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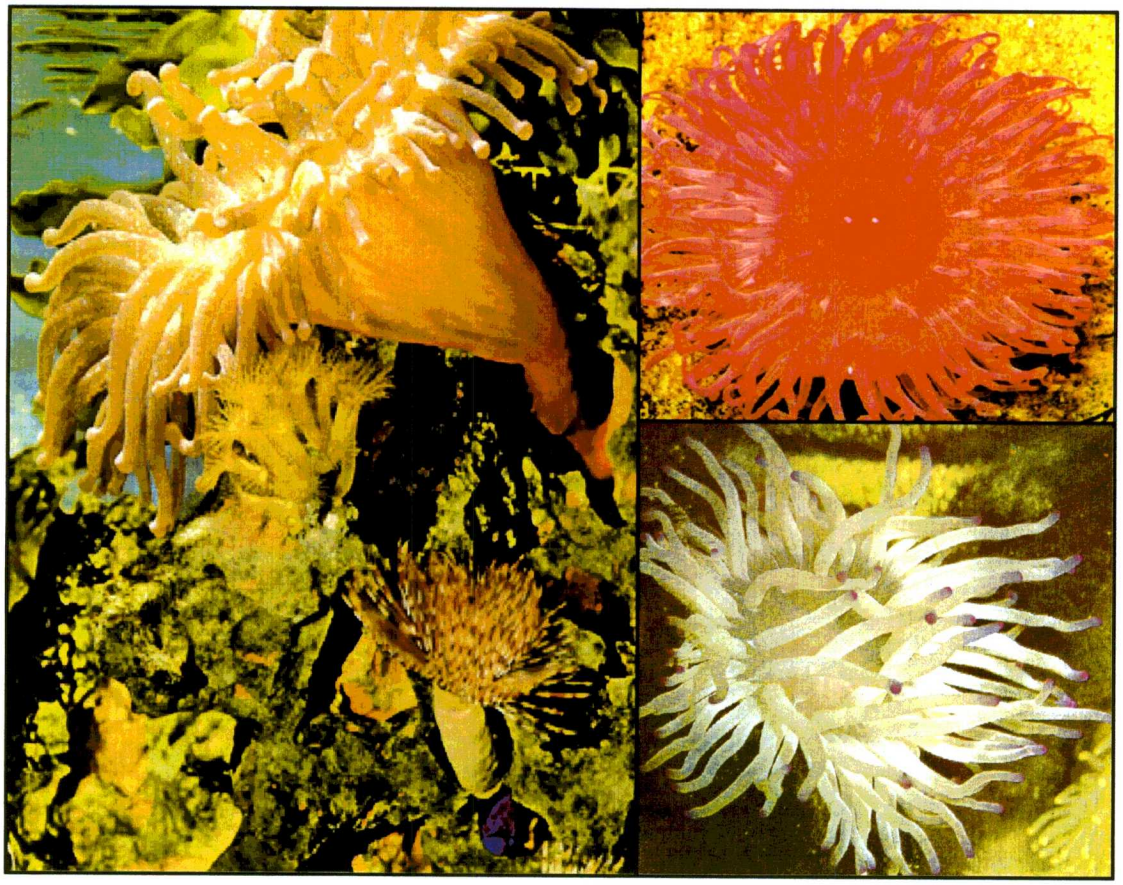
Coastal Marine Institute

Development and Characterization of Sea Anemones as Bioindicators of Offshore Resource Exploitation and Environmental Impact



MMS U.S. Department of the Interior
Minerals Management Service
Gulf of Mexico OCS Region

Cooperative Agreement
Coastal Marine Institute
Louisiana State University

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Development and Characterization of Sea Anemones as Bioindicators of Offshore Resource Exploitation and Environmental Impact

