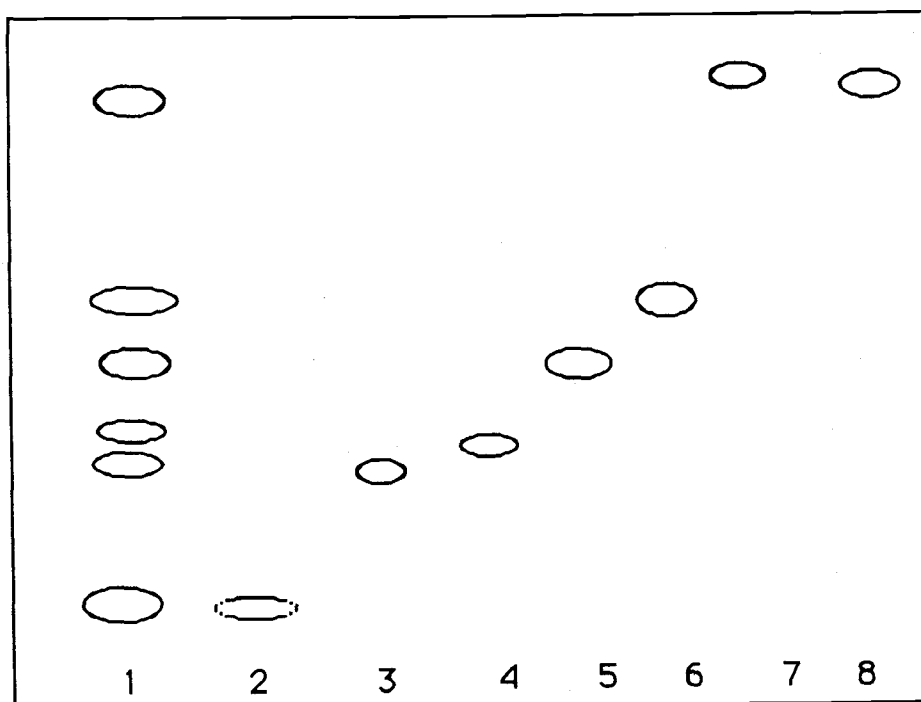
Figure 19.   Superimposition of radioactive metabolites from
             incubations at pH 7.6 + NADPH and UV 2-AF standards.

Figure 19
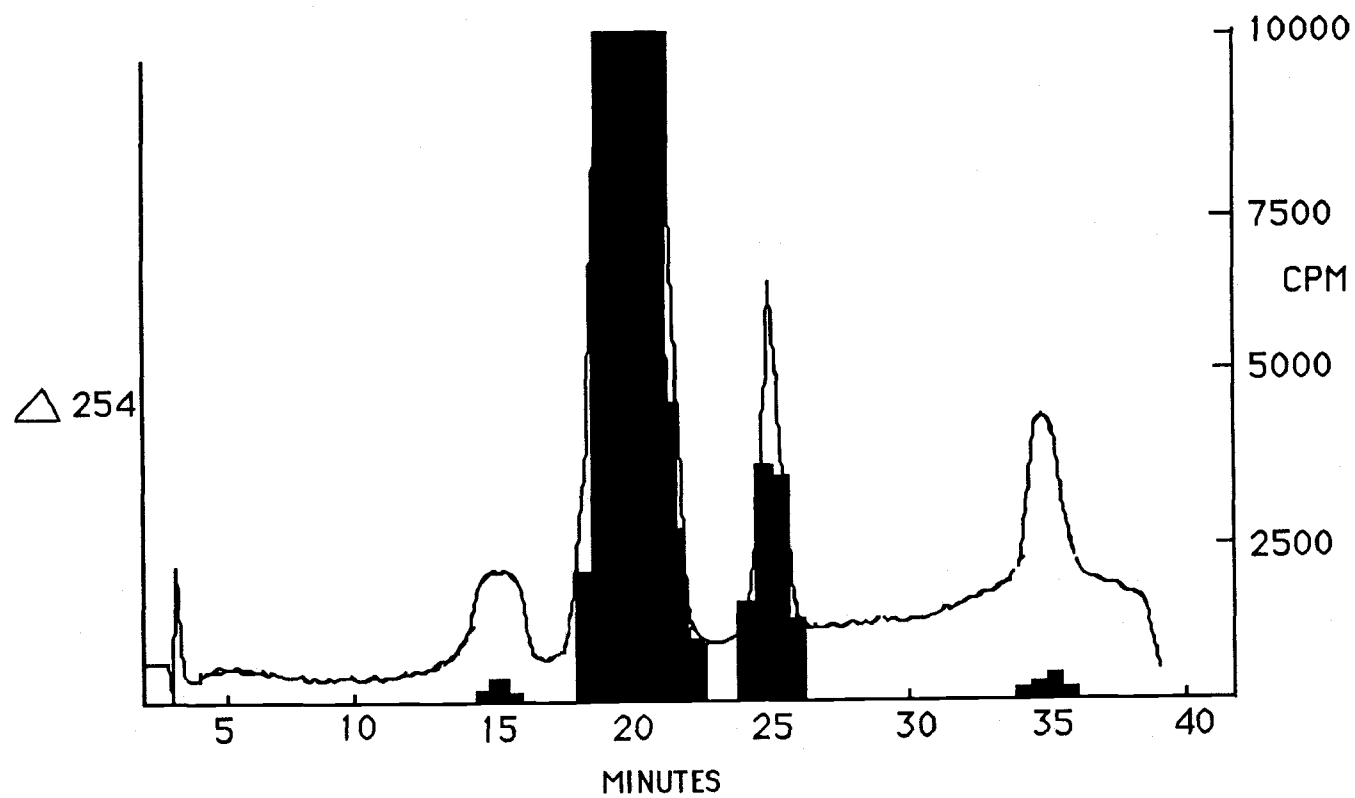
Figure 20. The effect of various inhibitors on 2-AF percent
conversion, N-oxidation and N-formylation in
microsomes from oyster digestive gland at pH 7.6

Percent conversion was defined as the percentage of
total radioactivity above background, not including 2-
AF which emerged in defined metabolite peaks. All
values represent the mean of three replications ±
S.D..

N-oxidation was defined as the radioactivity which
coeluted with N-hydroxy-2-AF, N-nitroso-2-AF and 2-
nitrofluorene standards. All values represent the
mean of three replications ± S.D..

N-formyl-2-AF formation was expressed as the amount
of radioactivity which coeluted with standard.

[a] 1.0 mM GSH.
[b] 1.0 mM EDTA.
[c] 1.0 mg SOD and 0.1 mg catalase.

Figure 20

Figure 21. The effect of various inhibitors on 2-AF percent
conversion, N-oxidation and N-formylation in
microsomes from oyster digestive gland at pH 8.4.

Percent conversion was defined as the percentage of
total radioactivity above background, not including 2-
AF which emerged in defined metabolite peaks. All
values represent the mean of three replications ±
S.D..

N-oxidation was defined as the radioactivity which
coeluted with N-hydroxy-2-AF, N-nitroso-2-AF and 2-
nitrofluorene standards. All values represent the
mean of three replications ± S.D..

N-formyl-2-AF formation was expressed as the amount
of radioactivity which coeluted with standard.

a 1.0 mM GSH.
b 1.0 mM EDTA.
c 1.0 mg SOD and 0.1 mg catalase.

Figure 21

Figure 22. The effect of various stimulators on 2-AF percent
conversion, N-oxidation and N-formylation in
microsomes from oyster digestive gland at pH 7.6.

Percent conversion was defined as the percentage of
total radioactivity above background, not including 2-
AF which emerged in defined metabolite peaks. All
values represent the mean of three replications ±
S.D..

N-oxidation was defined as the radioactivity which
coeluted with N-hydroxy-2-AF, N-nitroso-2-AF and 2-
nitrofluorene standards. All values represent the
mean of three replications ± S.D..

N-formyl-2-AF formation was expressed as the amount
of radioactivity which coeluted with standard.

[a] 1.0 mM hydrogen peroxide.
[b] 4.0 mM ADP, 15 μM Fe^{3+} and 1.0 mM ascorbate.
[c] 1.0 mM arachidonate.

Figure 22

Figure 23. The effect of various stimulators on 2-AF percent
conversion, N-oxidation and N-formylation in
microsomes from oyster digestive gland at pH 8.4.

Percent conversion was defined as the percentage of
total radioactivity above background, not including 2-
AF which emerged in defined metabolite peaks. All
values represent the mean of three replications $\pm$
S.D..

N-oxidation was defined as the radioactivity which
coeluted with N-hydroxy-2-AF, N-nitroso-2-AF and 2-
nitrofluorene standards. All values represent the
mean of three replications $\pm$ S.D..

N-formyl-2-AF formation was expressed as the amount
of radioactivity which coeluted with standard.

[a] 1.0 mM hydrogen peroxide.
[b] 4.0 mM ADP, 15 μM Fe^{3+} and 1.0 mM ascorbate.
[c] 1.0 mM arachidonate.

Figure 23

Table 7.   The effect of NADPH, pH and methimazole on 2-AF
           percent conversion and metabolite formation by
           microsomes from oyster visceral mass.

[a] Percent conversion was defined as the percentage of
total radioactivity above background, not including 2-
AF which emerged in defined metabolite peaks. All
values represent the mean of three replications ±
S.D..

[b] N-oxidation was defined as the radioactivity which
coeluted with N-hydroxy-2-AF, N-nitroso-2-AF and 2-
nitrofluorene standards. All values represent the
mean of three replications ± S.D..

[c] N-formyl-2-AF formation was expressed as the amount
of radioactivity which coeluted with standard.

[d] 1.0 mM methimazole + 0.5 mM NADPH.

Table 7

Incubation	Per. Conv[a]	N-oxidation[b]	N-Formylation[c]
7.6 + NADPH	5.3 ± 0.5	4.5 ± 1.7	28.4 ± 1.7
7.6 - NADPH	5.8 ± 1.8	7.3 ± 3.7	28.7 ± 3.7
8.4 + NADPH	5.1 ± 0.7	6.1 ± 3.0	25.5 ± 3.0
8.4 - NADPH	5.4 ± 1.6	8.3 ± 2.6	25.4 ± 2.6
8.4 + Meth +NADPH[d]	4.1 ± 0.4	4.2 ± 0.6	21.4 ± 0.6

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Chapter 5

Flavin-containing Monooxygenase Activity

in Liver Microsomes from

the Rainbow Trout (<u>Oncorhynchus</u> <u>mykiss</u>).

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ABSTRACT

Flavin-containing monooxygenase (FMO) is an important microsomal enzyme which may affect the toxicity of nitrogen and sulfur-containing chemicals such as organophosphate and carbamate pesticides encountered by aquatic organisms. FMO activity as determined via N,N-dimethylaniline (DMA) N-oxide formation was observed and characterized in liver microsomes from rainbow trout (Oncorhynchus mykiss). Microsomal DMA N-oxidase activity was optimal at pH 8.4-9.2, 20°C and required NADPH. Addition of the cytochrome P-450 inhibitor, N-benzylimidazole failed to alter DMA N-oxidase activity but coincubation with methimazole, trimethylamine (TMA) or aldicarb significantly inhibited DMA oxidase. DMA oxidase demonstrated Michaelis-Menten kinetics and was competitively inhibited by TMA and aldicarb. These data suggest that aldicarb and TMA are substrates for FMO in this organism.

INTRODUCTION

Flavin-containing monooxygenase is an enzyme associated with the endoplasmic reticulum which primarily catalyzes heteroatom oxidations on sulfur and nitrogen containing substrates (Ziegler, 1988). Along with cytochrome(s) P-450, FMO serves an important role in Phase I enzymatic biotransformations (Ziegler, 1980). There are many similarities between the FMO and cytochrome(s) P-450 systems that make it difficult to determine their relative contributions towards metabolism. For example, both cytochrome(s) P-450 and FMO require NADPH and oxygen as cofactors for catalysis, both are found in the endoplasmic reticulum, and each perform similar nitro and sulfo-oxidations. However, FMO differs from cytochrome P-450 in several respects. Cytochrome(s) P-450 monooxygenase is catalyzed via a heme-oxygen complex that can be inactivated by certain suicide substrate inhibitors (Ortiz de Montalleno, 1986). Conversely, FMO possesses a 4-alpha hydroperoxy-moiety which is highly susceptible to nucleophilic attack, but is not irreversibly inhibited (Ziegler, 1988). Since cytochrome(s) P-450 activity can be irreversibly inhibited in vitro by suicide substrates and FMO can similarly be inhibited competitively by other FMO substrates for the enzyme, it is possible, therefore, to determine the relative contribution of the two enzymes towards the metabolism of a given substrate in vitro (Tynes and Hodgson, 1984).

Several cytochrome P-450 isozymes have been isolated and

purified from trout liver microsomes (Williams and Buhler, 1982; Miranda et al., 1989). However, FMO has yet to be studied in detail in rainbow trout. Goldstein and Dewitt-Harley (1973) have suggested that trimethylamine oxidase activity, which has been observed in numerous fish species, is mediated via FMO. In an aquatic environment, fish are likely to be exposed to many nitrogen and sulfur-containing chemicals such as organophosphate and carbamate pesticides due to agricultural runoff (Briggs and Lord, 1983). Several of these pesticides have been shown to be _in vitro_ substrates for FMO in mammals (Hajjar and Hodgson, 1980). Consequently, in order to better understand the mechanisms for nitrogen and sulfur-containing xenobiotic activation and detoxification in fishes, it is essential to determine the contribution of the FMO system to biotransformation.

METHODS AND MATERIALS

Chemicals

N,N-Dimethylaniline (DMA) hydrochloride was recrystallized as

previously described (Schlenk and Buhler, 1989a). Anhydrous

diethyl ether was purchased from Baker Chemical Corporation

(Phillipsburg, NJ). CHAPS was purchased from Calbiochem (San

Diego, CA). All remaining chemicals were obtained from Sigma

Chemical Co. (St. Louis, MO).

Tissure preparation and enzyme assays

Mount Shasta strain rainbow trout (Oncorhynchus mykiss) (14

month-old) were maintained in tanks with free flowing well water

at 12°C. Twenty fish were each killed by a blow to the head and

liver microsomes were prepared by the method of Williams and

Buhler (1982). Livers from individual fish were removed, weighed,

and washed in 1.15% KCl. The livers were then pooled, minced and

homogenized in 4 volumes of ice cold buffer A [0.1 M Tris-acetate

pH 7.4, 0.1 M KCl, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl

fluoride (PMSF)]. A microsomal pellet was obtained by

centrifugation at 9,000 x g for 20 min and centrifugation of this

supernatant twice for 90 min at 100,000 x g. The resulting

microsomes were resuspended in buffer B (0.1 M potassium phosphate

pH 7.25, 20 % glycerol and 1 mM EDTA) to a protein concentration

of approximately 20 mg/ml, frozen in liquid nitrogen, and stored

at ⁻80°C until used.

DMA N-oxidation was determined by a modification of the method

of Ziegler and Pettit (1964). After several buffers were

evaluated to obtain maximal activity, a 1.0 ml incubation mixture

was chosen containing 0.25 M potassium phosphate (at various pH),

0.1 mM NADPH, and 0.5-1.0 mg microsomal protein. The reaction

was started with the addition of DMA (4.0 mM for optimum) and then

incubated at various temperatures for 30 min. After extraction

with anhydrous diethyl ether to remove unreacted substrate, DMA N-

oxide was reduced back to DMA and then converted to the nitroso

compound which was colorimetrically quantitated at 420 nm on a

Cary 219 Spectrophotometer (extinction coefficient 8.2 $mmol^{-1}$).

Initial controls consisted of boiled microsomes. Since

incubations not including NADPH gave the same background values as

boiled microsomes, incubations without NADPH were subsequently

used as controls.

RESULTS

Rainbow trout liver microsomal DMA oxidase was optimal at pH

8.4-9.2 and required NADPH as a cofactor for catalysis (Figures 24

and 25). Enzyme activity was linear up to 50 min (Figure 26).

Attempts to solubilize the microsomes for purification with the

addition of the detergent CHAPS drastically reduced activity.

Both phosphate and tricene buffers provided optimal conditions for

activity while tris-buffered incubations possessed lower rates of

DMA oxidase (Figure 25). Optimum temperature was approximately

20°C (Figure 26). If microsomes were warmed to 37°C for three

minutes and then added to incubations at 20°C, DMA oxidase

activity was not observed (data not shown). Likewise, if tissue

homogenates were not maintained at 4°C during microsomal

preparation, enzyme activity was destroyed.

The addition of methimazole, TMA, or aldicarb, led to

significant decreases in DMA oxidase (Figure 27). However,

inclusion of the cytochrome P-450 suicide substrate, N-

benzylimidazole, failed to alter enzyme activity. Coincubation

with n-octylamine caused a slight decrease in activity which was

not statistically significant ($p \geq 0.1$).

DMA N-oxidase demonstrated Michaelis Menten kinetics possessing

a Vmax of 1.31 nmol/min and a Km of 1.2 mM (Figure 28). The

addition of TMA or aldicarb failed to alter the Vmax (1.25

nmol/min) and increased the Km (2.3 mM and 7.6 mM, respectively).

Coincubation with n-octylamine caused a slight decrease in Vmax

(1.12 nmol/min) and Km (0.86) (Figure 29). The Km was unchanged

in incubations containing methimazole (1.25 mM). However,

methimazole caused a reduction in the Vmax (0.625 nmol/min).

DISCUSSION

In mammalian systems, the oxidation of most nitrogen- and sulfur-containing chemicals is catalyzed via FMO (Ziegler, 1980) and cytochrome(s) P-450 (Yang and Lu, 1987). In the past it has been difficult to separate the activities of the individual enzymes due to the occurrence of many shared characteristics. Although DMA has been shown to be a substrate for both enzymes in mammals, the enzyme-catalyzed product is very different. DMA oxidase (N-oxide formation) and DMA demethylase (formaldehyde formation) have been shown to be accurate indicators of FMO and cytochrome(s) P-450, respectively (Houdi and Damani, 1985). Consequently, the formation of DMA N-oxide in trout liver microsomes strongly suggests a significant presence of FMO in this species. FMO activity (DMA N-oxidase) has been observed previously in mammals (Ziegler, 1988), a protozoan (Agosin and Ankley, 1987), marine invertebrates (Kurelec, 1985; Schlenk and Buhler, 1989a) marine elasmobranchs (Goldstein and Dewitt-Harley, 1973), and freshwater fish (Arillio et al., 1984).

The subcellular location of FMO activity in liver microsomes from rainbow trout is consistent with previous studies in mammals and fish (Goldstein and Dewitt-Harley, 1973; Ziegler, 1988). The pH optimum for trout liver FMO activity was approximately 8.4-9.2 which is very similar to the pH optimum in the porcine enzyme (pH 8.4) (Ziegler, 1988), TMA oxidase in liver microsomes from the

nurse shark (pH 9.0) (Goldstein and Dewitt-Harley, 1973), and TMA

oxidase in cod liver microsomes (8.2) (Agustsson and Strom, 1981).

Another trait of the trout liver enzyme consistent with previous

characterizations in mammals (Ziegler, 1988) and fish (Bakker et

al., 1963) was the requirement of NADPH for catalysis. NADPH is

thought to directly reduce the flavin moiety of the enzyme prior

to the binding of oxygen and substrate (Ziegler, 1988). Trout

liver FMO activity was also very sensitive to temperatures above

its optimum of 20°C. Williams et al. (1985) have reported that,

without NADPH, the purified pig liver FMO is very unstable at

temperatures above 30°C, whereas the purified rabbit lung enzyme

seems to be more resistent to inactivation at moderate

temperatures.

Since DMA N-oxide formation was significantly decreased in

incubations containing the zwitterionic detergent CHAPS,

subsequent attempts to purify the enzyme utilizing this detergent

were aborted. TMA oxidase was shown to be destroyed in cod liver

microsomes following treatment with the Triton series of

detergents (X-45, X102, and N-101; Sigma). This contrasts results

seen in rat liver where the Triton series was used in

solubilization and subsequent purification of the enzyme (Kimura

et al., 1983). The most popular detergent used for FMO

purification has been the Emulgen series (911 and 913; Kao)

(Sabourin et al., 1984). Cholate, an anionic detergent, had

little effect on the rabbit lung enzyme (Williams et al., 1985).

However, the pig liver enzyme activity has been shown to be

destroyed using anionic detergents (Ziegler, 1988).

The trout liver enzyme displayed Michaelis-Menten kinetics, and the apparent Km value for DMA was 1.2 mM. Although DMA oxidase is a specific assay for FMO, it has been criticized as being insensitive (Ziegler, 1988). Consequently, the apparent Km determined my this method may actually be overestimated and may be much lower. The apparent Km for DMA in the pig liver enzyme is 3 μM (Ziegler, 1988).

Since primary alkyl amines have been shown to have different effects on rabbit lung and pig liver FMO (Ziegler, 1988), the effect of n-octylamine on DMA oxidase in trout liver was examined. However, it was difficult to interpret our kinetic data for incubations containing n-octylamine. The data show that n-octylamine increases the affinity of the enzyme for DMA (decreased Km), but also causes a slight decrease in Vmax. Tertiary amine oxidation catalyzed by the pig liver (Ziegler, 1980) and fish enzymes (Agustsson and Strom, 1981) has been shown to be stimulated by the addition of lipophilic primary amines. Nevertheless, long-chain primary amines are substrates for the rabbit lung enzyme and competitively inhibit tertiary amine oxidation (Williams et al., 1985). It is unclear whether n-octylamine is a substrate or stimulator of trout liver FMO. It is possible that n-octylamine may act as a stimulator at low concentrations and as an inhibitor at high concentrations, but more experimentation is required to ascertain its interaction with the trout liver enzyme.

Also intriguing were the kinetic results which examined the effect of methimazole on trout liver DMA oxidase. Since the rate-limiting step of FMO is the release of NADP and not oxygenated substrate, every substrate should theoretically possess the same Vmax (Ziegler, 1988). Consequently, if two FMO-substrates are coincubated with enzyme and adequate quantities of cofactor, each substrate should compete for the same active site and demonstrate competitive inhibition kinetics. However, our data suggest a noncompetitive inhibition of the enzyme. Methimazole has been shown to be a relatively good substrate for all forms of the mammalian FMO (Ziegler, 1988). Methimazole oxidation to the very reactive sulfenic acid does occur in microsomes from trout liver as well as other trout tissue microsomes (Schlenk and Buhler, 1990a). Noncompetitive inhibition of DMA oxidase by methimazole may be due to conformational changes in the protein caused by a covalent interaction of the sulfenic acid of methimazole with the protein, thus preventing the access of DMA to the active site of the enzyme.

Since TMA oxidase has been suggested to be catalyzed by FMO in fish (Goldstein and Dewitt-Harley, 1973), we examined the effect of TMA on DMA oxidase activity. DMA oxidase kinetic data demonstrated that TMA competitively inhibited DMA oxidase in microsomes from trout liver. Although the mechanism was not suggested, DMA has been found to inhibit 98 % (Goldstein and Harley, 1973) and 70 % (Agustsson and Strom, 1981) of TMA oxidase activity in nurse shark and cod liver microsomes, respectively.

The competitive inhibition of DMA oxidase by TMA provides
reasonable evidence that both chemicals are substrates for the
same enzyme. In elasmobranchs, the oxidation of trimethylamine to
TMA N-oxide serves an important function in osmoregulation
(Goldstein and Dewitt-Harley, 1973). However, the role of TMA N-
oxide in teleosts is still unclear (Charest et al., 1988). TMA
oxidase has been observed in a large number of plants and animals
(Bakker et al., 1963), including marine (Goldstein and Dewitt-
Harley, 1973; Agustsson and Strom, 1981; Daikoku et al., 1987) and
freshwater fish (Daikoku et al., 1988). Although the endogenous
function of FMO in mammals is still uncertain, it appears that the
enzyme is involved in cysteamine oxidation and may play a part in
sulfide oxidation and protein crosslinking (Ziegler, 1988).

Although little is known regarding the endogenous function of
FMO in mammals, there have been many studies performed examining
its biotransformation of xenobiotics (Ziegler, 1980; Prough et
al., 1981; Tynes and Hodgson, 1984; Baba et al., 1987; Kinsler et
al., 1988; Ziegler, 1988). Hajjir and Hodgson (1980) have shown
that FMO is able to oxidize several thiophosphate insecticides
including the carbamate, aldicarb. Sulfoxidation of these
compounds generally enhances their anticholinesterase activity and
subsequent toxicity (Ziegler, 1988). Since aldicarb and its
metabolites are extremely water soluble, fish and other aquatic
organisms are prime targets for exposure to these chemicals as a
result of agricultural runoff (Briggs and Lord, 1983).
Consequently, FMO may be important in the bioactivation of

aldicarb to the more potent acetylcholinesterase inhibitor, aldicarb sulfoxide. Like TMA, aldicarb competitively inhibited DMA oxidase in trout liver microsomes. Thus, it can be concluded that aldicarb is also a substrate for this enzyme and trout liver FMO may be responsible for the bioactivation of aldicarb in this organism. We have recently examined both the _in vitro_ and _in vivo_ biotransformation of aldicarb and its interaction with FMO in rainbow trout and found that aldicarb sulfoxide is produced _in vivo_ and by liver, kidney and gill microsomes _in vitro_ (Schlenk and Buhler, 1989b). Liver aldicarb sulfoxide formation was competitively inhibited _in vitro_ by DMA providing further evidence that FMO plays an important role in xenobiotic biotransformation within rainbow trout.

The rainbow trout is currently being utilized as an alternate vertebrate model to study carcinogenesis mechanisms (Sinnhuber et al., 1977). Cytochrome P-450 has been shown to be responsible for the bioactivation of aflatoxin B_1 to aflatoxin B_1 2,3-epoxide leading to DNA-adduct formation in rainbow trout (Loveland et al., 1987). In mammals, FMO has been shown to catalyze the N-oxidation of 2-aminofluorene to the carcinogen, N-hydroxy 2-aminofluorene (Frederick et al., 1982). Consequently, the occurrence of FMO activity in this model necessitates further research to better characterize this enzyme and determine its role in xenobiotic biotransformation within the rainbow trout.

ACKNOWLEDGEMENTS

Figure 24. The effect of pH on DMA N-oxide formation in liver
microsomes from trout.
Each value represents the mean of 2 assay
replications from the same preparation.

Figure 24
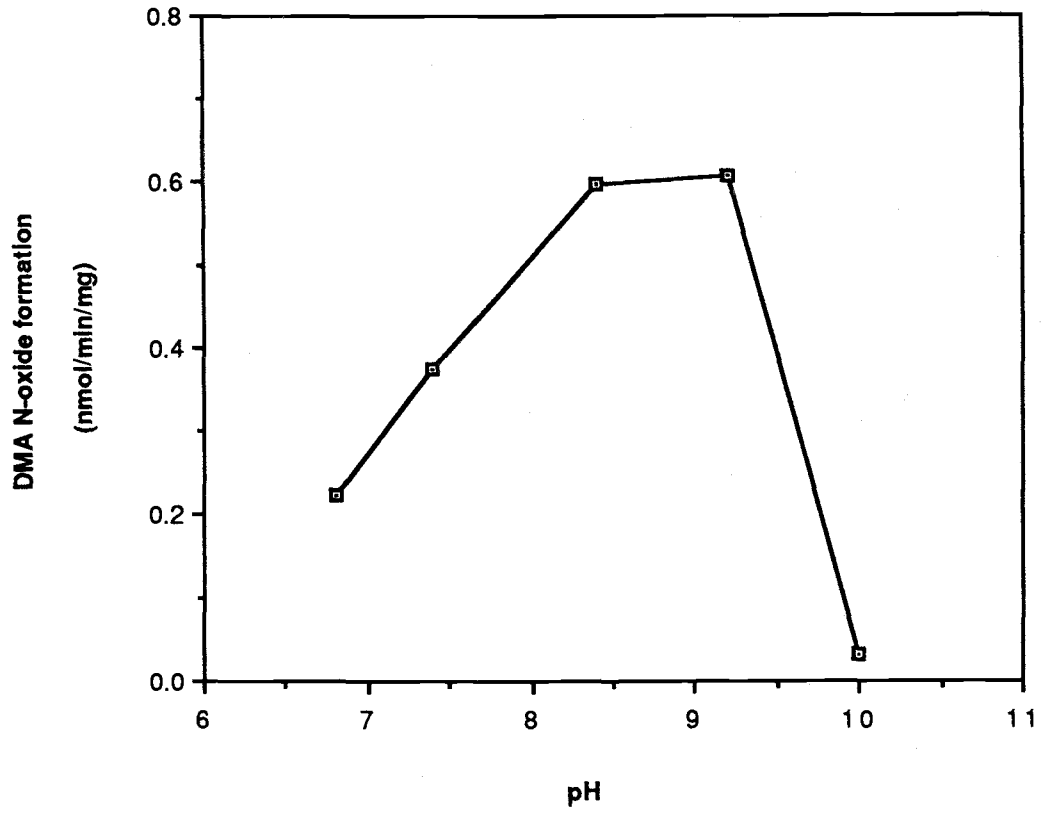
Figure 25. The effect of boiling, NADPH, chaps and various
buffers on DMA N-oxide formation in liver microsomes
from trout.
Each value represents the mean of 4 assay
replications from the same preparation ± S.D.

Figure 25
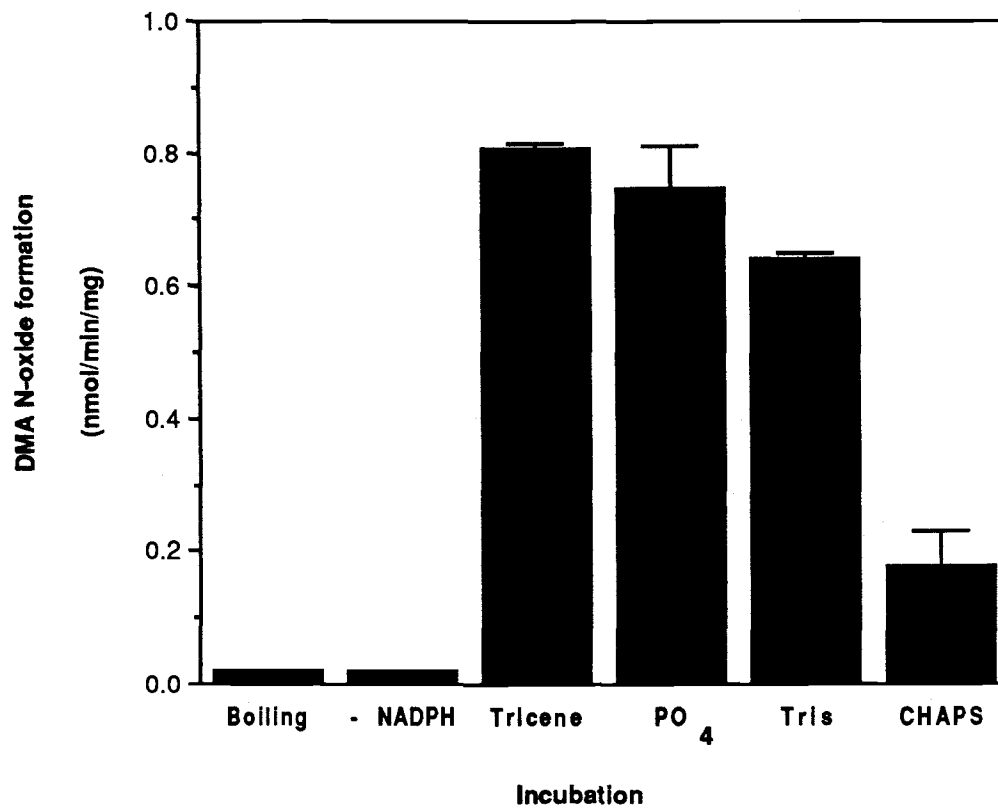
Figure 26. The effect of time and temperature on DMA N-oxide
            formation in liver microsomes from trout.
            Each value represents the mean of 2 assay
            replications from the same preparation.

Figure 26
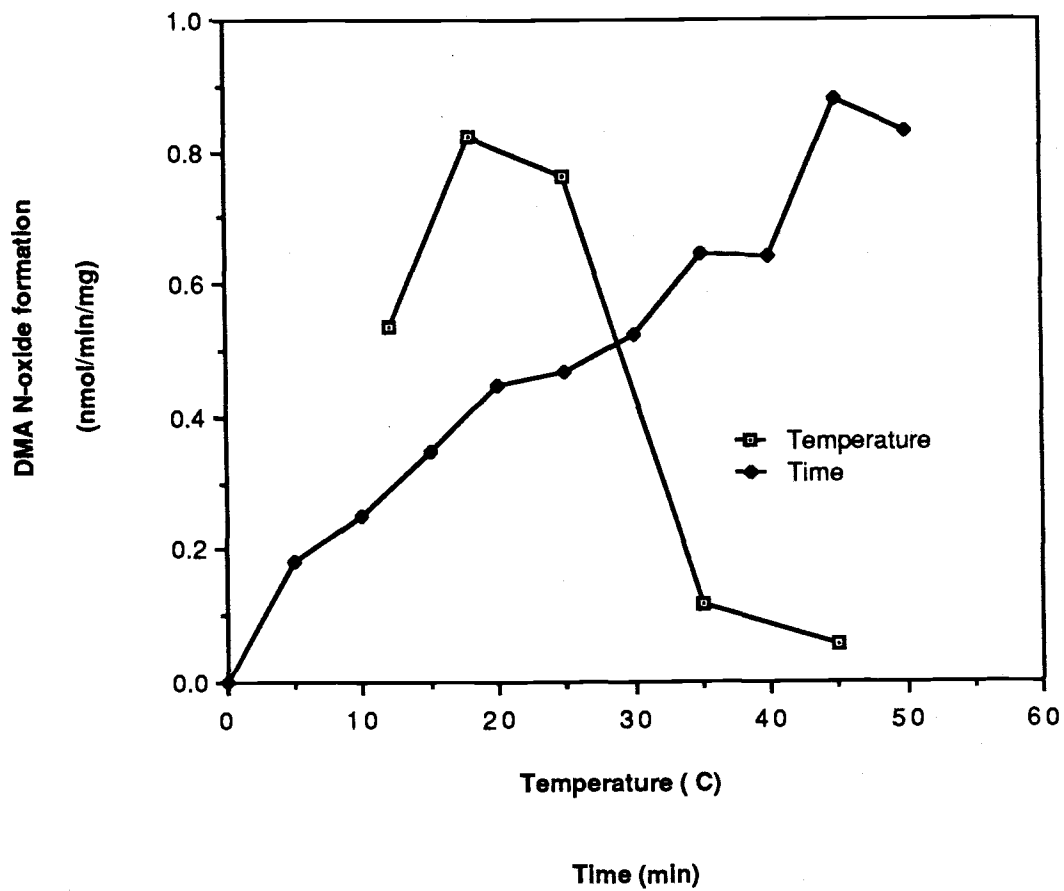
Figure 27. The effect of various enzyme inhibitors on DMA N-
oxide formation in liver microsomes from trout.
Each value represents the mean of 4 assay
replications from the same preparation ± S.D.
Each incubation was preincubated with 4 mM of
inhibitor for 15 min before the addition of DMA.
*Statistical significance was determined utilizing
Student's t-test.
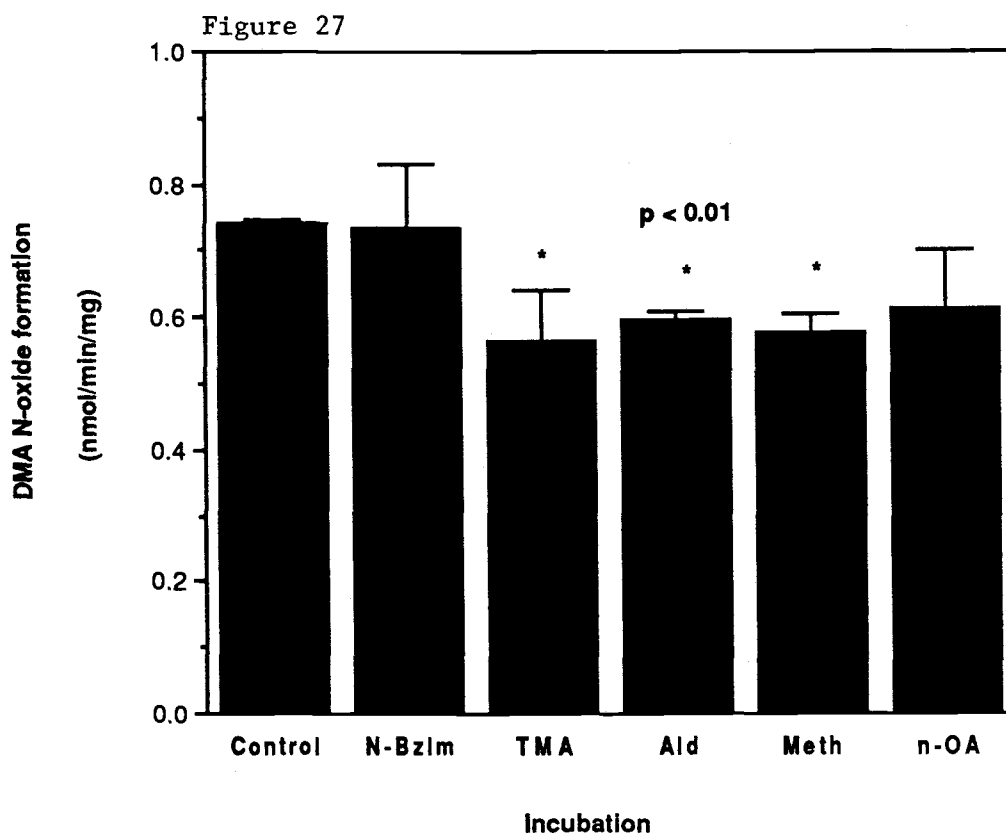
Figure 27

Figure 28. Double reciprocal plot demonstrating the effect of
            TMA and aldicarb on DMA N-oxidase formation.
            Each value represents the mean of 2 assay
            replications from the same preparation.
            Each incubation was preincubated with 4 mM of
            inhibitor for 15 min before the addition of DMA.
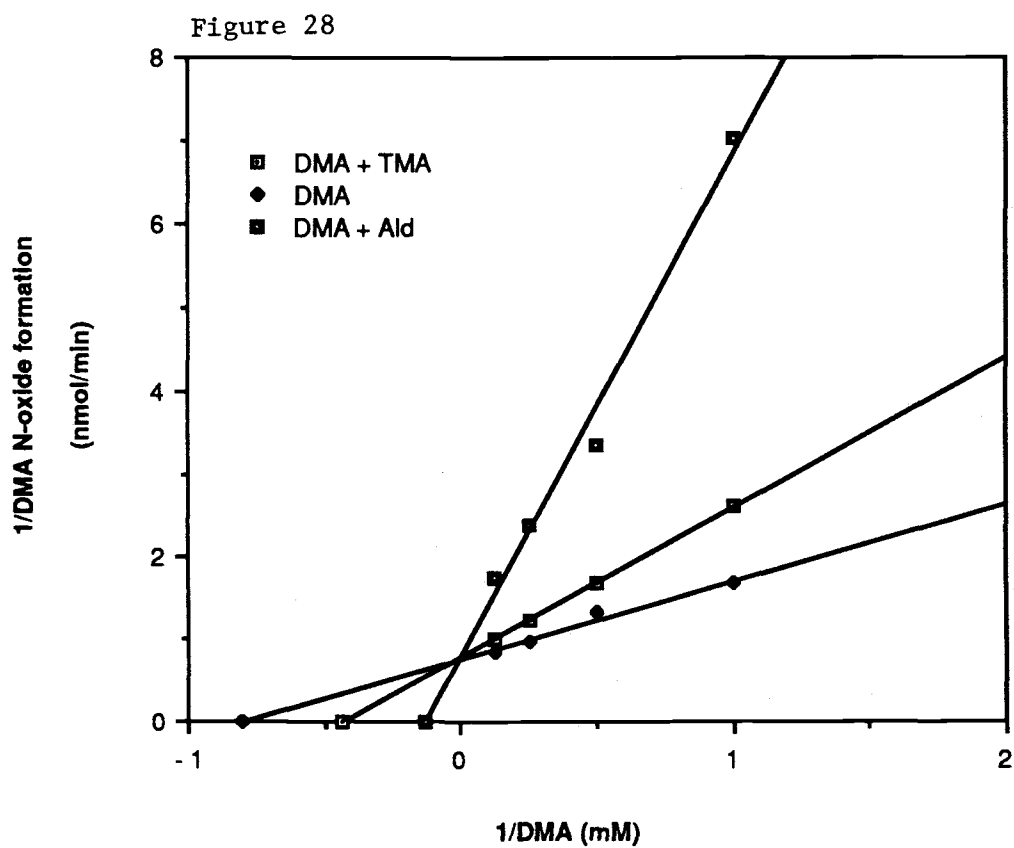
Figure 28

Figure 29. Double reciprocal plot of the effect of methimazole
and N-octylamine on DMA N-oxidase formation.
Each value represents the mean of 2 assay
replications from the same preparation.
Each incubation was preincubated with 4 mM of
inhibitor for 15 min before the addition of DMA.