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ABSTRACT

Cnidarians are primarily marine organisms with wide habitat diversity. Detrimental effects of exposure to environmental pollutants, such as pesticides and petroleum products, are known for corals, the only cnidarians to be so studied in the field and laboratory. Proteins of 50-60 KDa crossreact with several antibodies raised against fish or mammalian cytochromes P450 (CYP) in microsomes of 5 species of sea anemones. The difference spectrum of dithionite (DTN)-reduced, CO-liganded sea anemone microsomes contained a major peak with λ_{max} of ~418 nm and a lower amplitude peak of 450 nm. NAD(P)H-cytochrome *c* reductase and NADH-ferricyanide (b_5) reductase activities were detected in sea anemone microsomes. Microsomal ethoxyresorufin O-deethyase (EROD) increased linearly for up to 90 min. NADPH was the preferred cofactor for EROD. NAD(P)H-EROD was higher in *Anthopleura elegantissima* than *A. xanthogrammica*. *Bunodosoma cavernata* NADPH-EROD activity was near the detection limit and NADH-EROD activity was not detected. The results are consistent with functional P450-dependent MFO in sea anemones, with characteristics similar to and unique from other marine invertebrates. CYP content and EROD activity were distributed relatively evenly among the following tissue regions of *A. xanthogrammica:* 1) the outer muscular wall, 2) inner fibrous material & muscle, 3) soft digestive sac, nerve network, gonads, & mesentery, and 4) tentacles including algal symbiont. *B. cavernata* microsomes catalyzed NAD(P)H- or *tert*-butyl hydroperoxide- (*t*-BHP) dependent benzo[a]pyrene (B[a]P) oxidation with formation of B[a]P phenols and diols; B[a]P-diones; B[a]P-tetrols; and B[a]P-diol epoxides. NADPH-dependent B[a]P metabolism was about 70% greater than with NADH. *t*-BHP-dependent B[a]P oxidation was 70-fold greater than NAD(P)H-dependent reactions and produced primarily diols. *B. cavernata* and *A. elegantissima* microsomes catalyze NAD(P)H-dependent epoxidation of aldrin to dieldrin. Rates of aldrin epoxidation were similar to other marine invertebrates. Sea anemone microsomes catalyzed 1-electron anaerobic reduction of 4-nitropyridine-N-oxide (4NPO) to nitroanion and hydronitroxyl radicals and aerobic reduction to superoxide radical. A new method is described for measuring Total Oxyradical Scavenging Capacity (TOSC) of tissues from marine invertebrates. TOSC was measured in digestive gland cytosol of mussel *Mytilus edulis*, pyloric caeca of starfish *Leptasterias epichlora* and basal stalk of *B. cavernata*. These invertebrates showed higher TOSC than rat liver, and in all species TOSC was higher in soluble than in protein matrix. The highest TOSC was found in the sea anemones, indicating a significant antioxidative potential from oxidative stress caused by organic chemicals and metals. This method is useful for assessing the biological resistance to oxidative stress and relationship to environmental pollutants. Exposure of sea anemones to a model PAH and cadmium induced a protein of ~70KDa, which was recognized in sea anemone cytosol by a monoclonal anti-heat shock protein 70 IgG. Induction of a protein of ~90 KDa by exposure to 25 and 250 μg/liter of $CdCl_2$ was detected by a monoclonal Ab raised to scup CYP1A1. This is the first report of induction by PAH or cadmium in the sea anemone. As a whole our results indicate sea anemone species to have significant potential as sentinel organisms with respect to various biological endpoints. Although biochemical responses are the most rapid to obtain and the most sensitive it is recognized that they are of limited ecological relevance. Thus, a suite of biomarkers would promote the most comprehensive evaluation of the impact of environmental upsets, whether natural or inadvertant.

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TABLE OF CONTENTS

TABLE OF CONTENTS
(continued)

LIST OF FIGURES

LIST OF FIGURES
(continued)

LIST OF FIGURES
(continued)

LIST OF TABLES

EXECUTIVE SUMMARY

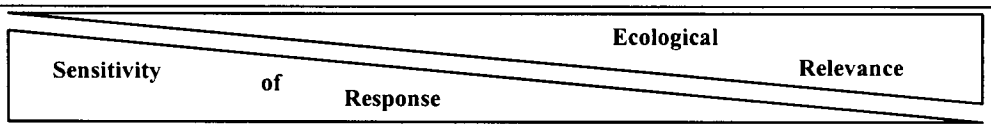
Cnidarians are primarily marine organisms that live in a wide diversity of habitats from tropical to arctic regions and from intertidal to pelagic zones. These organisms play important roles in marine environments, particularly in the coral reef communities. Cnidarians lack discrete organs having evolved only to the tissue level of development.