Figure 29
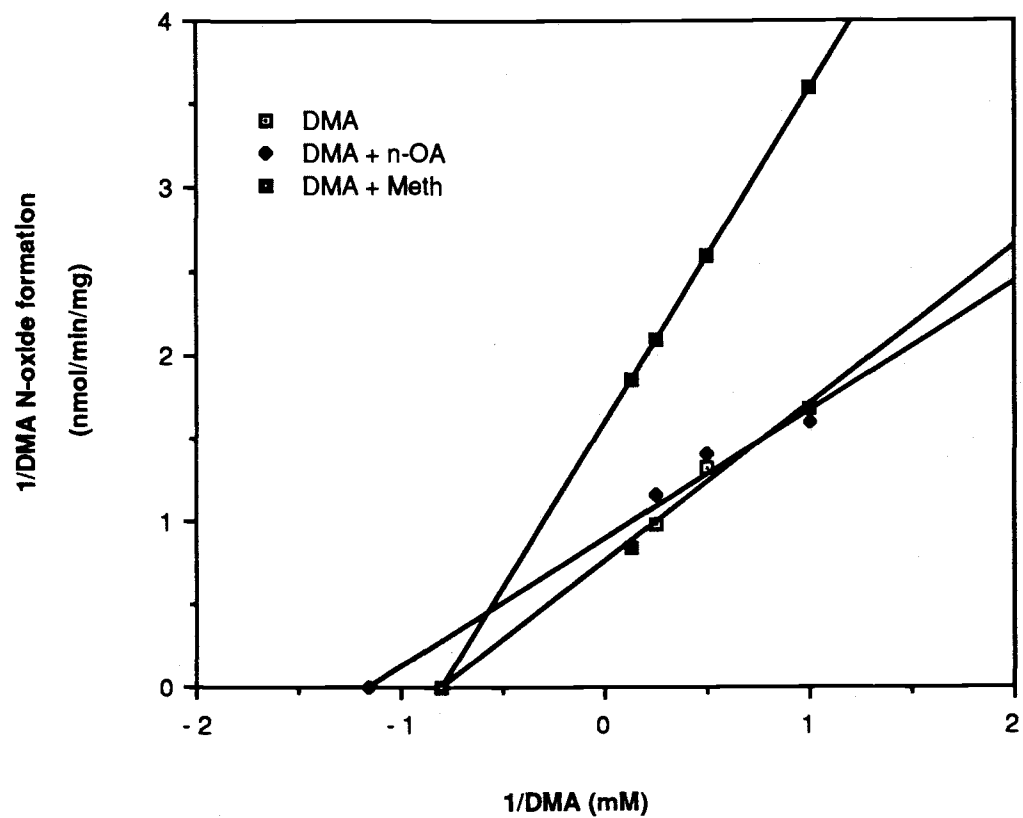
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Chapter 6

Role of Flavin-containing Monooxygenase

in the In Vitro and In Vivo

Biotransformation of Aldicarb

in the Rainbow Trout

(Oncorhynchus mykiss).

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ABSTRACT

Aldicarb (Temik[R]), a sulfur-containing pesticide, was a substrate for flavin-containing monooxygenase (FMO) in rainbow trout (Oncorhynchus mykiss). The in vitro biotransformation of [14C]-aldicarb was examined in microsomes from trout liver, kidney, and gill. In all tissues, the major metabolite was aldicarb sulfoxide. Addition of the cytochrome P-450 inhibitor, N-benzylimidazole, failed to significantly alter aldicarb sulfoxide levels, while coincubation with the FMO substrates N,N-dimethylaniline (DMA) or methimazole caused significant reductions of sulfoxide formation in liver and gill microsomes. Aldicarb sulfoxide formation was optimal at pH 8.0 and demonstrated Michaelis-Menten kinetics possessing an apparent Km of 46.7 μM and a Vmax of 0.216 nmol/min/mg. Aldicarb sulfoxide was competitively inhibited by coincubation of DMA in liver microsomes. The in vivo biotransformation of [14C]-aldicarb was also examined utilizing spinal transected trout held in a static metabolic chamber. [14C]-Aldicarb was administered via intraperitoneal injection and there were no significant differences in half-lives or tissue distribution between transected and normal trout. Elimination of [14C]-aldicarb was primarily via the gills with 85% of the radioactivity recovered 24 hr postinjection. The major metabolites in 24 hr were found to be aldicarb sulfoxide (7.6 %) with lesser amounts of aldicarb oxime (5.4 %). These data suggest that FMO plays an important role in the in vitro and in

vivo biotransformation of aldicarb in rainbow trout.

INTRODUCTION

Aldicarb (Temik[R]) is a carbamate pesticide which is used throughout the world to control insects and nematodes (Risher et al., 1987). Aldicarb exerts its toxicity through inactivation of the enzyme, acetylcholinesterase (Coppage, 1977). Although the parent is capable of enzyme inactivation, sulfoxidation to aldicarb sulfoxide or sulfone enhances acetylcholinesterase inhibition seventy-six fold (NIH, 1979). Since aldicarb and its metabolites are relatively water soluble, aquatic organisms have a great potential for exposure to these chemicals as a result of agricultural runoff. Although aldicarb biotransformation has been examined in mammals, little is known about its fate in aquatic animals.

Bioactivation of aldicarb to the sulfoxide or sulfone has been suggested to be catalyzed by cytochrome P-450 (Kulkarni and Hodgson, 1980) and FMO (Hajjar and Hodgson, 1982) in mammals. In fish, cytochrome P-450 has been shown to be the dominant enzyme system responsible for xenobiotic biotransformation (Kleinow et al., 1987). Recent studies in our laboratory with the rainbow trout indicate that fish also possess FMO (Schlenk and Buhler, 1990a). Having been shown to bioactivate a number of nitrogen and sulfur-containing chemicals, FMO has been purified and well characterized in several mammalian species (Ziegler, 1988), but has yet to be well characterized in fish. Consequently, the role that FMO plays in xenobiotic biotransformation in fish is not

known.

N,N-Dimethylaniline (DMA) N-oxidase activity has been found to be an appropriate assay for FMO activity (Ziegler, 1988). Our previous studies have shown that aldicarb competitively inhibits DMA N-oxidase activity in rainbow trout liver microsomes suggesting that aldicarb is a substrate for FMO (Schlenk and Buhler, 1990a). We have also shown that liver, trunk kidney and gill possess the highest levels of FMO activity and enzyme content (Schlenk and Buhler, 1990b).

The purpose of this study was to determine the role of FMO in the in vitro biotransformation of aldicarb in liver, kidney and gill microsomes. The in vivo biotransformation of aldicarb was also examined to compare with in vitro metabolite profiles and determine the relative importance of FMO in xenobiotic biotransformation in the rainbow trout.

METHODS AND MATERIALS

Chemicals

Aldicarb, aldicarb sulfoxide and aldicarb sulfone were obtained from the U.S. Environmental Protection Agency's Chemical Repository (Research Triangle Park, NC). [^{14}C]-Aldicarb (8 mCi/mmol), methimazole, and XAD-4 resin were purchased from Sigma Chemical Co. (St. Louis, MO). N-Benzylimidazole was purchased from Aldrich Chemical Co. (Milwaukee, WS). Aldicarb oxime and nitrile were synthesized via base catalyzed hydrolysis of aldicarb (Payne et al., 1966). Aldicarb sulfoxide oxime and aldicarb sulfoxide nitrile were products of base-catalyzed hydrolysis of aldicarb sulfoxide. DMA HCl was recrystallized as previously described (Schlenk and Buhler, 1990a).

Microsome preparation

Microsomes were prepared as previously described (Schlenk and Buhler, 1990a). Rainbow trout, 14 month-old of the Mount Shasta strain (Oncorhynchus mykiss) were maintained in tanks with free flowing well water at 12°C. Twenty fish were killed by a blow to the head. Tissues from individual fish were removed, weighed, and washed in 1.15% KCl. The tissues were then pooled, minced and homogenized in 4 volumes of ice cold buffer A [0.1 M Tris-acetate

pH 7.4, 0.1 M KCl, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl

fluoride (PMSF)]. A microsomal pellet was obtained by

centrifugation at 9,000 x g for 20 min and centrifugation of the

supernatant twice for 90 min at 100,000 x g. The resulting

microsomes were resuspended in buffer B (0.1 M potassium phosphate

pH 7.25, 20 % glycerol and 1 mM EDTA) to a protein concentration

of approximately 20 mg/ml and frozen in liquid nitrogen and stored

at ⁻80°C until used.

In vitro studies

Incubations contained 60 μM [^{14}C]-aldicarb (1.0 mcui/mmol) 0.1

mM NADPH, 0.5-3.0 mg protein and 10 mM potassium phosphate buffer

(pH 7.2, 7.6, 8.0, 8.6 and 9.2) in a total volume of 1.0 ml at

20°C. Reactions were initiated with the addition of substrate

and stopped with the addition of 2.0 ml dichloromethane.

Following three extractions with 2.0 ml of dichloromethane (85 $\pm$ 6

% recovery for all samples), the solvent was evaporated to dryness

under nitrogen gas. The residue was taken up in 0.25 ml methanol

and injected onto a Whatman C-8 column utilizing a Spectra Physics

8000 HPLC system. The solvent system, a modification of Miles and

Delfino (1984) was 12% acetonitrile and water for 10 min and a 2

min gradient increase to 40% acetonitrile for 8 min. Column

effluent was collected at 1.0 min intervals in scintillation vials

and counted on a Packard scintillation counter. Radioactive

metabolites were also quantitated utilizing a Beckman 171

radioisotope detector on a Shimadzu LC-6A HPLC system. Recovered

radioactivity was compared with retention time of known standards.

Metabolites were also separated and identified utilizing TLC

employing an acetone: benzene (3:1) solvent system and iodine

crystals for visual detection.

In vivo studies

Rainbow trout (250 $\pm$ 10 g) were isolated individually in

separate aquaria, not fed for 24 hr and immobilized by spinal

transection utilizing MS-222 as anesthetic (McKim et al., 1985).

Postoperative fish were housed in a rectangular plexiglass

metabolism chamber 18 x 6 x 4 in (See Figure 30) with an

approximate water depth of 5 inches. After spinal transection and

catheterization of the urinary bladder, fish were allowed to

equilibrate braced in an upright position within the chamber for

2-3 days before chemical administration. By placing valves in the

anterior and posterior sections of the chamber, well water could

flow through the chamber at approximately 500 ml/min allowing

equilibration. However, upon injection of the fish with [^{14}C]-

aldicarb, the valves were closed and air stones were added to

create a static system.

Pharmacokinetic and distribution studies

Three transected fish and three nontransected fish were

injected (intraperitoneal) with 1 mg/kg [^{14}C]-aldicarb (0.5

mCi/mmol) dissolved in ethanol. The dose was determined by

dividing the Hazardous Substances Data Base LC_{50} value (8 mg/l; 96

hr) by 8. The fish did not display overt signs of toxicity such

as convulsions. However there appeared to be a consistent

occurrence of mucus release from the gills. Fish were maintained

in aerated static chambers during the experiments. The total

volume of water was siphoned out of the chambers at various time

points (0.5, 1.0, 2.0, 4.0, 8.0, 13.0 and 24.0 hr postinjection)

an aliquot was counted and then an equivalent volume of fresh

water was added to the chamber. After 24 hr, the fish were killed

by a blow to the head and frozen at -20°C for tissue distribution

studies. Kinetic elimination phases were calculated by curve

stripping using a two-compartment body model (O'Flaherty, 1981).

Various tissues were dissected from thawed fish and weighed. A

portion of each tissue was removed, diluted with 4 volumes of

distilled water, and homogenized using either a teflon Potter-

Elvehnjeim or polytron homogenizer. An aliquot of this homogenate

was then placed in scintillation vials and incubated overnight in

NCS tissue solubilizer (Amersham Corp; Arlington Heights, Il).

Following the addition of 30% hydrogen peroxide and isopropyl

alcohol the following day, scintillation fluid was added and the

samples were then counted using a Packard scintillation counter.

Excretory route studies

Following a 2-3 day equilibration period, two transected trout with urinary catheters (PE 60 tubing) were injected (intraperitoneal) with 1.0 mg/kg [^{14}C]-aldicarb (0.5 mCi/mmol). Epidermal mucus was scraped from the dorsal potion of the lateral line at 1.0, 2.0, 4.0, 8.0, 13.0 and 24.0 hr postinjection, placed in preweighed glass scintillation vials and subsequently counted. Aliquots of collected urine and bile samples were taken 24 hr postinjection and counted via scintillation.

Metabolism studies

A recirculating chemical extraction system was set up immediately after injection to determine metabolite composition of the radioactivity. Water was pumped at approximately 15-20 ml/min via PVC tubing through a glass column containing 20 ml of methanol extracted XAD-4 resin (Junk et al., 1974). Trapping efficiency of the resin was determined by periodically collecting water samples from the chamber after administration of a known quantity of radiolabel and counted via scintillation. The chamber was partially submerged in free flowing well water to maintain a constant temperature of 12 °C. After 24 hr, the resin was then sequentially extracted with 50 ml of methanol, 50 ml of dichloromethane: methanol (1:1), 50 ml of dichloromethane, and a final 100 ml wash with methanol. Each of these fractions was

evaporated to dryness and taken up in 0.25 ml of methanol. A 0.05 ml aliquot of each fraction was then analyzed utilizing the above HPLC and TLC procedures. Urine was filtered through a 0.5 μm Millipore filter and a 0.05 ml aliquot was directly analyzed via HPLC.

RESULTS

In vitro studies

Aldicarb and its metabolites were separated utilizing an
acetonitrile: water solvent system (Figure 31). The retention
times for the metabolites were as follows: aldicarb sulfoxide
oxime (4 min); aldicarb sulfoxide nitrile (6 min); aldicarb
sulfoxide (8 min); aldicarb sulfone (13.5 min); aldicarb nitrile
(20 min); aldicarb oxime (22 min); aldicarb (25 min). Aldicarb,
aldicarb nitrile and aldicarb oxime possessed slightly lower
retention times on the Shimadzu LC-6A HPLC system utilizing the
Beckman 171 radioisotope detector.

Initial in vitro experiments were performed using microsomes
from trout liver, kidney (trunk kidney) and gill at a pH of 8.0.
The major metabolite in these tissues was aldicarb sulfoxide
(Figure 32). There were also trace levels of aldicarb sulfoxide
oxime and aldicarb oxime in liver microsomal incubations and small
quantities of aldicarb oxime in kidney microsomal samples.
Dichloromethane, ethyl acetate, and diethyl ether were employed as
extraction solvents. When dichloromethane was used, percent
recovery was the highest at 85.0 $\pm$ 6 %. Coincubation with the
cytochrome P-450 suicide substrate inhibitor, N-benzylimidazole,
failed to alter aldicarb sulfoxide formation in microsomes from
liver, kidney, and gill (Figure 33). The addition of DMA and

methimazole caused a significant decrease in aldicarb sulfoxide formation in liver microsomes. Methimazole also led to a significant decrease in aldicarb sulfoxide formation in gill microsomes. However, methimazole and DMA had no significant effect on sulfoxide formation in kidney microsomes. In all tissues, aldicarb sulfoxide formation was dependent upon NADPH. Sulfoxide formation was also unaltered after coincubation with indomethacin or glutathione (Figure 34).

The formation of aldicarb sulfoxide in liver microsomes was optimal at pH 8.0 (Figure 35). At more alkaline pH, there did appear to be some NADPH-independent sulfoxide formation, but this was subtracted from sulfoxide rates in NADPH-containing incubations. Aldicarb sulfoxide formation was linear for approximately 15 min and was directly proportional to the amount of microsomal protein (Figures 36 and 37). Liver microsomal aldicarb sulfoxide formation also demonstrated Michaelis-Menten kinetics possessing an apparent Km of 46.7 μM and a Vmax of 0.216 nmol/min/mg (Figure 38). Aldicarb sulfoxide formation was competitively inhibited by coincubation with 3 mM DMA (Km = 98 μM; Vmax = 0.234).

In vivo studies

Whole-body elimination of radioactivity was similar for transected and nontransected rainbow trout (Figure 39). Elimination of radiolabel appeared to be biphasic. A rapid

elimination phase was observed in the first 5 hr followed by a slower rate of elimination. The mean elimination halflives (N = 3) for intact (2.32 hr) and transected (3.24 hr) fish for the first phase (α) and the elimination half-lives of the second phase (β) for nontransected (28.4 hr) and transected (25.3 hr) fish were not statistically different (p $\geq$ 0.4; Student's t-test). After 24 hr, 84.6 $\pm$ 6 % of the injected dose was eliminated by both groups of fish. Consequently, subsequent metabolite collection procedures were performed for 24 hr.

There were no significant differences in tissue distribution of radioactivity in transected and intact animals (Figure 40). Tissues possessing the highest levels of residual radioactivity were stomach, caeca, liver, heart, and gall bladder. Total percent recovery of injected radioactivity (tissue levels + urine metabolites + XAD resin metabolites) was 94.7 + 5 %.

Excretion of radioactivity appeared to be primarily via the gills (96.2 %) (Figure 41). Branchial elimination was determined indirectly by process of elimination. There was no radioactivity detected above background from dermal scrapping and very little radioactivity present in the bile sampled 24 hr after injection (0.2 %). Since a urinary catheter was used to collect urinary radioactivity (3.6 %), the remaining possible alternatives for elimination were intestinal and branchial elimination pathways. However, since no fecal material was observed and rapid elimination was demonstrated in the previous pharmacokinetic studies, branchial elimination was the most logical route. The

mean urinary flow rate (N = 2) was 12.2 ml collected per 24 hr.

Prior to the addition of fish, the metabolic chamber was filled with water and [^{14}C]-aldicarb was added to determine the extraction efficiency of the XAD-4 resin and to examine the possibility of breakdown of the pesticide to form other products. Extraction efficiency was 100 % and no other radioactive products were observed upon HPLC analysis. The average percent conversion (N = 2) of [^{14}C]-aldicarb to metabolites by the fish was 14.3 %. The predominant metabolite observed in urine and water was aldicarb sulfoxide (Figure 42). The sulfoxide accounted for an average of 7.6 % of the total dose of [^{14}C]-aldicarb (N = 2). Other metabolites present in vivo were aldicarb sulfoxide oxime (0.88 %) and the hydrolytic products aldicarb oxime (5.4 %) and aldicarb nitrile (0.56 %).

DISCUSSION

Aldicarb is a widely used pesticide which is employed to
control insects and nematodes in agricultural settings (Risher et
al., 1987). Since aldicarb and its metabolites are water soluble,
fish and other aquatic organisms which coinhabit areas where
aldicarb may be used would be extremely susceptible to exposure to
the pesticide due to agricultural runoff (Briggs and Lord, 1983).
Several studies have examined the toxicity of aldicarb to various
species of fish (Kumar and Pant, 1984; Landau and Tuken, 1984; Ali
et al., 1985; Pant et al., 1987). However, we have found no
report in the literature examining the biotransformation pathways
of aldicarb in fish. Establishment of the metabolic pathways of
aldicarb is crucial in understanding its toxicity.

Aldicarb toxicity in fish is mediated by its inhibition of
acetylcholinesterase leading to enhanced levels of the
neurotransmitter, acetylcholine, subsequent cholinergic overload
and, depending on the dose, death of the organism (Coppage, 1977).
S-Oxidation of aldicarb to the sulfoxide has been shown to
potentiate inhibition of acetylcholinesterase 76-fold (NIH,
1979). Consequently, S-oxidation of aldicarb can be considered a
pathway of bioactivation producing a more toxic chemical. To
evaluate this conclusion, 4 rainbow trout (250 g) were
administered aldicarb sulfoxide at the same dose used for [^{14}C]-
aldicarb in our metabolism studies (1 mg/kg). Within 10 min, all

of the fish died exhibiting severe convulsions prior to death.

Since hydrolysis of aldicarb to aldicarb nitrile or aldicarb oxime

essentially eliminates its acetylcholinesterase inhibition

properties, this metabolic pathway can be considered a route of

detoxification.

The proposed metabolic pathway for aldicarb in the rainbow

trout is summarized in Figure 43. Aldicarb sulfoxide constituted

7.6 % of the injected dose of $[^{14}C]$-aldicarb with lesser amounts

of aldicarb oxime, aldicarb sulfoxide oxime, and aldicarb nitrile.

The _in vivo_ formation of the two oximes and aldicarb nitrile was

probably a result of the hydrolytic action of plasma esterases

since there were minimal amounts of hydrolytic metabolites

observed _in vitro_. The low levels of hydrolysis observed with

aldicarb biotransformation in this study were very similar to the

low plasma esterase activity observed in rainbow trout by Glickman

and Lech (1981).

Even though aldicarb sulfoxide was the major metabolite

observed, the levels of sulfoxide detected were probably

underestimated due to the occurrence of thioreductases throughout

the organism which could catalyze reduction of aldicarb sulfoxide

back to the sulfide (Mannervik, 1982; Tatsumi, 1982; Ziegler,

1988). However, the formation of aldicarb sulfoxide _in vivo_ is

consistent with the _in vitro_ biotransformation profile of aldicarb

in microsomes from trout liver, kidney, and gill.

The enzyme responsible for the oxidation of aldicarb to

aldicarb sulfoxide in rainbow trout appears to be FMO. Aldicarb

sulfoxide formation was dependent on NADPH suggesting that

cytochrome P-450 and/or FMO might be involved in the reaction.

However, the addition of the cytochrome P-450 inhibitor, N-

benzylimidazole, to microsomal incubations prepared from liver,

kidney, and gill failed to alter sulfoxide formation in these

tissues. N-Benzylimidazole has been suggested by Ziegler (1988)

as being one of the best cytochrome P-450 inhibitors to be used in

FMO characterization studies. In microsomes from trout liver,

benzo(a)pyrene hydroxylase activity was significantly inhibited

after coincubation with N-benzylimidazole (data not shown).

Since the cyclooxygenase inhibitor, indomethacin did not change

sulfoxidation levels, it appears co-oxidation via prostaglandin

synthase is not involved in aldicarb sulfoxidation. Prostaglandin

cyclooxygenase hydroperoxidase has been shown to catalyze

sulfoxide formation of methylphenyl sulfide (Egan et al., 1981).

An oxidation mechanism involving free radicals also appears to be

ruled out due to unaltered sulfoxide formation observed with the

addition of the free radical scavenger, glutathione. However, it

should be mentioned that these pathways may play an important role

in the oxidation of other xenobiotics in trout.

Other evidence supporting an FMO-catalyzed reaction included an

pH 8.0 optimum for aldicarb sulfoxide formation in trout liver

which is a characteristic consistent with FMO described previously

in trout (Schlenk and Buhler, 1990a). In gill and liver

microsomes, aldicarb sulfoxide formation was suppressed by

coincubation of the FMO substrates, DMA and methimazole. In trout

liver microsomes, the observed decrease in sulfoxide formation was due to a competitive inhibition of aldicarb sulfoxidation by DMA. In previous studies in our laboratory, aldicarb competitively inhibited DMA N-oxidase activity which demonstrated that DMA and aldicarb were substrates for the same enzyme, FMO (Schlenk and Buhler, 1990a). Trout liver microsomes have also been shown to possess the highest levels of FMO activity and enzyme content in this organism (Schlenk and Buhler, 1990b). It was surprising that there were no significant differences in kidney microsomes after the addition of FMO substrates. The lack of statistical significance was due to the large variance between assay replications. This variability was probably due to the occurrence of rapid emulsion formation during metabolite extraction procedures using dichloromethane. Kidney microsomal samples appeared to be more susceptible to emulsion formation during extraction procedures than either gill or liver microsomes. In addition, percent recovery of radioactivity was extremely poor in these samples (71 $\pm$ 13 %).

In mammalian organisms, both cytochrome P-450 and FMO appear to be involved in aldicarb oxidation. Kulkarni and Hodgson (1980) have shown that aldicarb is a substrate for cytochrome P-450 demonstrating a type I binding spectrum. Hajjar and Hodgson (1982) have demonstrated that aldicarb is also a substrate for FMO in pig liver microsomes. The formation of a thioether sulfoxide via FMO is consistent with other work in Hodgson's group involving the organophosphate, phorate. Levi and Hodgson (1988) showed that

purified mouse liver FMO catalyzed the formation of (-) phorate

sulfoxide while cytochrome P-450 isozymes produced (+) phorate

sulfoxide. It would have been interesting to examine the

stereospecificity of aldicarb oxidation in trout tissue

microsomes. Unfortunately, we were unable to obtain a large

enough quantity of aldicarb sulfoxide to determine its optical

activity.

Although the focus of this work was to compare the in vivo and

in vitro biotransformation profiles of aldicarb, the routes of

elimination and certain pharmacokinetic parameters such as half-

life and rates of distribution were necessary to optimize the in

vivo metabolism portion of this study. Aldicarb appeared to be

rapidly absorbed and distributed to all tissues in both free-

swimming and spinally transected rainbow trout. Since there were

no significant dispositional differences between transected and

free-swimming fish, transected fish could be used to examine the

in vivo metabolism of aldicarb. Previous studies have

demonstrated that spinal transection does not alter bilary

(Schmidt and Weber, 1973) or urinary (McKim, personal

communication) flow rates in the rainbow trout.

The major route of elimination of aldicarb in the trout was via

the gill which was consistent with the rapid distribution rate

constants observed in both groups of animals. The gill appears to

be an important organ for xenobiotic elimination in fish possibly

due to its high surface area and large cardiac output. Dauble and

Curtis (1989) have shown that quinoline elimination in the rainbow

trout was rapid and primarily via the branchial route. Since aldicarb possesses a log P value of 1.13, it would be expected to readily cross the gill membrane and be eliminated. McKim et al. (1985) have shown that chemicals having log P values less than 3 rapidly crossed the gill membrane in rainbow trout. In mammals, the main route of aldicarb elimination is via the urine (Andrawes et al., 1967). This was not the case in trout. However, upon examination of the urinary metabolites, only aldicarb sulfoxide, aldicarb sulfoxide oxime, and little free aldicarb were observed. Since FMO levels were shown to be relatively high in kidney (Schlenk and Buhler, 1990b), it is possible that the remaining parent compound which is not excreted via the gill may be metabolized by FMO in the kidney to the sulfoxide and be eliminated through the urine. There may also be active transport processes that may excrete aldicarb sulfoxide, but not aldicarb. Although very little radioactivity was present in fresh bile samples taken from fish 24 hr postinjection, it is important to note that the samples obtained were just above the limits of detection due to the quenching effect of bile in the scintillation process. Thus, the aldicarb and aldicarb metabolite content of these samples may have been underestimated. Erickson and Lech (unpublished data) found high levels of radioactivity accumulated in the gallbladder following dietary administration of radiolabeled aldicarb. In our study, the gall bladders also had relatively high radioactivity compared to other tissues. But due to the freeze-thaw processing of these fish, gallbladders were

extremely fragile and often disintegrated during dissection.
Consequently, total radioactivity in these tissues may also have
been underestimated. However, since overall percent recovery of
administered [^{14}C]-aldicarb was approximately 95 %, this
underestimation was probably not large.

Although a large portion of aldicarb was rapidly eliminated,
approximately 15 % of the injected dose remained in the fish,
suggesting bioaccumulation. This may have been due to oral
reabsorption of aldicarb in the static system. The occurrence of
high levels of radioactivity in the stomach and caeca support the
possibility of reuptake. Although the tissue radioactivity levels
were normalized according to weight, muscle appeared to be a prime
target for aldicarb bioaccumulation. Erickson and Lech
(unpublished data) also noted bioaccumulation of aldicarb in
muscle from rainbow trout given oral administration of aldicarb.

Since aldicarb sulfoxide was observed to be the major
metabolite of aldicarb _in vitro_ and _in vivo_ and this oxidation was
mediated through FMO, this enzyme appears to play an important
role in the bioactivation of certain xenobiotics to more toxic
chemicals in the rainbow trout. In mammals, FMO may be involved
in disulfide bond formation between sulfur-containing amino acids
(Ziegler, 1988). The endogenous substrate for this enzyme in fish
appears to be trimethylamine (Schlenk and Buhler, 1990a;
Goldstein and Dewitt-Harley, 1973). More nitrogen and sulfur-
containing chemicals need to be examined in the trout to determine
the relative contribution of FMO to xenobiotic biotransformation.

Since many laboratories are utilizing the rainbow trout as an alternative vertebrate model to study carcinogenesis (Sinnhuber et al., 1977), the enzymes responsible for bioactivation and detoxification of xenobiotics need to be better characterized in this organism.

ACKNOWLEGDEMENTS

Figure 30. Diagrammatic view of the _in vivo_ metabolism chamber.

Figure 30
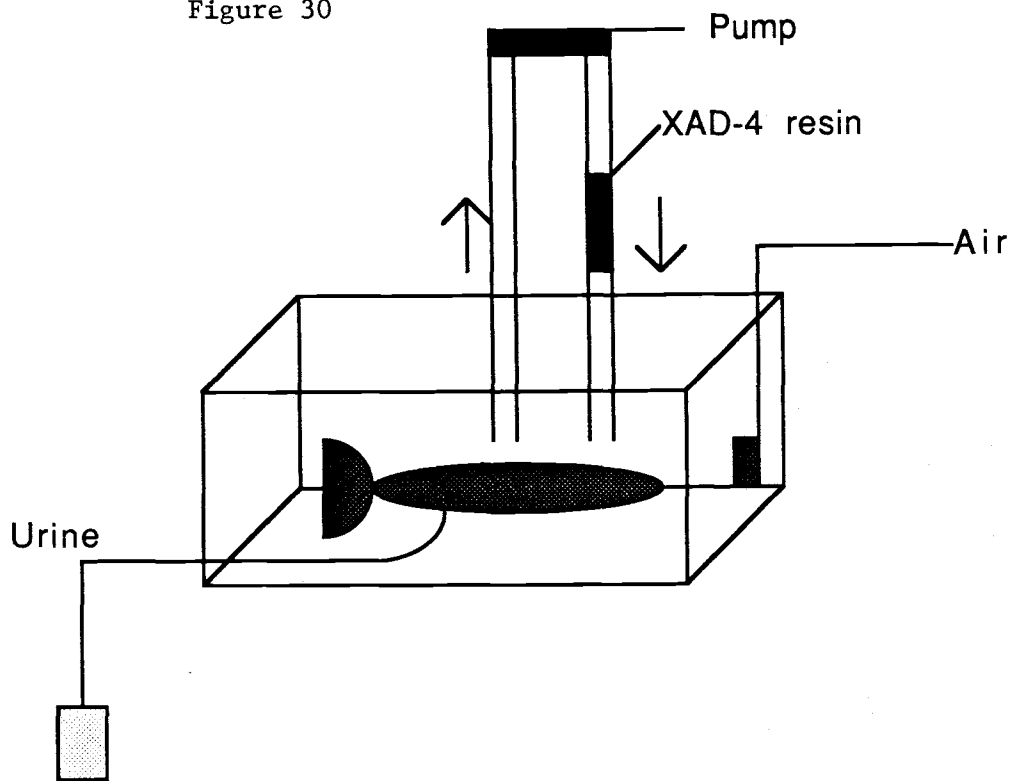
Figure 31. HPLC trace demonstrating the separation of aldicarb
           and metabolites.

Figure 31
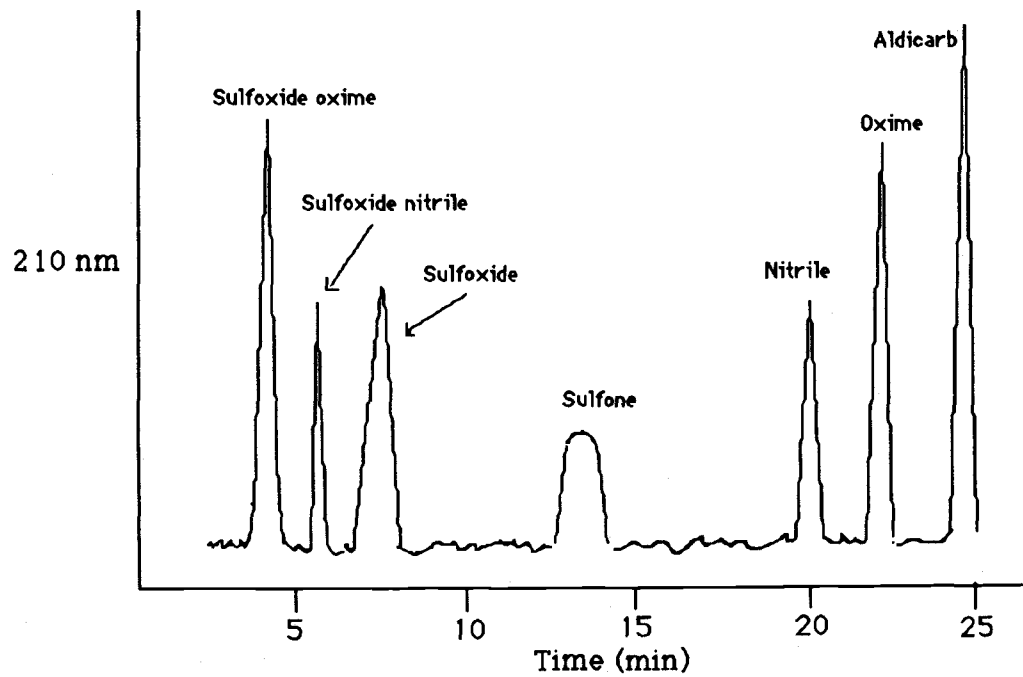
Figure 32. In vitro biotransformation of [^{14}C] aldicarb in
            liver, kidney and gill microsomes.
            Each value represents the mean of n assay
            replications from the same preparation ± S.D. (liver
            n=8; kidney n=5; gill n=3).

Figure 32

Metabolite
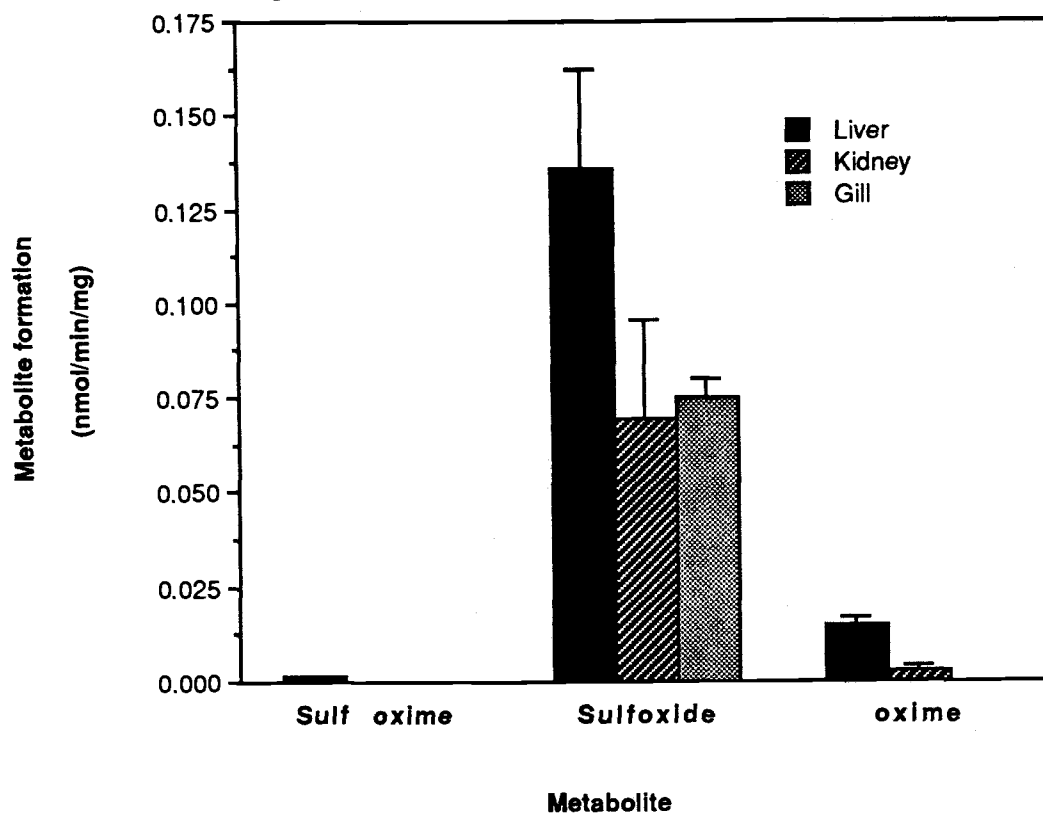
Figure 33. Effect of N-benzylimidazole, DMA and methimazole on
            aldicarb sulfoxide formation in liver, kidney and
            gill microsomes from rainbow trout.
            Each value represents the mean of n assay
            replications from the same preparation ± S.D. (liver
            n=15; kidney n=8; gill n=3).
            Each incubation was preincubated for 15 min with 3 mM
            of inhibitor before the addition of 0.06 mM aldicarb.
             Statistical significance was determined utilizing
            student's t-test.
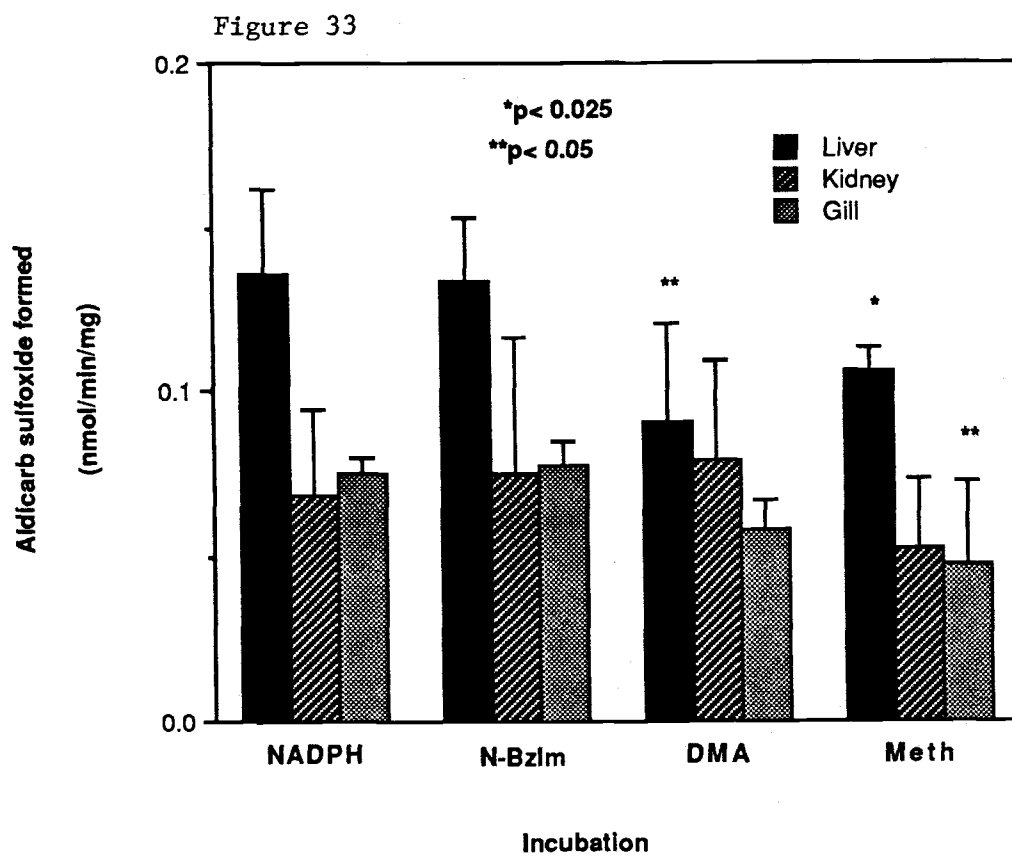
Figure 33

Figure 34. Effect of indomethacin and glutathione on aldicarb
            sulfoxide formation in liver, kidney and gill
            microsomes from rainbow trout.
            Each value represents the mean of 3 assay
            replications from the same preparation ± S.D.
            Each incubation was preincubated for 15 min with 3 mM
            of inhibition before the addition of 0.06 mM
            aldicarb.
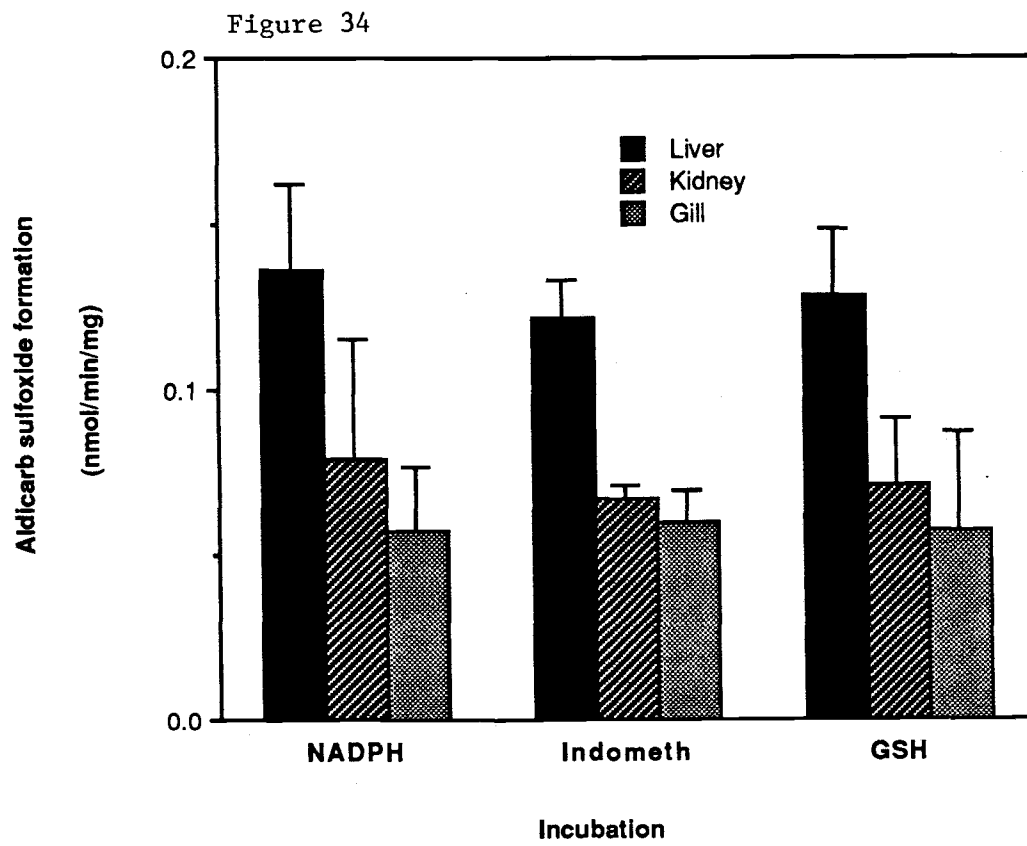
Figure 34

Figure 35. The effect of pH on aldicarb sulfoxide formation in
            liver microsomes from trout.
            Each value represents the mean of 2 assay
            replications from the same preparation.
            [1] percent of recovered radioactivity which coeluted
            with aldicarb sulfoxide. All incubations possessed 1
            mg protein.