The detrimental effects of anthropogenic compounds, such as pesticides and petroleum products, have been clearly documented in corals, the only representative cnidarians to be so studied, through both field and laboratory studies (Burns and Knap, 1989). Several experiments have demonstrated rapid uptake and slow elimination rates of xenobiotics in cnidarians as compared to other marine invertebrates (Khan et al., 1972; Knap et al., 1982). The overall objective of this work is to characterize the biochemical reponsiveness of sea anemones for use in environmental monitoring programs. To this end we have examined specific known stress-related parameters that can provide environmental managers, researchers and regulatory agencies with: (1) sublethal, early warning detection of environmental stress; (2) a means of demonstrating, assigning and delineating zones of ecological impact from point- and nonpoint-source pollution; and (3) quantifiable biomarkers of integrative stress load and ecological health through characterization of biochemical responses with high potential for assessing the limits of natural and inadvertant stress.

Detection and evaluation of biological changes caused by exposures to various chemicals are of the utmost importance in evaluating the effects of contaminant exposure (McCarthy and Shugart 1990). Certain biological changes can indicate exposure to specific chemicals or adverse effects of exposures. A biological response can range from biochemical to behavioral responses, to changes in species composition effecting populations and ecosystems. Biomarkers have potential for a number of applications of benefit to regulatory agencies. The use of biomarkers might be envisaged on several levels: (1) establishing cause associated with an observed malady in organisms, populations or communities; (2) documenting exposure to organisms including the range and zones of delineation of that exposure; (3) documenting the effects (reversible or irreversible) caused by contamination to organisms, populations or communities.

Integrated Relationships of Biomarkers and Their Levels of Biological Organization

	Biochemical	Organismic	Population	Community
Response Parameter	MFO hsp SOD DNA	behavior growth reproduction survivorship	energy flow interspecific abundance	abundance diversity interactions
Response Time	h	h/days	wks/mos/yrs	mos/yrs



Biomarker research is rooted in the biochemistry, physiology, behavior, systematics and population dynamics of organisms. These integrated relationships are illustrated above in the context of the limits of understanding that biomarker research can provide. Thus, for any given set of response parameters under any level of organization (biochemical, organismic, population or community), there is an inverse heirarchy of sensitivity of response and ecological relevance of the response. Information on the ecological relevance of a response may be at the sacrifice of sensitivity and *visa versa*. There are two major advantages of using biochemical level changes as biomarkers of environmental contamination; 1) they are generally the first responses to contamination that can be detected and quantified, and 2) biochemical changes can reflect both exposure and effect, i.e. a change in the biochemistry of an organism upon exposure to a chemical *is an effect.*

The present summary is of studies to characterize the cytochrome P450-dependent microsomal mixed function oxidase (MFO) system of sea anemones to better understand the biochemistry of these systems for use as bioindicators of environmental pollution. The seven chapters presented in this report are thoroughly integrated. The interrelationships between the various chapters are shown schematically in the Conclusions section following Chapter 7. Therein, the concepts of the scheme are related to the specific chapters in which they are discussed. In this report results of experiments to determine the responsiveness of two classes of proteins to organic and metal exposures. The first class of proteins are those of the *cytochrome P450-dependent microsomal monooxygenases,* a complex membrane-associated, multienzyme family that is of critical importance in both the detoxification and activation of xenobiotic chemicals. A classical feature of cytochromes P450 in many organisms is their induction by xenobiotic chemicals, a process which involves *de novo* synthesis of mRNA and its translation to P450 protein. This ability to be induced by chemicals has prompted great interest in the characterization of cytochromes P450 as potential biomarkers for environmental pollutants. The other class is the stress proteins or heat shock proteins, (HSP), which are also induced by various chemical agents and other stress factors such as heat or oxidative stress. These proteins are believed to protect other proteins and nucleic acids from environmental damage. Their usefulness as biomarkers therefore are as determinants of the degree to which the organism responds defensively to environmental stress.