Figure 35
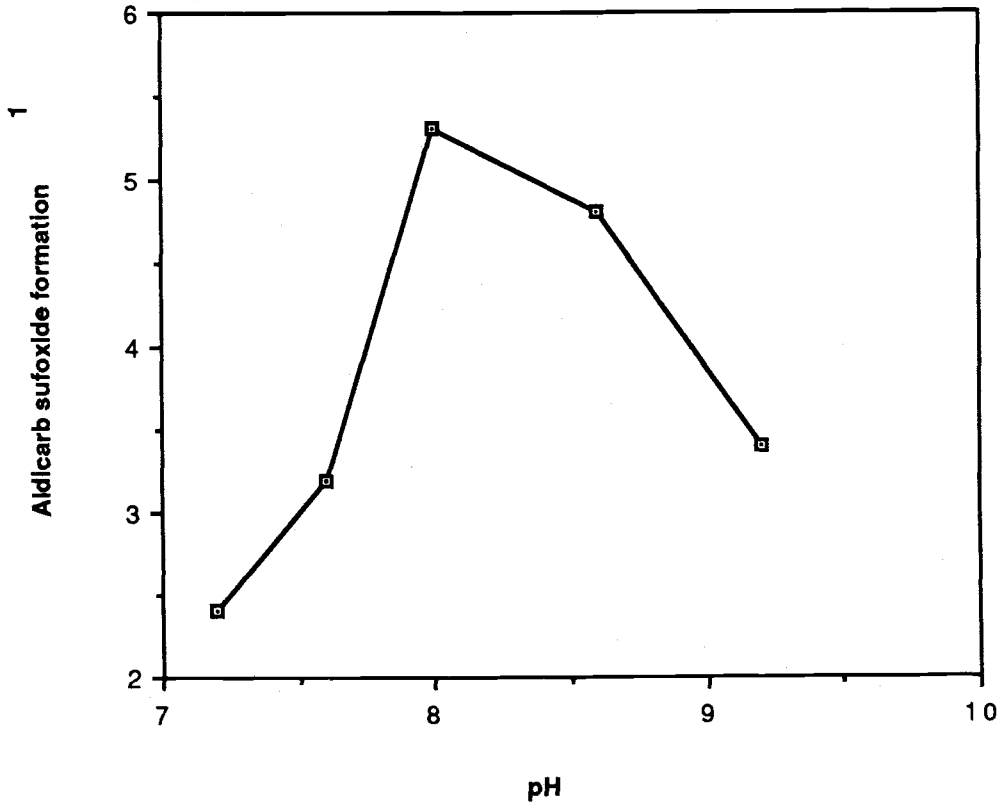
Figure 36. Time profile of aldicarb sulfoxide formation in liver
microsomes from trout.
Each value represents the mean of 2 assay
replications from the same preparation.
[1] percent of recovered radioactivity with coeluted
with aldicarb sulfoxide. All incubations possessed 1
mg protein.

Figure 36
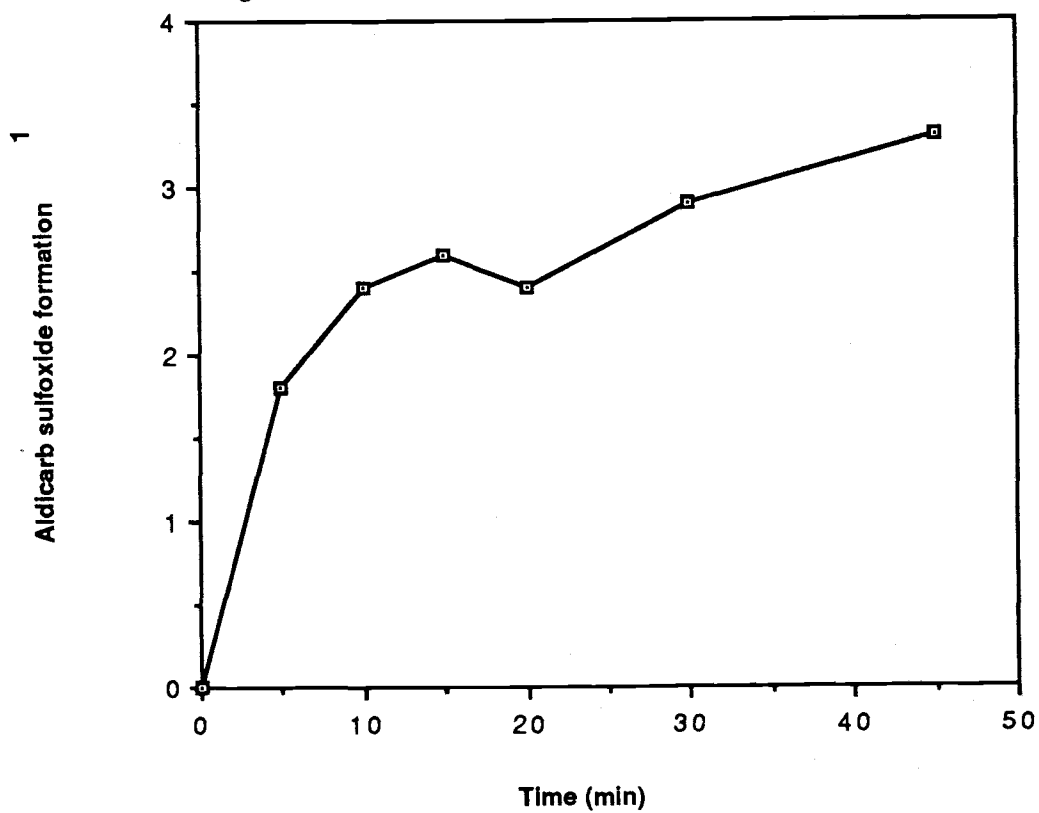
Figure 37. The effect of protein content on aldicarb sulfoxide
formation in liver microsomes form trout.
Each value represents the mean of 2 assay
replications from the same preparation.
[1] percent of recovered radioactivity with coeluted
with aldicarb sulfoxide. All incubations possessed 1
mg protein.
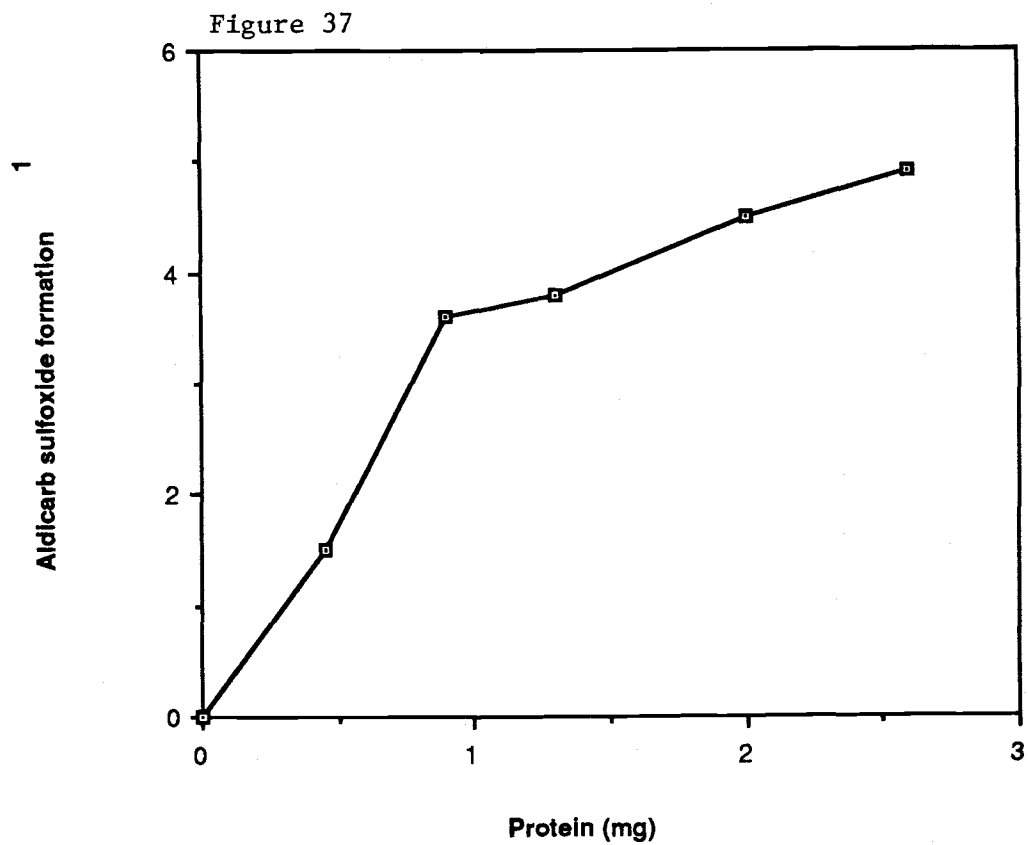
Figure 37

Figure 38. Double Reciprocal plot demonstrating the effect of
DMA on aldicarb sulfoxide formation in liver
microsomes from trout.
Each value represents the mean of 2 assay
replications from the same preparation.
Each incubation was preincubated with 3 mM DMA for 15
min before the addition of 0.06 mM aldicarb.
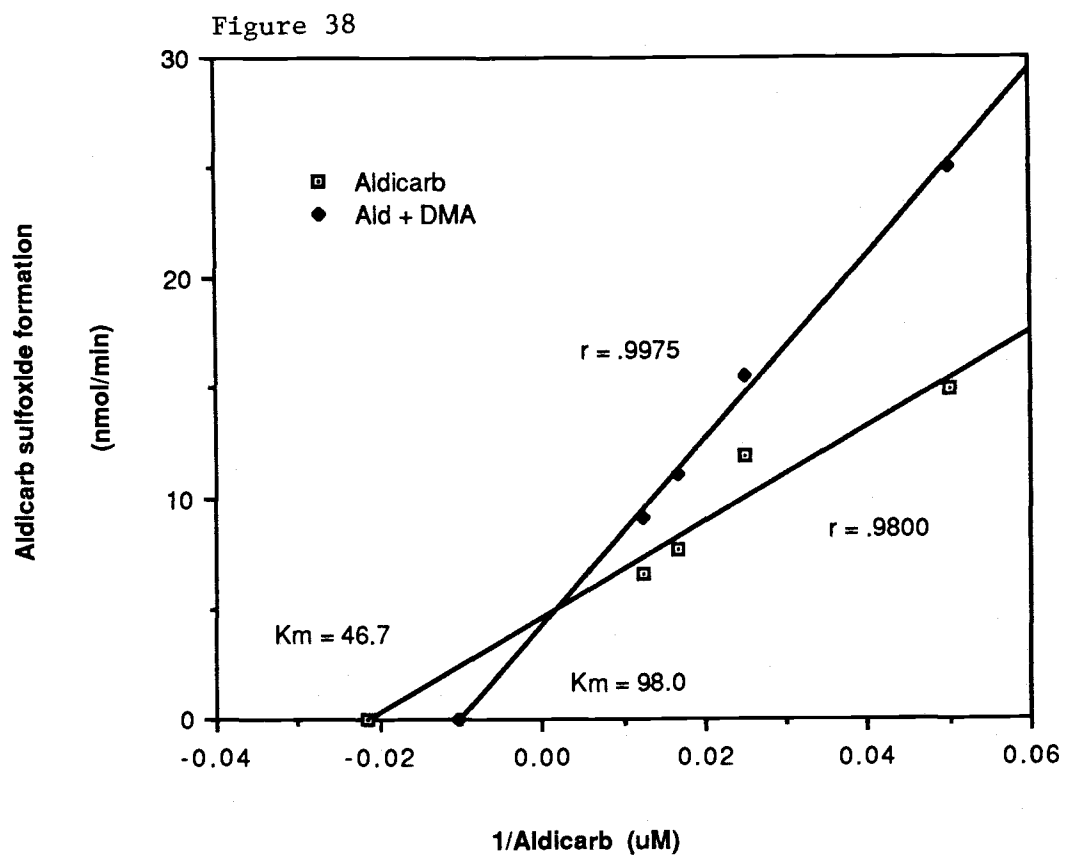
Figure 38

Figure 39. The elimination profiles of [^{14}C] aldicarb in
transected and freeswimming trout.
Each value represents the mean of 3 individual fish ±
S.D.
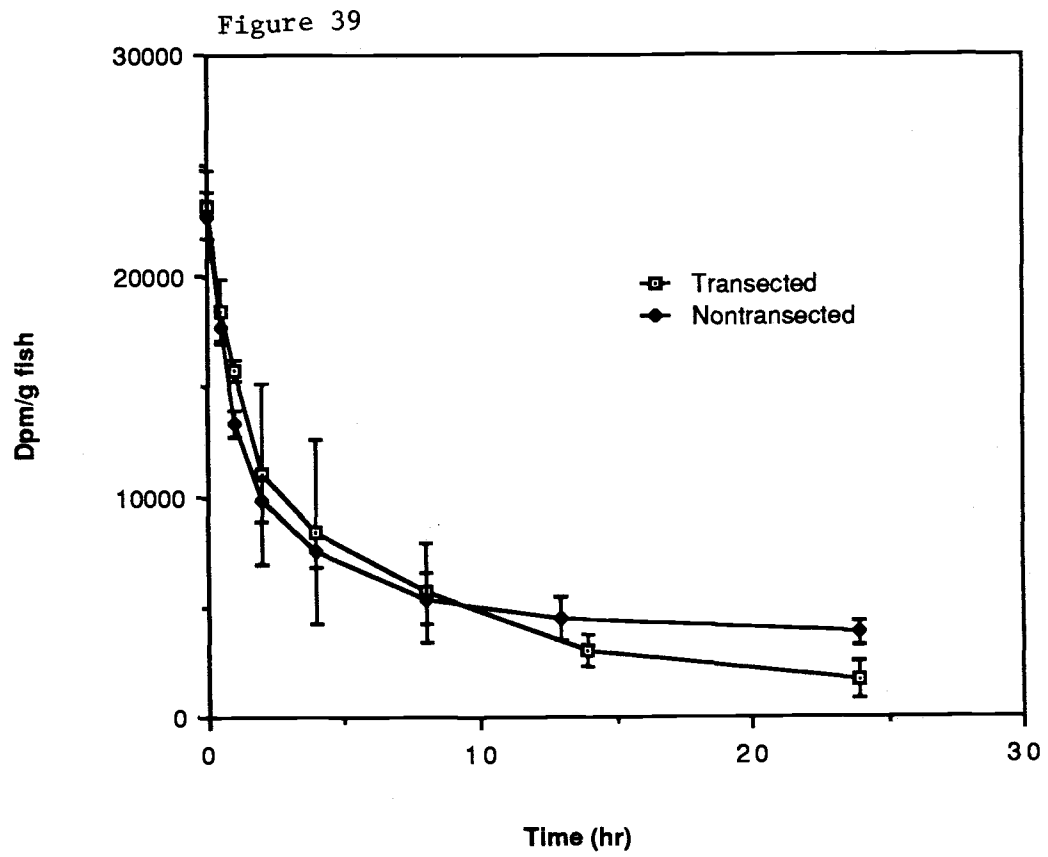
Figure 39

Figure 40. The tissue distribution of [^{14}C] aldicarb in
transected and freeswimming trout.
Each value represents the mean of 3 individual fish
killed 24 hr postinjection $\pm$ S.D.
(GB = Gallbladder; G = Gill; L = Liver; HK = Head
Kidney; ST = Stomach; I = Intestine; H = Heart; TK =
Trunk Kidney; SP = Spleen; B = Brain; M = Muscle; E =
Eye; C = Caeca)

Figure 40
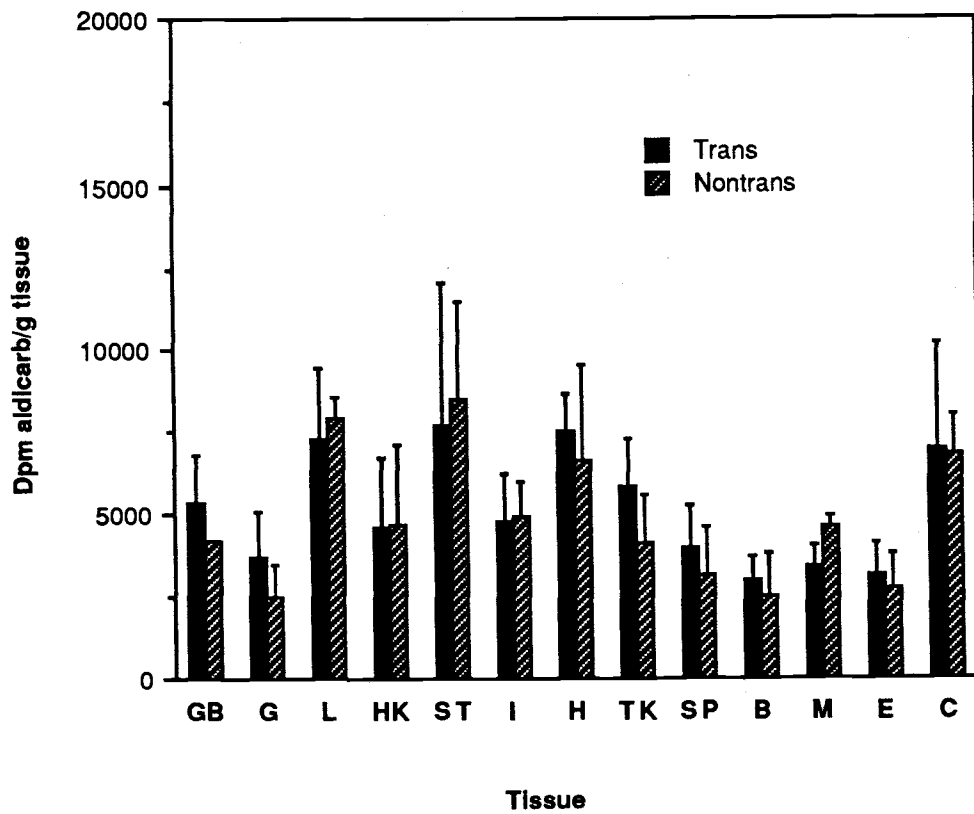
Figure 41. [^{14}C]-Aldicarb excretion profile 24 hr after 1 mg/kg
intraperitoneal injection.
Each value represents the mean of 3 individual fish $\pm$
S.D.