Chapter 1 presents immunochemical evidence for the existence of multiple forms of P450 in sea anemone species. The studies in chapter 1 were undertaken to characterize the presence of a cytochrome P450-dependent microsomal mixed function oxygenase system in five sea anemone species using immuno blotting (western blotting) techniques. Western blots indicated the presence of immunoreactive proteins to several cytochromes P450 (CYP) antibodies in the columnar or whole body microsomal fraction of *Anthapleura elegantissima, Aiptasia pallida, and Bunodosoma cavernata, Anthapleura xanthogrammica, Condylactis gigantea*. Columnar microsomes of *A. elegantissima* and *B. cavernata* contain proteins that cross-reacted with a monoclonal antibody derived from scup liver CYP 1A1 (~70KDa), a polyclonal anti-trout CYP 2K1 (~40KDa), and polyclonal anti-trout CYP 3A1 (~30KDa). *A. pallida* whole body microsomes contain various proteins that cross-reacted with scup CYP 1A1, trout CYP 2K1, and trout CYP 3A1 antibodies, which depending on the antibody used ranged from 72-47 KDa. Pretreatment by direct injection of 3-methylcholantrene (20mg/kg) into the body cavity of *A. pallida* did not induce protein recognized by scup anti-CYP 1A1. Anti-trout P450 LMC2 (CYP 2K1), a P450 isozyme involved in steroid and fatty acid metabolism, recognizes a very intense band in sea anemone microsomes (10 μg per well), at approximately 39 KDa in two of the species, i.e. *Anthapleura elegantissima* and *Bunodusoma cavernata. Aiptasia palluda* , contains a protein that is strongly recognized by anti-LMC2 at about 67 KDa with a fainter band also seen at about 50 KDa. Anti-LMC2 was also strongly immunoreactive with a protein in rat liver microsomes in the 50 KDa range, i.e. the expected range for cytochrome P450. These data suggest very unique proteins in sea anemones with epitope regions similar to those of higher vertebrates, e.g., fish and mammals.

In light of the presence of P450 antibody immunoreactive proteins in sea anemone microsomes, it was of interest to determine if these microsomes were catalytically active with respect to oxidation of PAH, as this would be indicative of the capacity of sea anemones to clear petroleum-derived PAH from the body. Furthermore, because PAH metabolism is inducible in certain organisms, such activity might be useful as a biomarker of PAH exposure. The benchmark PAH benzo[a]pyrene (B[a]P) was selected for these studies. The study results are presented in Chapter 2 where it is shown that incubations of *Bunodosoma cavernata* microsomes with B[a]P, a benchmark PAH used for such study, and either NAD(P)H or *tert*-butyl hydroperoxide (*t*-BHP) produced several oxidative metabolites including: 3-OH-B[a]P; 4,5-, 7,8-, and 9,10-B[a]P-diols; 1,6-, 3,6-, and 6,12-B[a]P-diones; B[a]P-tetrols; and B[a]P-diol epoxides. NADPH-dependent rates of B[a]P metabolism were approximately 70% greater than with NADH. The rate of *t*-BHP-dependent B[a]P oxidation was approximately 67-fold faster than NAD(P)H-dependent oxidation and produced primarily diols. The production of oxidative metabolites of B[a]P is consistent with a catalytically active cytochromes P450-mediated microsomal mixed-function oxygenase system in the sea anemone, albeit the metabolic profiles observed in this study are likely unique to *B. cavernata* suggesting that comparative studies of B[a]P metabolism by other species of sea anemones and other Cnidarians would be of value. Nevertheless, our data represents the first evidence of the capability of body burdon clearance of PAH by sea anemones.