Figure 41
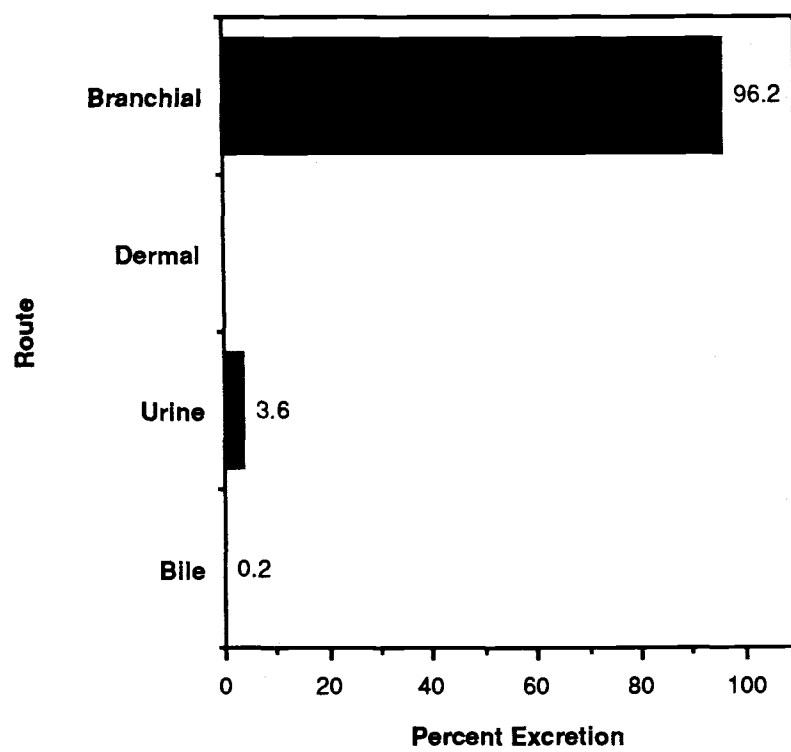
Figure 42. In *vivo* metabolite profile of [^{14}C] aldicarb in trout
24 hr after 1 mg/kg intraperitoneal injection.
Each value represents the mean of 3 individual fish ±
S.D.
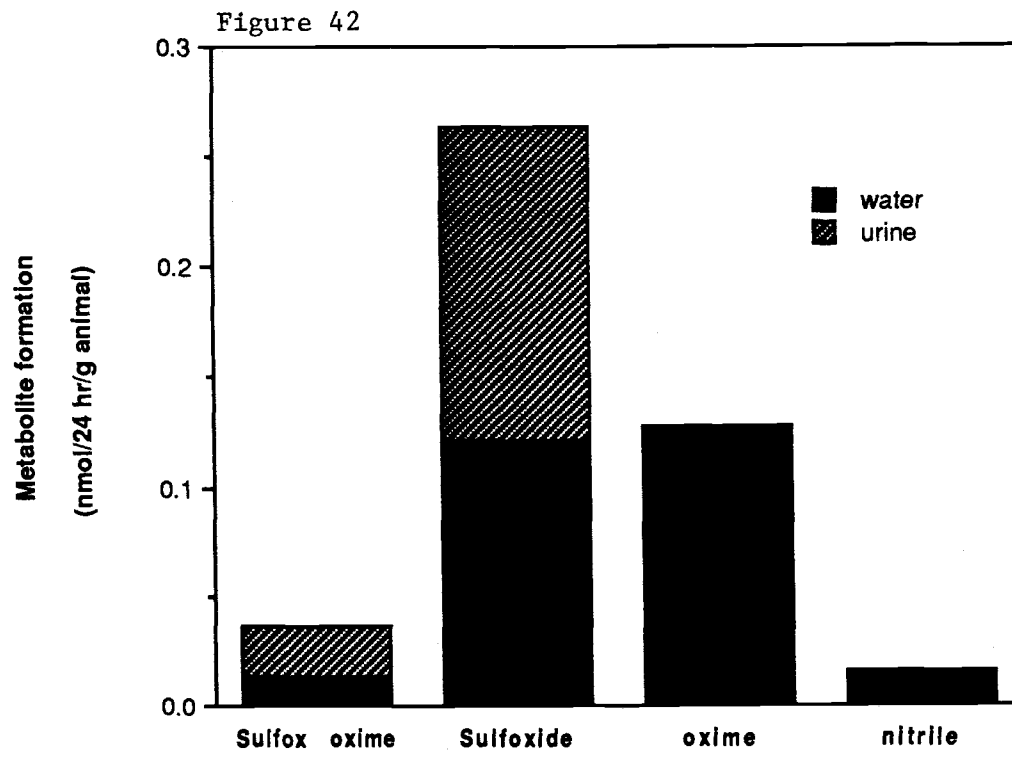
Figure 42

Figure 43. Proposed metabolic pathway of [^{14}C] aldicarb in
            trout.

Figure 43
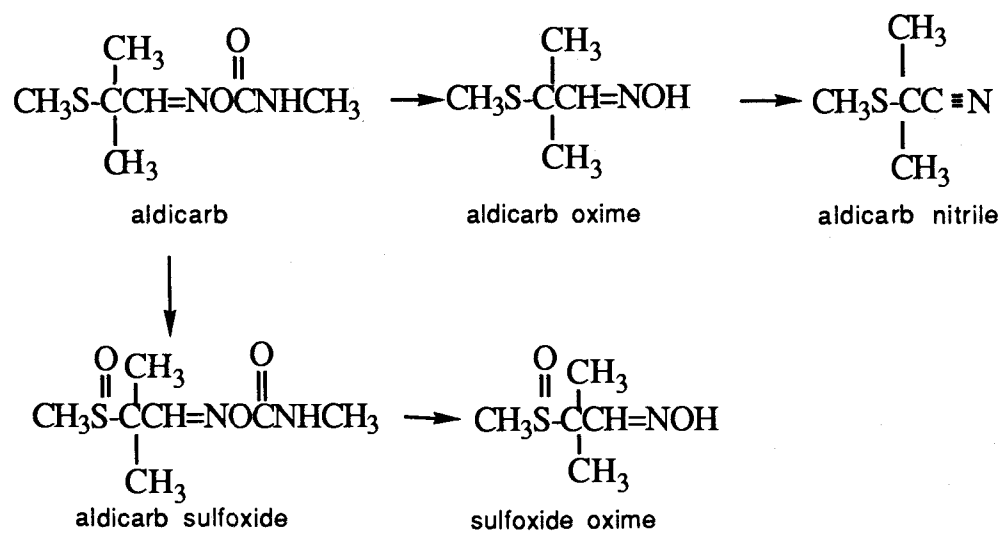
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Chapter 7

Further Characterization of

Flavin-containing Monooxygenase

in Rainbow Trout (<u>Oncorhynchus</u> <u>mykiss</u>).

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ABSTRACT

Polyclonal antibodies raised against flavin-containing

monooxygenase (FMO) enzymes purified from pig liver and rabbit

lung were used in conjunction with N,N-dimethylaniline (DMA) N-

oxidase to better characterize FMO in the rainbow trout

(Oncorhynchus mykiss). The tissue distribution of DMA oxidase

and methimazole oxidation activities paralleled FMO

immunoquantitation. Two proteins appeared to react with anti-pig

liver FMO (PL-1 and PL-2) and anti-rabbit lung FMO (RL-1 and RL-

2). Although there was no difference in DMA oxidase observed

between mature male and female trout liver microsomes, RL-2 and

PL-2 were significantly less than RL-1 and PL-1, respectively, in

mature females. Inconclusive results were obtained after trout

were pretreated with estradiol or testosterone to determine the

contribution of hormonal regulation. FMO is directly correlated

with an increase in age in trout liver. DMA oxidase and FMO

isozymes were unaltered after pretreatment with the endogenous

substrate trimethylamine.

INTRODUCTION

As an alternative vertebrate model, the rainbow trout
Oncorhynchus mykiss has been utilized in several laboratories to
study carcinogenesis (Sinnhuber et al., 1977) and physiological
toxicology (McKim et al, 1985). Consequently, due to augmented
use of the trout, there has been growing interest in xenobiotic
biotransformation mechanisms in these animals. In mammalian
systems, cytochrome P-450 and flavin-containing monooxygenase
(FMO) play dominant roles in chemical detoxification but also can
bioactivate chemicals to more toxic species (Miller and Miller,
1985). Several cytochrome P-450 isozymes have been purified and
well characterized from various tissues in the rainbow trout
(Williams and Buhler, 1984; Williams et al., 1986; Miranda et al.,
1989). Sexual differences, isozyme induction, tissue
distribution, and enzyme development during growth have all been
previously examined in the rainbow trout (Williams and Buhler,
1984; Williams et al., 1986). However, very little work has been
done regarding FMO in this species.

FMO activity (N,N-dimethylaniline N-oxidase) has been observed
in liver microsomes from rainbow trout (Arillo et al., 1984) and
appears to be involved in the oxidation of endogenous
trimethylamine (Agustsson and Strom, 1981; Schlenk and Buhler,
1990a). FMO has also been shown to be responsible for the
oxidation and subsequent bioactivation of the carbamate thioether,
aldicarb to aldicarb sulfoxide in rainbow trout (Schlenk and

Buhler, 1990b). Virtually nothing is known about this enzyme in trout.

Consequently, the purpose of this paper is to better characterize this important enzyme system in the trout. Although the distribution of the enzyme will be determined in various tissues from the trout, most of the studies will focus on the form found in liver microsomes. With this form, the possibility of enzyme induction will be addressed as well as sexual and age-related differences in enzyme content and activity. Polyclonal antibodies raised against the purified rabbit lung (anti-RL) and pig liver (anti-PL) enzymes have been used to determine the relative content of microsomal FMO. The results will help provide a more complete understanding of xenobiotic metabolizing pathways in trout and better characterize FMO in this organism.

METHODS AND MATERIALS

Chemicals

Unless otherwise noted all chemicals were purchased from Sigma

Chemical Co. (St. Louis, MO).

Animal maintenance and treatment

Rainbow trout (Oncorhynchus mykiss) were maintained in tanks at

the Food Toxicology and Nutrition laboratory of the Oregon State

University Marine Freshwater Biomedical Center with free flowing

well water at 12°C. To compare sexual differences in FMO, 10

mature males and 10 mature females (24 mo) were each killed by a

blow to the head. Livers were excised and pooled according to

sex. To examine enzyme development with age, 10 fish from each

age group (6 mo, 14 mo, 18 mo, and 24 mo) were likewise killed by

a blow to the head and livers were dissected and pooled according

to age group.

In order to examine the effects of testosterone and estradiol

pretreatment on FMO, three treatment groups (testosterone,

estradiol and control) of 20 fish per group (6 mo) were placed

into three individual aquaria. The animals received 3 mg/kg

intraperitoneal injections of testosterone and estradiol while

controls were given the same volume of corn oil. Injections were

made on day 1, day 3, day 7 and the fish were killed on day 10.

Livers were dissected and pooled within each treatment group.

The effect of TMA on FMO was also examined by injecting (ip) 10

trout (18 mo) with 100 mg/kg TMA (10 controls in a separate

aquarium received the same volume of 0.9 % saline) on day 1, day

3, and day 5. The fish were then killed on day 7 and the

dissected livers were pooled into control and TMA-treated groups.

Microsome preparation

Microsomes were prepared as previously described (Schlenk and

Buhler, 1990a). Livers from individual fish were removed,

weighed, and washed in 1.15% KCl. The livers were then pooled,

minced and homogenized in 4 vol. of ice cold buffer A [0.1 M Tris-

acetate pH 7.4, 0.1 M KCl, 1 mM EDTA, and 0.1 mM

phenylmethylsulfonyl fluoride (PMSF)]. A microsomal pellet was

obtained by centrifugation at 9,000 x g for 20 min and

centrifugation of this supernatant twice for 90 min at 100,000 x

g. The resulting microsomes were resuspended in buffer B (0.1 M

potassium phosphate pH 7.25, 20 % glycerol and 1 mM EDTA) to a

protein concentration of approximately 20 mg/ml and frozen in

liquid nitrogen and stored at $^-$80°C until used.

Assay of FMO

DMA N-oxidation was determined by the method of Ziegler and

Pettit (1964). DMA HCl was prepared as previously described (Schlenk and Buhler, 1990a). A 1 ml incubation mixture contained 0.25 M potassium phosphate (pH 8.4), 0.1 mM NADPH, and 1-2 mg microsomal protein. The reaction was started with the addition of 4 mM DMA and then incubated at 25°C for 30 min. After extraction with ether to remove unreacted substrate, DMA N-oxide was reduced back to DMA and then converted to the nitroso compound which was colorimetrically quantitated at 420 nm on a Cary 219 Spectrophotometer (extinction coefficient 8.2 mmol^{-1}). Initial controls consisted of boiled microsomes. Since incubations not including NADPH gave the same background values as boiled microsomes, incubations without NADPH were subsequently used as controls.

Methimazole oxidation catalyzed by FMO was measured by the spectrophotometric assay of Dixit and Roche (1984). Assay mixtures contained 0.1 M Tricine HCl (pH 8.4), 1.0 mM EDTA, 0.06 mM 5,5'-dithiobis(2-nitrobenzoate) (DTNB), 6.0 mM potassium phosphate (pH 8.0), 0.025 mM dithiothreitol (DTT), 0.10 mM NADPH, 1.0 mg microsomal protein, and 1.0 mM methimazole in a final volume of 1.0 ml. Enzyme activity was initiated by addition of methimazole to one cuvette, and reaction rates were measured with a Cary 219 spectrophotometer as the rate of decrease in absorbance at 412 nm between identical assay mixtures with and without methimazole.

Electrophoresis, immunoblotting, and immunostaining

Electrophoresis of microsomal proteins was performed using 8.0 %
separating and 3.0 % stacking polyacrylamide gels in the presence
of sodium dodecyl sulfate (SDS)(Laemmli, 1970). All materials
were purchased from BioRad (Richmond, CA). Samples were combined
with buffer (60 mM Tris HCl, pH 6.8; 2 % SDS; 10 % glycerol; 5 %
β-mercaptoethanol; 0.0025 % pyronin Y) and boiled for 3 min prior
to electrophoresis. Electrophoresis was performed utilizing a
BioRad Protean^{TM} II system and power source. Gels were run for 4
hr at 30 mV. Following electrophoresis, the separated proteins
were transferred via electroblot to nitrocellulose sheets
following a modification of the method of Towbin et al. (1979) and
stained by a modification of the method of Burnette (1981). A
BioRad transblot cell was utilized to transfer the protein from
the polyacrylamide gel to the nitrocellulose paper. The transfer
buffer contained 20 mM Tris base, 0.15 M glycine in 25 % methanol.
The transfer was performed overnight at 30 mV. After transfer,
the nitrocellulose sheets were incubated 60 min with constant
shaking in phosphate buffered saline solution (PBS), (20 mM
potassium phosphate, pH 7.4, 0.8 % sodium chloride) containing 2 %
bovine serum albumin (BSA). Transfers were then incubated for 90
min with FMO antibody (20 μg/ml) in PBS containing 2 % BSA.
Following antibody treatment, the sheets were sequentially washed
with PBS, PBS containing 0.05 % triton X-100, a second PBS wash
and then incubated with constant shaking for 60 min with a PBS

solution containing [^{125}I]-protein A at 2 x 10^5 cpm/ml. After a second sequential wash as above, the nitrocellulose sheets were wrapped in cellophane plastic and exposed to Kodak XAR-5 X-ray film for 7 days at - 80°C. Relative quantitation of autoradiograms was performed utilizing laser densitometry (Zeineh).

RESULTS

Tissue distribution

Microsomes from trunk kidney and liver possessed the highest
rates of FMO activity (Figure 44). Lesser amounts of FMO activity
were observed in gill and head kidney. Several proteins from
tissue microsomes gave a positive reaction with polyclonal
antibodies during Western blot analysis (Figures 45 and 46). A
single band of staining was observed at 59,000 daltons in
microsomes from liver, trunk kidney, head kidney and gill when
anti-RL were utilized. The band of staining present in liver
microsomal samples possessed the greatest reactivity with rabbit
lung FMO antibodies. However, these samples underwent
electrophoresis for only 2 hrs at 70 mV. Subsequent
electrophoretic experiments demonstrated that if the gels were
allowed to run at 4 hrs using only 30 mV, two bands of staining
were detected with both anti-PL and anti-RL. The top band (PL-1 or
RL-1) possessed a consistent molecular weight of 61,000 while the
lower band (PL-2 or RL-2) was approximately 57,000 daltons. Two
bands of staining were detected in microsomes from liver, trunk
kidney and head kidney utilizing anti-PL. Upon densitometric
quantitation, the bands were combined and compared against the
single bands observed in anti-RL analyses (Figure 47).

Sex differences

A difference in relative quantities of the two bands of staining was observed between male and female samples (Figures 48 and 49). RL-2 and PL-2 were significantly lower in female liver microsomes compared to males. However, there were no statistically significant differences in RL-1 and PL-1 between males and females. DMA oxidase activity in liver microsomes from mature males was also not significantly different from that observed in mature females (Figure 50).

Effect of TMA pretreatment

Administration of TMA in 18 month-old rainbow trout did not significantly alter DMA oxidase in treated fish relative to control trout which received saline (Figure 51). There were also no differences observed in protein band staining between controls and treated fish (Figures 48 and 49).

Effects of testosterone or estradiol pretreatment

Unlike the experiments performed with mature males and females, upper and lower bands of staining in microsomes from juvenile trout using anti-PL and anti-RL were inconsistent. Juvenile trout (6 mo) pretreated with testosterone led to decreases in DMA oxidase and RL-1 but increases were observed in PL-1, RL-2 and PL-

2 relative to controls (Figures 52, 53 and 54). Although an
increase in PL-1 was observed after pretreatment with estradiol,
DMA oxidase activity and RL-1, RL-2 and PL-2 were reduced relative
to controls.

Developmental studies

DMA oxidase activity and both bands of staining were directly
related to increases in trout age (Figures 55, 56 and 57). A
sixfold increase in protein content and activity was observed in
microsomes from trout between 6 to 14 months of age. Although
protein content was also augmented over this time, only a two-fold
increase was observed in bandstaining with the exception of PL-2
where only a 30 % increase was seen. Protein content appears to
reach consistent levels at approximately 18 mo while activity
continues to increase with the age of the animal. Amounts of the
lower band (RL-2 and PL-2) generally appeared to be less than
quantities of the upper band (RL-1 and PL-2) throughout the life
of the animal.

DISCUSSION

The reactivity of microsomal proteins from trout tissues with polyclonal antibodies from higher vertebrates and the co-migration of these proteins with purified standards suggests that the observed bands of staining represent FMO in microsomes from trout tissues. Antibodies against various forms of FMO have been shown to react with forms from similar tissues in various species in mammals (Tynes and Philpot, 1987). However, the observation of a crossreaction between FMO in fish and antibodies raised against mammalian forms of FMO suggests that the structure of FMO must be somewhat conserved through evolution, at least from fish to mammals. This evolutionary conservation of enzyme structure also appears to be observed with certain cytochrome P-450 isozymes. Antibodies raised against rat liver cytochrome P-450c, the β-naphthoflavone (BNF) inducible form, have been shown to react with the purified cytochrome P-450 LM_{4b} isozyme in trout liver which is also BNF-inducible (Miranda et al., 1990).

The presence of two bands of staining in trout tissue microsomes was unexpected. Although most purification studies of FMO in mammals have identified only a single form within each tissue, Tynes and Philpot (1987) have reported that there may in fact be multiple forms of FMO in certain tissues. Ziegler (unpublished data) has noted that over a certain period of time partial degradation of the purified porcine enzyme does occur

leading to the formation of multiple protein bands which are still immunochemically active, but catalytically inactive. Although DMA oxidase activity was greater in male liver microsomes than in female samples in our studies, the upper bands of staining at 61,000 daltons (PL-1 and RL-1) were actually greater in female liver microsomes. Conversely, the lower band of staining at 57,000 daltons (PL-2 and RL-2) was significantly less than these bands in male liver microsomes. However, when the staining intensities of PL-1 and PL-2 or RL-1 and RL-2 were combined in microsomes from male and females, respectively, the males consistently possessed a slightly greater total content of enzyme than the female, but the difference was not statistically significant ($p \geq 0.375$; Student's t-test). Consequently, since the relationship of isozyme content is consistent with DMA oxidase activity and assuming each band represents a distinct FMO isozyme, it appears that each band may exist as a catalytically active isozyme. Of course, these explanations remain speculative until purification of the enzyme can be accomplished.

The lack of statistically significant differences between sexes observed in trout contrasts results reported in mammalian studies. Adult male mice were shown to express one-half to one-third of the FMO activity levels of adult females, mostly due to repression by testosterone (Duffel et al., 1981). The exact opposite was observed in rats where livers from males had three times greater levels of FMO than female livers (Dannan et al., 1986). In the rabbit, liver and lung microsomes from males

possessed approximately twice the amount of FMO compared to females (Dannen and Guengerich, 1982). However, upon pregnancy, FMO in microsomes from the lung of female rabbits was shown to be enhanced fivefold that of controls (Williams et al., 1985).

Since several studies have shown that FMO appears to be affected by various hormones (see Ziegler, 1988 for review), trout were pretreated with testosterone and estradiol in order to see if FMO content or activity in liver was regulated hormonally. Administration of either testosterone or estradiol significantly reduced FMO activity, but a concomitant decrease in protein band staining was not consistently observed. Although the dose of testosterone and estradiol was identical to previous procedures which examined the effect of these compounds on cytochrome P-450 isozymes in fish (Stegeman et al., 1982), perhaps FMO is more sensitive to hormonal changes compared to cytochrome P-450 in the trout. Dannan et al. (1986) have shown that rat liver FMO was regulated positively by testosterone and negatively by estradiol. Conversely, hepatic FMO activity in gonadectized mice was shown to be reduced by testosterone pretreatment (Duffel et al., 1981). It appears that a host of steroids, possibly in given in combination, need to be examined in differing doses to establish the effect of these compounds on FMO in the trout.