Chapter 3 presents results of spectral analyses, NAD(P)H-oxidoreductase and ethoxyresorufin O-dealkylase (EROD) of sea anemone microsomal fractions. The predominant feature in the difference spectrum of dithionite (DTN)-reduced, CO-liganded sea anemone

microsomes was a peak with λ_{max} of 418 ± 1 nm, which slowly increased for ~20 min. A relatively lower amplitude 450 nm peak was attained within 5 min of CO addition and was stable for up to 90 min. The 450 nm peak did not decrease concomitant to the increase in the 418 nm peak suggesting that the latter is not denatured P450. A significantly larger 450 nm peak was obtained when DTN was added prior to CO. Chapter 4 presents more detailed studies of the spectral characteristics of sea anemone microsomes (see below). NADPH-dependent cytochrome c (P450) reductase of the sea anemones was 1.8-3.9 nmol/min/mg, which is at the lower end of the range observed in invertebrates. NADH-cytochrome c reductase was 9-25 nmol/min/mg protein, while the NADH-ferricyanide (b_5) reductase ranged from 73-232 nmol/min/mg. The latter was indicated to have marked potential as a biomarker of environmental exposure in molluscs; this potential is herein indicated for sea anemones. NADPH-dependent EROD of *Anthopleura elegantissima* was higher and less variable than of *A. xanthogrammic*. NADH-dependent EROD was lower than with NADPH in both species. NADPH-dependent EROD of *B. cavernata* was at the detection limit of the assay and NADH-dependent EROD was not detected. These results further substantiate the presence of functional P450-dependent MFO system in sea anemones however, variability between species is indicated. The components and catalytic activities associated with the MFO system are of sea anemones for the most part fall within the range of other marine invertebrates studied.

Chapter 4 is the first report of the tissue distribution of immunoreactive protein to P450 antibody, CO-binding chromophores and P450 content and EROD activity. Therein is discussed how interferences caused by the presence of other heme proteins could potentially hamper quantification of CYP content and how this might be avoided. Based on the spectral properties of the 500-700 nm region, additional heme-protein(s) appear to be present and variable between each tissue region. When KCN is added in place of CO a corresponding shift in the spectral properties contributed by heme was observed. The data with KCN indicates the presence of heme proteins in sea anemone microsomes with observable and alterable spectral properties upon binding of KCN ligand. Anti-CYP2K1-reactive protein and EROD activity were essentially evenly distributed throughout the different tissues of the sea anemone, a phenomenon suggested to be related to the lack of a circulatory system and hence the potential for indiscriminant distribution of pollutants within the body of sea anemones.

Chapter 5 contains the first direct evidence for the production of free radicals by sea anemone microsomal metabolism. The data is compared with that obtained from the marine mussel *Mytilus edulis,* a benchmark sentinel species of environmental contamination in marine ecosystems. Sea anemone and mussel microsomes can produce nitro anion radicals from aromatic nitro compounds, indicating the presence of single-electron transferring enzymes in these organisms. Sea anemone microsomes catalyzed very low level radical yield detectable only with NADH. The higher level of NADH- than NADPH-flavoprotein reductase activities found in various tissues of *M. edulis* and sea anemones are likely responsible for the generally higher rates of 1-electron with NADH than NADPH as evinced by the suppression of the mussel microsomal-mediated radical signal by $CuSO_4$ and PCMB, both inhibitors of flavoprotein reductases.

Chapter 6 extends the concept of free radical production and oxidative stress to marine invertebrates by chemical contaminants. A quantifiable index of antioxidant defenses in

such organisms had been lacking in the literature and thus, provided impetus for the development of a new, rapid and reliable approach for measuring the protective antioxidant potential of marine invertebrates. Initial experiments were designed to characterize pure antioxidants for use in budget analysis of antioxidant activity of biological tissues. The assay is simple and reliable for rapid quantification of relative biological resistance to oxyradical toxicity and indicates its usefulness as a biomarker of contaminant-mediated oxidative stress in sea anemones. Peroxyl radicals generated by thermal homolysis of 2,2'-azobis-amidinopropane (ABAP) cause the oxidation of α-keto-γ-methiolbutyric acid (KMBA) to ethylene, the formation of which is monitored by gas chromatographic analysis of head space from the reaction vessel. The partial inhibition of ethylene formation in the presence of antioxidants or biological tissues competing with KMBA for oxyradicals is the basis of the Total Oxyradical Scavenging Capacity Assay (TOSCA). Responses were linear over a wide range of sample concentrations and the TOSC values of classical soluble antioxidants showed the following relative order: Trolox > uric acid > ascorbic acid > GSH. The KMBA method appeared reliable also for biological tissues and the TOSC for rat liver cytosol was for 1 μg protein 0.40 ± 0.02. Soluble antioxidants accounted for 77% of the protective antioxidant potential in rat liver cytosol. Sea anemones had the highest oxyradical scavenging capacity of the marine organisms studied, which suggests that in a biomarker setting these organisms might be more resistant to oxidant challenge.