The tissue distribution of DMA oxidase activity paralleled immunoblot quantitation. Liver and trunk kidney microsomes possessed the overall greatest contents and activities. In all of the mammalian species examined, the highest concentration of FMO

is usually found in the liver (Ziegler, 1988). FMO activity has also been observed in kidney, urinary bladder mucosa, testicle, corpus luteum, thyroid, thymus, and adrenal microsomes in the hog as well as kidney and lung microsomes in rat, rabbit, hamster and mouse (Ziegler, 1980). Several groups have shown, however, that the forms expressed in separate tissues are immunochemically distinct even across species (Williams et al., 1985; Tynes and Philpot, 1987). For example, antibodies raised against the purified rabbit lung FMO do not react with the purified rabbit or pig liver FMO. This did not appear to be the case regarding trout tissues. Anti-RL reacted with proteins from several tissues all of similar molecular weight (59,000). Likewise, proteins from liver, trunk kidney and head kidney also reacted with anti-PL. The main reason for these results is probably the long exposure time during autoradiography needed to obtain a stain dark enough to quantitate by laser densitometry.

Pretreatment of trout with TMA failed to alter FMO activity or isozyme content. These results contrast reports of TMA oxygenase induction in eel and guppy after the fish were pretreated with TMA (Daikoku et al., 1988). TMA oxygenase and FMO have been shown to be the same enzyme (Schlenk and Buhler, 1990a). TMA is a ubiquitous chemical found in fish, and has been shown to be responsible for the "fishy" odor associated with the organisms after death. TMA is oxygenated in liver and stored as the N-oxide within fish muscle (Charest, 1988). The function of TMA oxidation in teleosts is unknown but is suggested to be involved in

osmoregulation in elasmobranchs (Pang et al., 1977).

Although FMO activity continues to increase up to at least 2 years of age, protein band staining appears to be maximal at 18 months. Enhanced isozyme content was generally accompanied by an increase in DMA oxidase activity as fish matured. However, it is possible that enzyme turnover may increase without an increase in isozyme content as the fish develops. In a similar relationship, Das and Ziegler (1970) showed that DMA oxidase activity in rat liver microsomes was directly related to the age of the animal. In mouse liver microsomes, DMA oxidase activity was shown to dramatically increase at approximately 3 days of age and remain essentially constant in both sexes until day 16 when a marked enhancement of activity occurred in liver microsomes from female mice (Wirth and Thorgeirsson, 1978).

The rainbow trout has been extensively utilized as a model to study the behavior of chemicals in aquatic systems (McKim et al., 1985) and also as an alternative vertebrate model to study carcinogenesis (Sinnhuber et al., 1977). In addition, fish are continually exposed in the aquatic environment to a host of nitrogen and sulfur-containing chemicals. Since FMO has been shown to be responsible for the activation and detoxification of chemicals of this nature, the occurrence of FMO in trout is significant. To better understand chemical toxicology in biological systems, the roles of all enzymes of xenobiotic biotransformation should be a primary area of research to be explored in future studies.

ACKNOWLEDGEMENTS

Figure 44. DMA N-oxide formation and methimazole oxidation in
various trout tissue microsomes.
Each value represents the mean of 2 assay
replications from the same preparation.

Figure 44
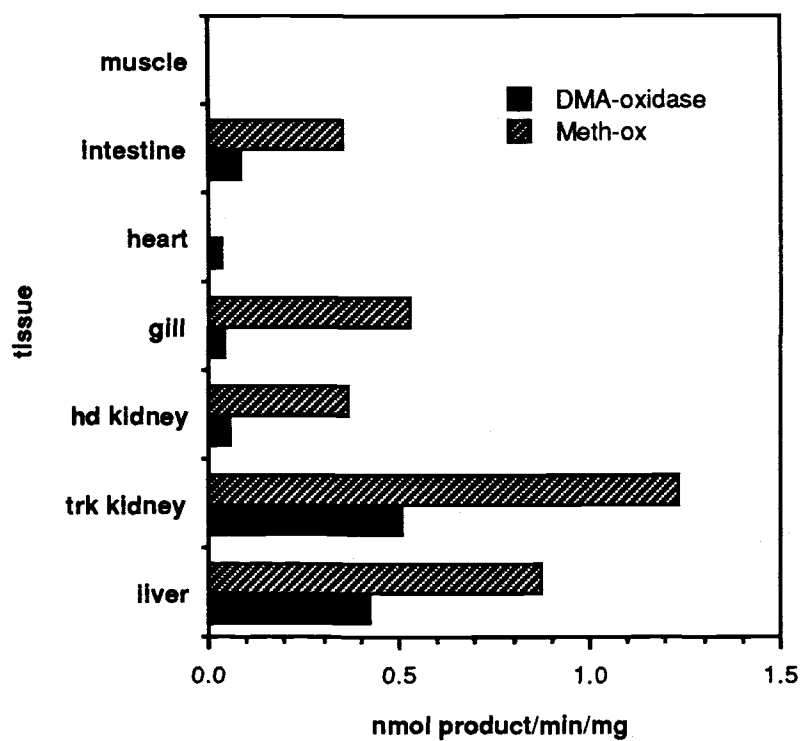
Figure 45. Western blot analysis of microsomes of various
tissues from trout stained with anti-rabbit lung FMO.
Lanes 1-2, intestine, 80 μg protein; Lanes 3-4, heart,
80 μg protein; Lanes 5-6, trunk kidney, 80 μg protein;
Lanes 7-8, liver, 80 μg protein; Lanes 9-10, gill, 80
μg protein; Lanes 11-12, head kidney, 110 μg protein;
Lanes 13-14, purified rabbit lung FMO, 0.1, 0.2 pmol.

Figure 45

274
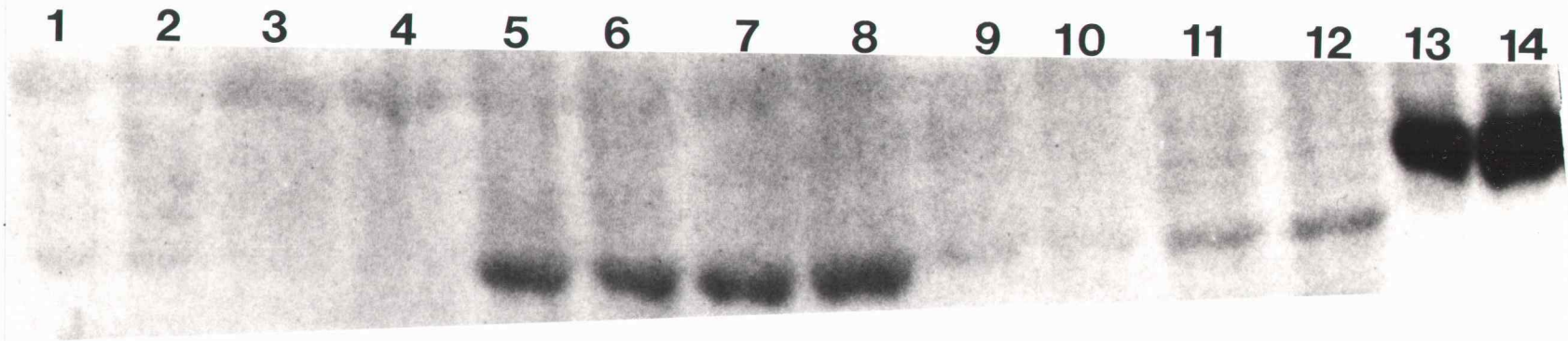
Figure 46. Western blot analysis of microsomes of various
tissues from trout stained with anti-pig liver FMO.
Lanes 1-2, head kidney, 110 μg protein; Lane 3, gill,
80 μg protein; Lanes 4-5 intestine, 100 μg protein;
Lanes 6-7, trunk kidney, 100 μg protein; Lanes 8-9,
heart, 80 μg protein; Lanes 10-11, liver, 80 μg
protein; Lanes 12-13, purified pig liver FMO, 0.1, 0.2
pmol.

Figure 46
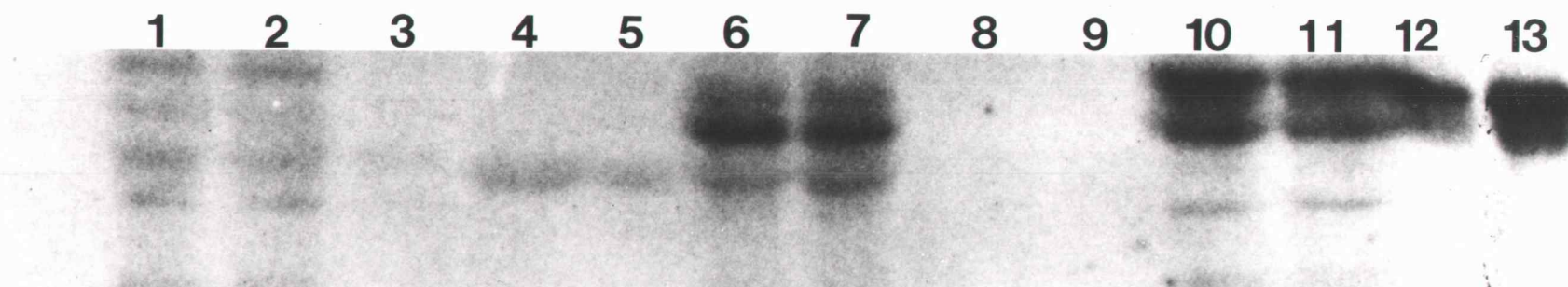
Figure 47. Relative stain intensities of microsomes of various
tissues from trout stained with anti-rabbit lung and
anti-pig liver FMO.
Each value represents the mean of 2 sample
replications.
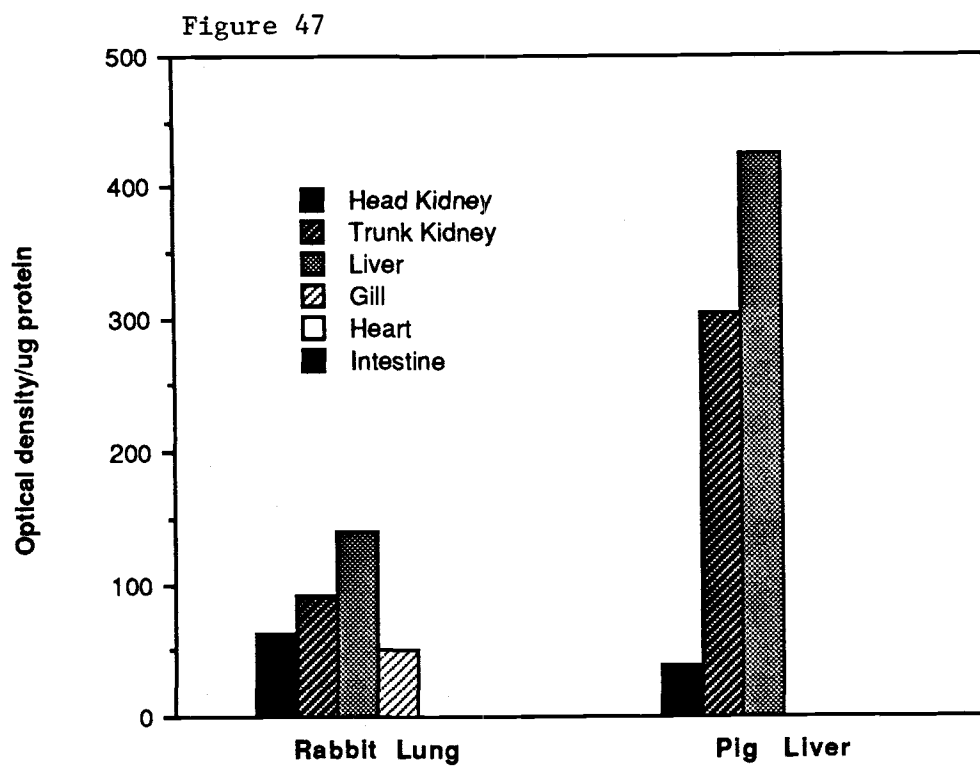
Figure 47

Figure 48. Western blot analysis of liver microsomes from
            control and TMA-treated trout as well as sexual
            mature male and female trout stained with anti-
            rabbit lung FMO.
            Lanes 1-3, control, 100 μg protein; Lanes 4-6, TMA,
            100 μg protein; Lanes 7-9, male, 100 μg protein;
            Lanes 10-12, female, 100 μg protein; Lanes 13-15,
            purified rabbit lung FMO, 0.01, 0.02, 0.05 pmol.
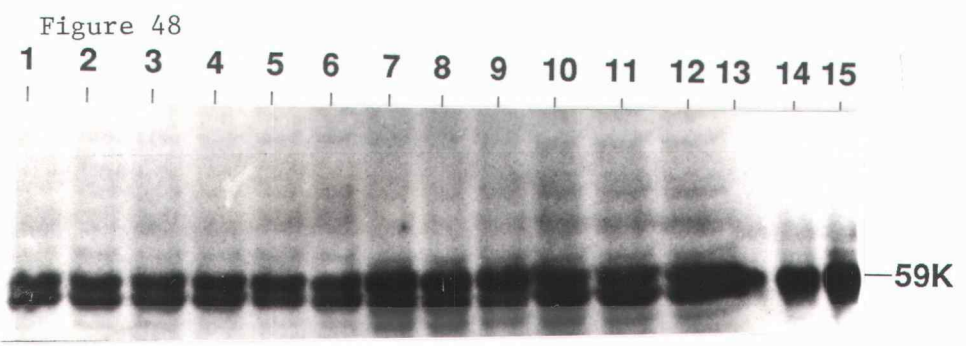
Figure 48

Figure 49. Western blot analysis of liver microsomes from
control and TMA-treated trout as well as sexual
mature male and female trout stained with anti-pig
liver FMO.
Lanes 1-3, control, 100 μg protein; Lanes 4-6, TMA,
100 μg protein; Lanes 7-9, male, 100 μg protein;
Lanes 10-12, female, 100 μg protein; Lanes 13-15,
purified pig liver FMO, 0.01, 0.02, 0.05 pmol.

Figure 49

Figure 50.   DMA N-oxide formation and relative staining
             intensities of liver microsomes from mature male and
             female trout.
             Each value represents the mean of 3 sample
             replication ± S.D.
             (RL-1 = anti-rabbit lung upper band; RL-2 = anti-
             rabbit lung lower band; PL-1 = anti-pig liver upper
             band; PL-2 anti-pig liver lower band)
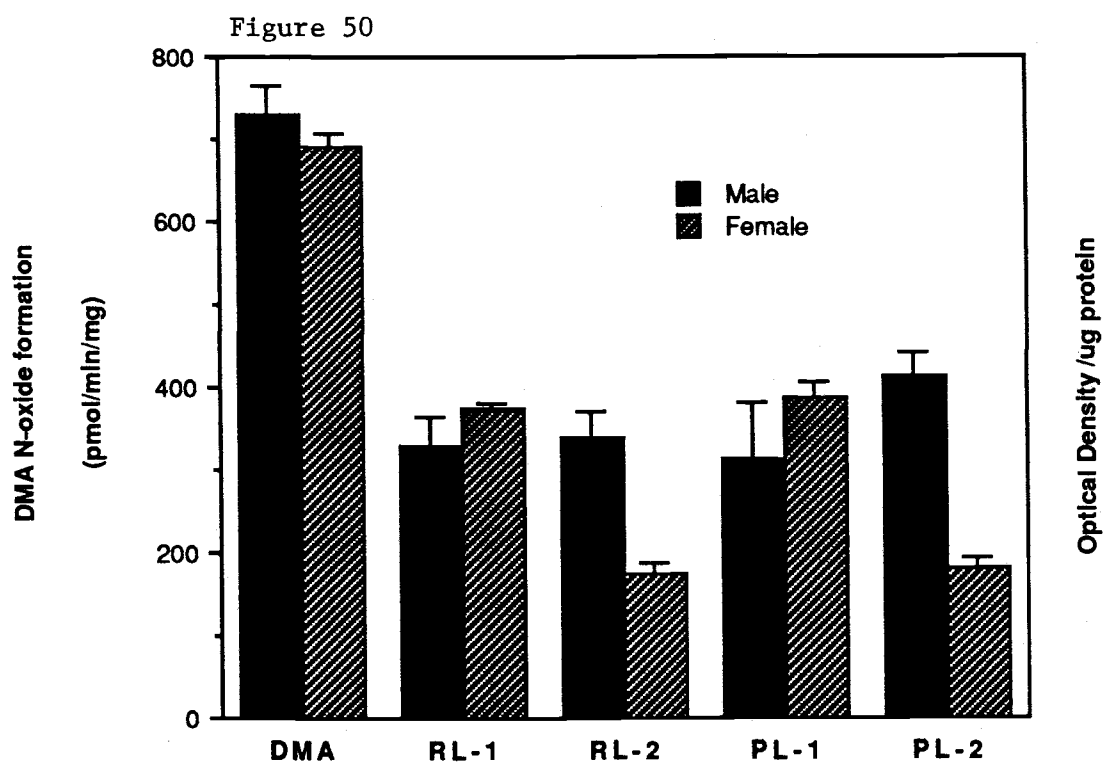
Figure 50

Figure 51. DMA N-oxide formation and relative staining
            intensities of liver microsomes from control and TMA
            treated trout.
            Each value represents the mean of 3 sample
            replication ± S.D.
            (RL-1 = anti-rabbit lung upper band; RL-2 = anti-
            rabbit lung lower band; PL-1 = anti-pig liver upper
            band; PL-2 anti-pig liver lower band)
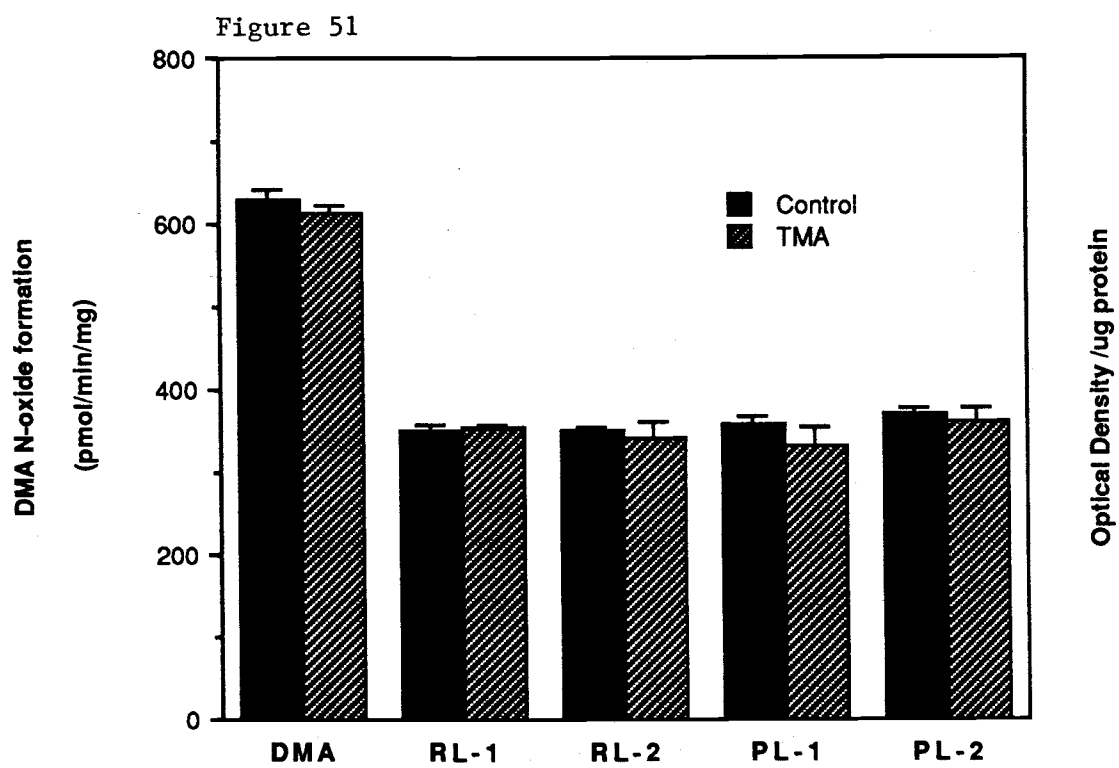
Figure 51

Figure 52. Western blot analysis of liver microsomes from
            control, testosterone and estradiol treated trout
            stained with anti-rabbit lung FMO.
            Lanes 1-2, estradiol, 75 μg protein; Lanes 3-4,
            testosterone, 75 μg protein; Lanes 5-6, control, 75
            μg protein.
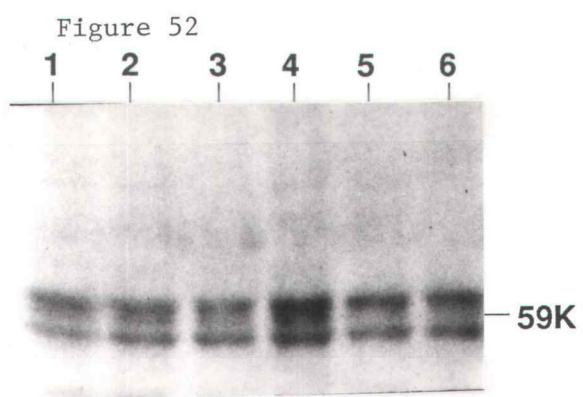
Figure 52

Figure 53. Western blot analysis of liver microsomes from
control, testosterone and estradiol treated trout
stained with anti-pig liver FMO.
Lanes 1-2, estradiol, 75 μg protein; Lanes 3-4,
testosterone, 75 μg protein; Lanes 5-6, control, 75
μg protein; Lane 7, purified rabbit lung FMO 0.02
pmol.