Chapter 7 presents results of analyses of cytosolic fractions to assess the presence of stress or heat shock proteins in these organisms as potential biomarkers of environmental contamination. Exposure of sea anemones to the model polynuclear aromatic hydrocarbon, 3-methycholanthrene (3MC) and cadmium caused an increase in a protein band of approximately 70KDa which was recognized in sea anemone cytosolic fractions by a monoclonal anti-heat shock protein 70 IgG. To the best of our knowledge this is the first demonstration of induction by a polycyclic aromatic hydrocarbon and cadmium in the sea anemone. A protein of about 90KDa that cross-reacted with a monoclonal anti-heat shock protein 90 IgM was also present in sea anemone cytosol, albeit this protein did not appear to be induced by exposure to either 3MC or cadmium. A striking result was the induction of an immunoreactive protein band of about 90 KDa by exposure to 25 and 250 μg/liter of $CdCl_2$ that was recognized by a monoclonal antibody raised against scup CYP1A1. Cadmium also induced a protein of about 96 KDa that was recognized by a trout polyclonal antibody to CYP3A1, a steroid-inducible CYP in mammals, which may aid understanding of pollutant impact on steroid hormone regulation and hence, reproductive processes of sea anemones and other marine invertebrates.

In conclusion sea anemone species show significant potential as sentinel organisms with respect to biological endpoints. Although biochemical responses are the most rapid to obtain and the most sensitive it is recognized that they are of limited ecological relevance. Thus, a suite of biomarkers would promote the most comprehensive evaluation of the impact of environmental upsets, whether natural or inadvertant. This is important if regulatory agencies are to be able to assess the impact of ecological perturbations on the higher levels of ecosystem development such as the population or community level. Conversely, ecosystem diversity has highly ecological relevance but suffers disadvantages in the time-frame to collect information and in the sensitivity of the response.

CHAPTER 1

IMMUNO-DETECTION OF PROTEINS BY CYTOCHROMES P450 ANTIBODIES IN FIVE SPECIES OF SEA ANEMONES

L.M. Heffernan and G.W. Winston

INTRODUCTION

Various marine invertebrate phyla, including Annelida, Arthropoda, Cnidaria, Echinodermata, Mollusca, and Porifera (Anderson, 1978; Moore *et al.*, 1980; Zahn *et al.*, 1982; Livingstone, 1991) catalyze enzymatic monooxygenation of organic xenobiotic chemicals and various endogenous substrates such as steroid hydroxylation, bile acid oxidation and fatty acid oxidation. Gassman & Kennedy (1992) found monooxygenase activity in coral *(Favia fragum)*, which is from the same phylum as the sea anemone.

In this chapter we have used immunochemical techniques, i.e., western blot analysis to probe the characteristics of the cytochromes P450-dependent monooxygenase system of sea anemones. Aside from understanding how these fragile organisms metabolize chemicals foreign to their makeup, thereby aiding our knowledge of the potential for anthropogenic deleterious impact on cnidarians, knowledge of cytochromes P450 in different organisms will engender greater understanding of the function, regulation, and evolution of this supergene family of proteins. Immunoblotting aids in the recognition and detection of cytochromes P450 by exploiting the epitope region, i.e., the immunoreactive recognition sites of proteins that have common properties and characterisitics of the antigenic proteins to which antibodies may be generated. It is noted that such recognition does not constitute positive assigment of a protein to a specific cytochrome P450; it is however evidence, to be weighed together with catalytic activities and gene sequence analysis in seeking positive identification. An immunoreactive protein band to a given antibody indicates proteins of some shared sequence homology to the protein from which the antibody was derived.