Figure 53
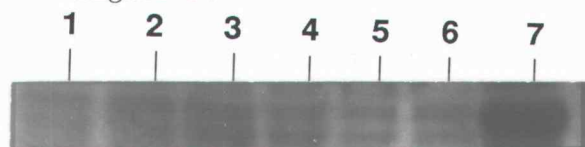
Figure 54. DMA N-oxide formation and relative staining
intensities of liver microsomes from control,
testosterone, and estradiol treated trout stained
with anti-rabbit lung and anti-pig liver FMO.
[1] Each value represents the mean of 3 assay
replication $\pm$ S.D.
[2] Each value represents the mean of 2 sample
replications.
(RL-1 = anti-rabbit lung upper band; RL-2 = anti-
rabbit lung lower band; PL-1 = anti-pig liver upper
band; PL-2 anti-pig liver lower band)
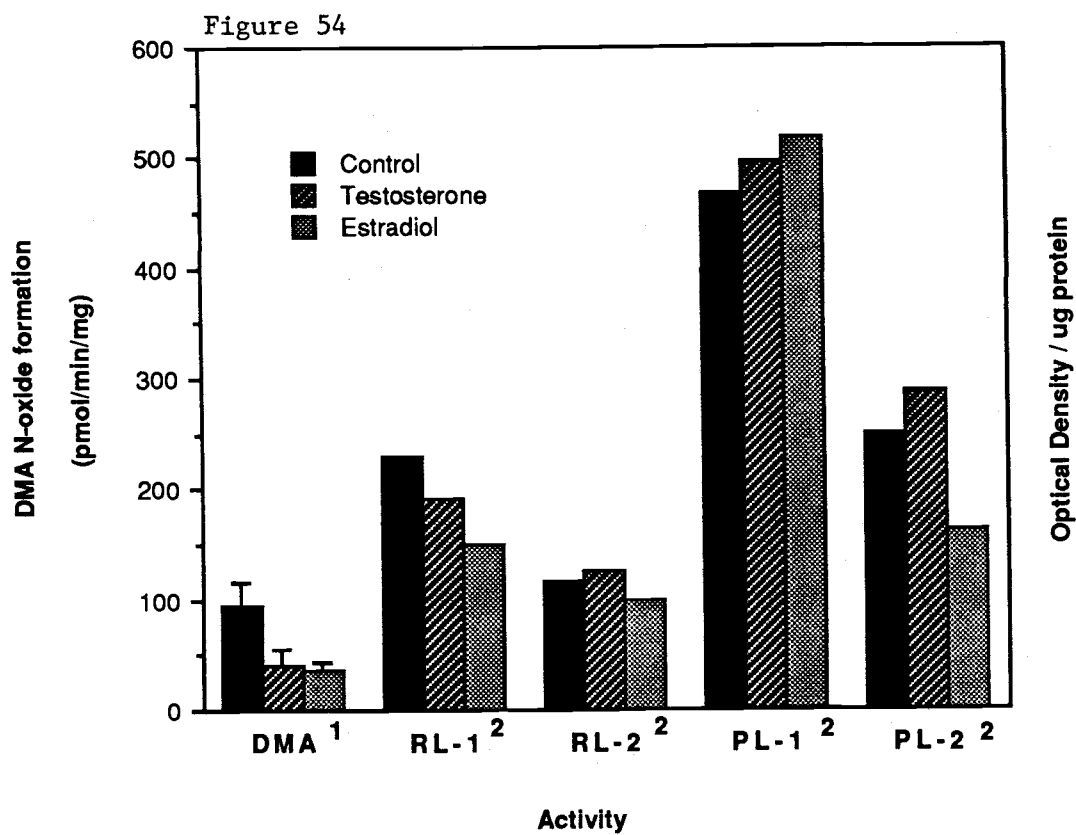
Figure 54

Figure 55. Western blot analysis of liver microsomes from trout
at various ages stained with anti-rabbit lung FMO.
Lanes 1-2, 6 month, 100 μg protein; Lanes 3-4, 14
month, 100 μg protein; Lanes 5-6, 18 month, 100 μg
protein; Lanes 7-8, 24 month, 100 μg protein; Lanes 9-
11, purified rabbit lung FMO, 0.01, 0.02, 0.05 pmol.
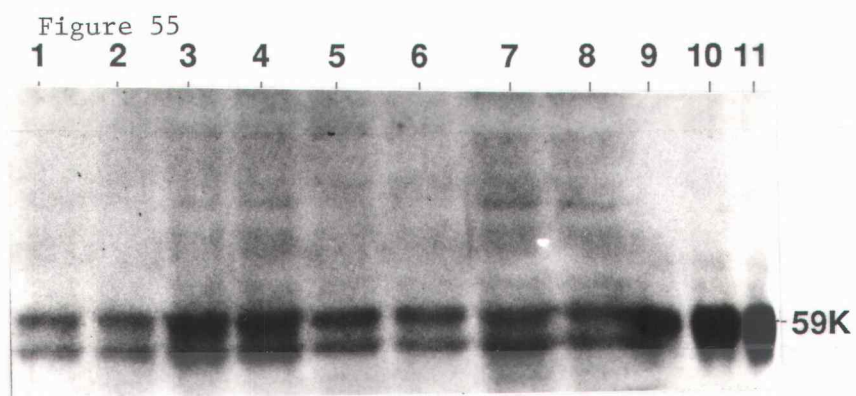
Figure 55

Figure 56.   Western blot analysis of liver microsomes from trout
at various ages stained with anti-pig liver FMO.
Lanes 1-2, 6 month, 100 μg protein; Lanes 3-4, 14
month, 100 μg protein; Lanes 5-6, 18 month, 100 μg
protein; Lanes 7-8, 24 month, 100 μg protein.

Figure 56
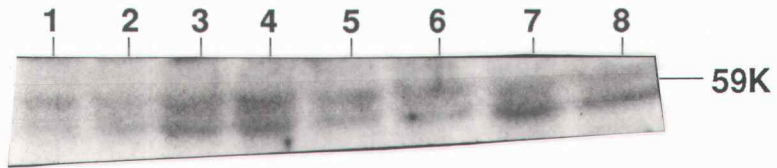
Figure 57.   DMA N-oxide formation and relative staining
             intensities of liver microsomes from trout of various
             ages stained with anti-rabbit lung and anti-pig liver
             FMO.
             [1] Each value represents the mean of 3 assay
             replication ± S.D.
             [2] Each value represents the mean of 2 sample
             replications.
             (RL-1 = anti-rabbit lung upper band; RL-2 = anti-
             rabbit lung lower band; PL-1 = anti-pig liver upper
   band; PL-2 anti-pig liver lower band)

Figure 57
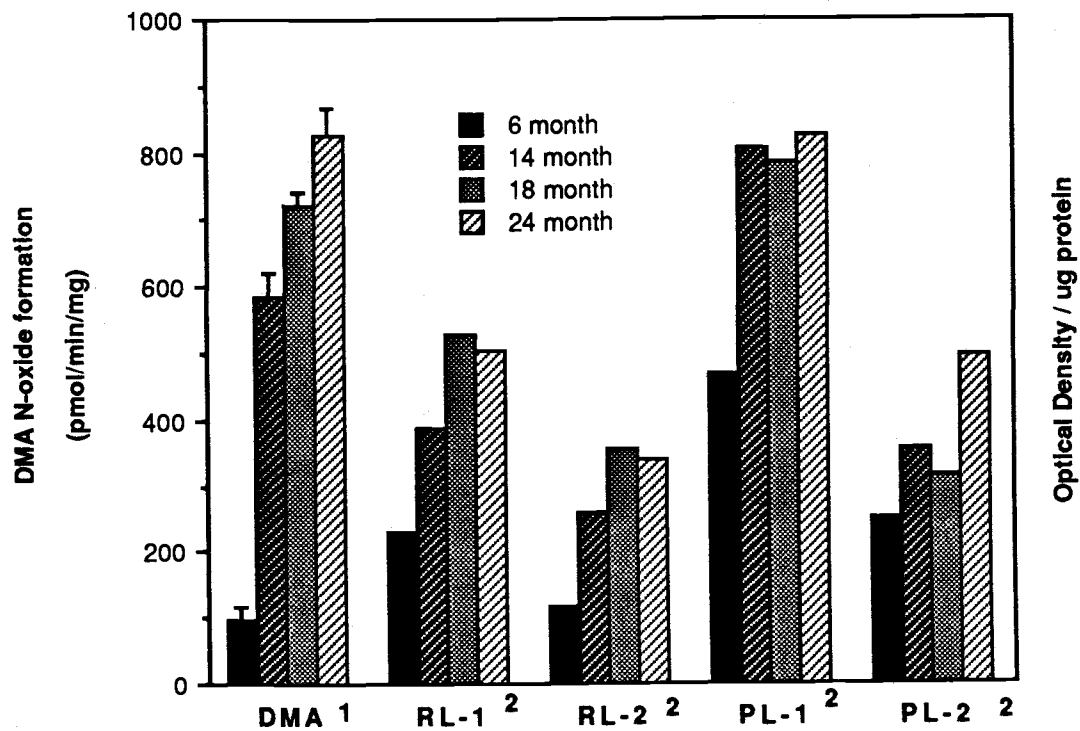
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CONCLUSIONS

Phase I biotransformation pathways have the potential to bioactivate as well as detoxify xenobiotics (Caldwell, 1980; Buhler and Williams, 1988). Determination of the ratio of bioactivation to detoxication is essential in understanding the toxicity of any xenobiotic. Therefore, assuming two animals in a given habitat are exposed to the same toxicant, the animal that is equipped with enzymes which lead to detoxication of the chemical may have an advantage over the animal that either does not possess enzymes of detoxication or has enzymes which bioactivate chemicals to more toxic species. Consequently, animals with detoxication enzymatic pathways would be better equipped to survive in a chemically dynamic environment such as the marine ecosystem.

Marine algae produce tremendous quantities of secondary metabolites providing an adaptive advantage over herbivorous organisms which feed upon them (Hay and Fenical, 1988). Several molluscan herbivores have been shown to consume specific species of algae containing alleochemicals and incorporate the chemicals unchanged or slightly biotransformed into the organism also serving an adaptive advantage as a chemical defense mechanism against predators of the herbivore (Stallard and Faulkner, 1974; Paul and Van Alstyne, 1988). Since other species of marine molluscan herbivores feed on toxic algae without incorporation of algal-derived chemicals into their tissues (Hay and Fenical,

1988), other herbivore species must be able to biotransform and/or excrete these chemicals.

Cryptochiton stelleri has been shown to feed on algae which contain a number of relatively toxic chemicals such as bromophenols (Yates, personal communication). In an attempt to better understand how this animal copes with the toxic chemicals that it encounters in its diet, the contribution of several xenobiotic biotransformation systems in this species was examined. Cytochrome(s) P-450 were shown to be present within digestive gland microsomes from the chiton. There appear to be at least two isozymes, one that is inducible by BNF and one that is reduced after treatment with BNF. Although enzyme content was shown to be relatively high, cytochrome P-450 activities, such as BPH and EROD were very low or not detected. The large content of cytochrome P-450 in a herbivore is consistent with the hypothesis of Nebert and Gonzalez (1987) who suggest that cytochrome P-450, already serving a function in steroid biosynthesis early in evolution, broadened its role to include xenobiotic metabolism when animals began to encounter plant metabolites. However, the minimal amount of cytochrome P-450 activity found suggests that the enzyme may have other roles besides biotransformation of the substrates that were examined.

Besides cytochrome P-450, other Phase I enzymes also appear to be involved in xenobiotic biotransformation in Cryptochiton. Although it did not have the levels of activity reported by Kurelec (1985), FMO activity in Cryptochiton was 10 times greater

than cytochrome P-450 activity. Britvic and Kurelec (1986) have stated that FMO is the primary enzyme of xenobiotic biotransformation in marine invertebrates. FMO may also play a role in osmoregulation by catalyzing the oxidation of TMA in marine invertebrates (Bakker et al., 1963). However, it is interesting that potential FMO substrates, such as nitrogen and sulfur-containing secondary metabolites are uncommon in macroalgae upon which Cryptochiton feeds (Faulkner, 1977). Perhaps evolution has broadened FMO substrate specificity in order to cope with nitrogen and sulfur-containing chemicals from anthropogenic or other natural sources in the same way that cytochrome P-450 has evolved to metabolize plant constituents.

The role of co-oxidation pathways in xenobiotic biotransformation in chitons is unclear. The high formation of quinone metabolites of BP suggests one electron oxidation reactions indicative of redox-cycling or co-oxidation processes. However, since NADPH-independent pathways were not observed in chiton digestive gland microsomes, it appears that co-oxidation via lipid peroxidation, lipoxygenase or peroxidase are not significant pathways in the chiton. Consequently, NADPH-cytochrome P-450 reductase may be responsible for the stimulation of redox-cycling and subsequent production of BP quinones. There has been strong evidence supporting a pathway of this nature in other marine molluscs (Livingstone et al., 1989).

Phase II conjugation pathways may also be an important way in which chitons cope with xenobiotics. The relatively high levels

of glutathione-S-transferase activities in the digestive glands of chiton are consistent with previous studies examining Phase II pathways in other molluscan herbivores (Lee, 1988). It has been suggested that Phase II pathways may be the more dominant detoxication enzymes rather than Phase I pathways (Lee, 1988). The high levels of glutathione-S-transferase activity and the electrophilic nature of the secondary metabolites encountered by the chiton when feeding upon certain species of macroalgae tend to support this view in molluscan herbivores.

In marine bivalves the relationship between Phase I and Phase II reactions is still unclear (Livingstone, 1985). In the oyster, Crassostrea gigas, the Phase II reaction of N-formylation of 2-AF was consistent with previous studies where N-formylation of toluidine was observed in vivo in the same species of oyster whereas in the mussel, Mytilus edulis, N-formylation was not observed (Knezovich and Crosby, 1985). Livingstone (1985) has reported that Phase I pathways such as cytochrome P-450 play an important role in xenobiotic biotransformation in Mytilus. However, in Crassostrea, cytochrome P-450 does not appear to play a major role in BP or 2-AF biotransformation. FMO and co-oxidation pathways may play a much more significant role than cytochrome P-450 dependent metabolism.

The natural histories of the oyster and chiton support the existence of differing contributions of Phase I pathways. For example, large amounts of dimethylsulfide have been reported to occur naturally in unicellular algae and bacteria which are common

dietary organisms for oysters (Stegeman, personal communication).

Dimethylsulfide has been shown to be a substrate for FMO (Ziegler,

1984). Therefore, since the oyster would naturally encounter

dietary chemicals that would better serve as FMO substrates rather

than cytochrome P-450, FMO activity would be expected to be

greater than cytochrome P-450 activity in the oyster. Likewise,

the occurrence of xenobiotic co-oxidation pathways through a lipid

peroxidase or lipoxygenase enzyme system is consistent with the

observation of immense stores of lipid within the oyster (Stegeman

and Teal, 1973). Clearly, more work examining enzymes associated

with lipid peroxidation and redox-cycling is needed to establish

the significance of these pathways in xenobiotic

biotransformation.

Since cytochrome P-450 isozymes in microsomes from chiton

digestive gland react with antibodies to two purified trout liver

cytochrome P-450 isozymes, there appears to be a degree of

structural homology between invertebrate and vertebrate FMO. It

is interesting, however, that only a faint reaction was observed

in microsomes from oyster digestive glands treated with the

antibodies raised against the trout protein. The class of

Amphineura (Polyplacophora) has been suggested to be one of the

more primitive classes of molluscs (Seed, 1983). As oysters

evolved from these primitive molluscs and changed from macroalgae

to dimethylsulfide-containing phytoplankton feeders, FMO may have

become more significant in its role of xenobiotic

biotransformation. In addition, since oysters would not normally

be exposed to possible dietary cytochrome P-450 substrates, the role of cytochrome P-450 in xenobiotic metabolism may have become secondary to FMO.

Although the function of cytochrome P-450 in invertebrates is unclear, cytochrome P-450 has been shown to play a central role in xenobiotic biotransformation in the trout (Kleinow et al., 1987). However, little is known regarding the occurrence and function of FMO in this organism. Since the trout has been used as an alternative vertebrate model to study carcinogenesis, enzymes other than cytochrome P-450 need to be examined and characterized. In addition, thioether pesticides have been shown to be good substrates for FMO in mammalian systems (Hajjar and Hodgson, 1980) and aquatic organisms, such as fish, can be exposed to these chemicals via agricultural runoff (Briggs and Lord, 1983). Consequently, the role of trout FMO in xenobiotic biotransformation was explored utilizing the thioether pesticide, aldicarb. FMO was shown to catalyze the bioactivation of aldicarb to the sulfoxide. Thus, it can be concluded that FMO is also an important xenobiotic biotransformation enzyme in the trout.

FMO has been shown to occur in human (Dannan and Guengerich, 1982) and unicellular organisms (Agosin and Ankley, 1987). Since FMO also occurs in fish and FMO activity was observed in chiton as well as oyster, FMO seems to be conserved throughout evolution. The reaction of trout FMO with antibodies raised to the mammalian protein suggests that FMO in the trout also possesses structural similarities to mammalian forms of the enzyme. This finding is

not uncommon since antibodies raised to purified rat cytochrome P-450 have been shown to react with purified trout cytochrome P-450 isozymes (Miranda et al., 1990). Although a reaction occurs between antibodies to the mammalian FMO and fish FMO, there was no reaction observed in digestive gland microsomes from oyster or chiton (data not shown). Consequently, the fish enzyme must be more structurally related to the mammalian form than are the proteins from invertebrates.

Besides possessing immunochemical similarities, fish and mammalian FMO share many common characteristics. Both appear in greatest levels within the livers of the organism (Ziegler, 1988). Each enzyme is optimal at pH 8.4-9.2 and requires NADPH and oxygen (Ziegler, 1988). DMA, TMA, methimazole, and aldicarb are also substrates for the mammalian enzyme (Ziegler, 1980). The sensitivity to temperature closely resembles characteristics of the purified pig liver enzyme (Ziegler, 1988). The development of the enzyme in each class of animals is directly correlated with age (Ziegler, 1980). As in mammals, there also appear to be some sexual differences in the trout; however, these are not as dramatic as that seen in mammals (Ziegler, 1980). Furthermore the enzyme in trout does not seem to be inducible which is consistent with previous reports in mammalian systems (Ziegler, 1980)

The differences observed between fish and mammal FMO include the large presence of enzyme activity and content in kidney microsomes from the trout. Although kidney has been shown to possess measurable amounts of FMO activity in mammals, the

relative levels are substantially less than that observed in the liver (Dannan and Guengerich, 1982). Another difference is the presence of multiple forms within single tissues. Although Tynes and Philpot (1987) have observed multiple forms in rabbit lung, generally a single form has been observed in mammalian tissues (Ziegler, 1988).

The endogenous function of FMO also appears to differ between mammals and fish. In fish, the endogenous substrate for FMO seems to be TMA. Oxidation of TMA to the N-oxide has been shown to be important in osmoregulation in elasmobranchs (Goldstein and Dewitt-Harley, 1973) and marine invertebrates (Bakker et al., 1963). However, although TMA oxidase is present in teleosts, its function remains unclear (Charest et al., 1988). The occurrence of TMA oxidase in invertebrates and fish supports evolutionary conservation of the enzyme between these species. In mammals, the enzyme has been suggested to function in the formation of disulfide linkages during protein synthesis (Ziegler, 1980). It is not known whether FMO is involved in protein synthesis in fish.

The goal of this dissertation was to better describe the enzymes involved in xenobiotic biotransformation within fish and aquatic invertebrates. Phase I activities have been characterized, compared and contrasted in two invertebrates representing two different classes in the phylum of Mollusca. Since biochemical indices from invertebrates have been used as biomonitors to assess pollution of various chemicals, the enzymes involved in chemical metabolism in these organisms must be better

characterized in order to understand the mechanisms responsible for the observed alterations. This research has laid a foundation upon which future studies can be performed to better understand how marine organisms cope with natural and/or anthropogenic xenobiotics. More research is needed to examine the mechanisms involved in redox cycling and oxygen activation. Furthermore, identification of endogenous and/or dietary substrates and their impact on xenobiotic biotransformation should provide a better understanding of these enzymes in marine molluscs.

The rainbow trout has been used as a model to study various aspects of mammalian toxicology. This dissertation has generally characterized FMO which, in addition to cytochrome P-450, plays an important role in xenobiotic biotransformation within this species. The activity of this enzyme has been characterized and compared to equivalent invertebrate and mammalian enzyme systems. Since FMO in fish has been shown to be very similar to the mammalian enzyme, these data support further utilization of the rainbow trout as a model to understand mammalian toxicology.

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