MATERIALS AND METHODS

Maintenance and care of sea anemones. *Anthapleura elegantissima* (AE) were collected from an intratidal zone in Alaska and California, *Aiptasia pallida* (AP) was collected from the Florida Gulf Coast, *Bunodosoma cavernata* (BC) was collected from rock jetties at Pass Fourchon on the Louisiana Gulf Coast, *Anthapleura xanthogrammica* (AX) was collected from the northern California coast and *Condylactis gigantea* (CG) from a site in the Florida Keys. The sea anemones were acclimated in artificial sea water (Instant Ocean) in aerated ten gallon tanks for three weeks prior to the preparation of microsomal fractions (post 105,000 x g pellet). Ambient salinities were adjusted to that of the collection sites of the various species. Sea anemones were fed fish three times a week kept on a daily light-dark cycle (light = 10 h).

Preparation of microsomes. Microsomes were prepared by homogenizing sea anemone columnar tissue with a Tekmar Polytron Tissuemizer in 50 mM Tris-HCl buffer, pH 7.4, containing 0.125 M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreotol (DTT), 1 mM phenylmethylsulfonyl flouride (PMSF) and 1 mg/ml soybean trypsin inhibitor. Homogenates were serially centrifuged for 10 min each at 500, 8,500, and 14,000 x g and the resulting pellets were discarded. The 14,000 x g supernatant was then centrifuged for 1 h at 105,000 x g to pellet the microsomal fraction. The microsomal pellet was washed once in 50 mM Tris-HCl/ 0.125 M sucrose, pH 7.4, centrifuged again at 105,000 x g for 1 h and the final pellet was resuspended and stored at -80°C in 0.25 mM sucrose/20% glycerol. Microsomal protein was estimated by the method of Lowry *et al.*, (1951).

Antibodies. Rabbit anti-trout P450 LMC2 (CYP2K1) and LMC5 (3A1) antibodies were prepared as described in Miranda *et al.*, (1989) and were a generous gift from Dr. Donald.R. Buhler, Department of Agricultural Chemistry, Oregon State University, Corvallis. Mouse anti-scup CYP1A1 monoclonal antibody was prepared as described in Park *et al.*, (1986) and provided by Dr. J.J. Stegeman, Department of Biology, Woods Hole Oceanographic Institution, Woods Hole, MA. Anti-CYP 2C7, 2C11 and 2C13 were generous gifts of Dr. S.M. Bandiera, Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, BC. Anti-CYP2E1 was also a gift from Dr. A.I. Cederbaum, Department of Biochemistry, Mount Sinai School of Medicine, New York, NY.

Immunoblotting. Western blot analysis was performed as described by Towbin *et al.*, (1979). Essentially, microsomes were reduced with β-mercaptoethanol, boiled for 4 min and protein bands separated by SDS/polyacrylamide (10%) gel electrophoresis. Gels were transferred to nitrocellulose filters in a Transblot apparatus (BioRad) for 50 min at 100V, filters were blocked with 5% powdered milk in 20mM Tris-buffered saline (TBS), pH 7.5, overnight, incubated with primary cytochrome P450 antibody in TBS with 1% gelatin for 2 h, incubated with an appropriate immunoglobulin biotin conjugate for 2 h, and again incubated with extraavidin alkaline phosphatase (Sigma) for 1 h, all at 37°C. Filters were washed 4 times with TBS between each step and rinsed with water after the final step. Blots were developed in 100mM $NaHCO_3$/1mM $MgCl_2$, pH 9.8, containing 3.0% NBT (v/v 70% N,N-dimethyl formamide (DMF) and 1.5% BCIP (v/v 100% DMF) and then soaked for 10 min in H_2O. Primary antibodies used were: monoclonal mouse anti-scup cytochrome P450 (CYP) 1A1; polyclonal rabbit anti-trout LMC2 (CYP2K1); and, polyclonal rabbit anti-trout LMC5 (CYP3A1). Each of these antibodies cross-reacted with microsomal proteins in the five sea anemones studied.

RESULTS

Immunoblotting with anti-cytochromes P450 antibodies. Proteins in sea anemone microsomal fractions that contain common epitopes to various cytochromes P450 isoforms were probed with the following primary antibodies: 1) mouse anti-*scup* CYP1A monoclonal IgG (this antibody is monospecific for a single CYP1A protein bans of about 50 Kda that is inducible by Ah-locus agonists in scup and other fish microsomes and similar proteins in birds, reptiles and mammals), 2) goat anti-rabbit CYP1A IgG (Oxford Biomedical, Oxford, MI; this antibody

recognizes CYP1A1 and 1A2 in rabbit), 3) goat anti-rabbit CYP2B1&2 (Oxford Biomedical; note: Oxford sells this product as anti-CYP2B1&2 albeit, this rabbit antibody is generally referred to as anti-CYP2B4&5. This antibody cross reacts with CYP2B1 and 2 in rat as well as CYP2B4 and 5 in rabbit), 4) rabbit anti-*scup* P450B (putative CYP2B; cross reacts with a protein in scup that has high sequence homology with mammalian CYP2B), 5) rabbit anti-rat CYP2E1 (this antibody cross reacts with CYP2E1/2 in rabbit liver), 6) rabbit anti-trout CYP LMC2 polyclonal IgG (2K1; this antibody cross reacts with a single 59 kDa protein in trout liver microsomes and a 51 Kda protein in rat liver microsomes) (Buhler *et al.*, 1994; Miranda *et al.*, 1989), rabbit anti-rat CYP2C7 (this antibody cross reacts with several CYP2C enzymes in rabbit liver), 7) rabbit anti-rat CYP2C11 (this antibody cross reacts with several CYP2C enzymes in rabbit liver), 8) rabbit anti-trout LMC5 (this antibody, recently designated CYP3A27, crossreacts with with CYP3A enzymes in trout liver) (Buhler *et al.*, 1994), and 9) a rabbit anti-trout LMC5 (CYP3A1) polyclonal IgG; this antibody recognizes a protein of about 50 KDa in rabbit liver microsomes. Each of these cross-reacted with various microsomal proteins isolated from the five sea anemones, albeit with varying degrees of intensity, and indicates the presence of proteins with epitope region(s) common to several families of cytochromes P450 in sea anemone microsomes. Interestingly, the antibody probes cross-reacted with various proteins of molecular weight that differed markedly from those of proteins recognized by these antibodies in microsomal fractions of fish and mammalian species.

Studies with rabbit anti-trout LMC2 (CYP 2K1) IgG. Of particular interest was the very strong recognition of a major protein band observed with the trout LMC2 (CYP2K1) antibody. At 10 μg protein per lane, this antibody detected a single intense band at approximately 40 KDa in *B. cavernata*, *A. elegantissima*, *A xanthogrammica* and *C. gigantea* (Fig. 1.1).

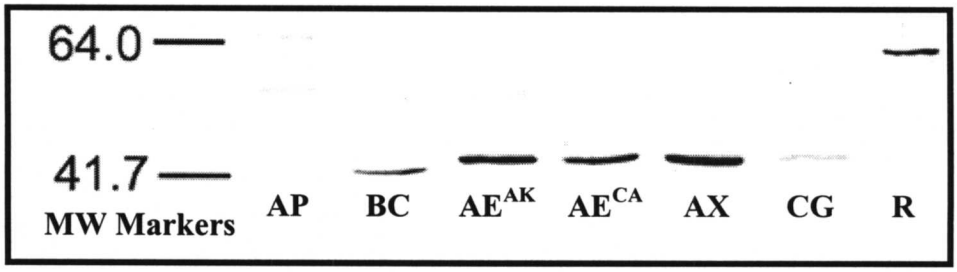


Figure 1.1. Immunoblots of Columnar Microsomes from Five Sea Anemone Species Probed with Anti-CYP2K1.

In *A. pallida*, there was a conspicuous absence of a 40 KDa band. A protein band at approximately 70 KDa was recognized in all of the sea anemones except *C. gigantea* and some lighter bands at about 68, 60 and 66 KDa were detected in *A. pallida* that were not present in the other species (Data not shown). In view of the very low intensity of these bands as compared to the 40 Kda band the specificity of the higher molecular weight proteins by this antibody is not conclusive. A protein was recognized by the CYP2K1 antibody in rat liver microsomes of about 55 KDa, which is in the molecular weight range expected for a mammalian cytochrome P450.

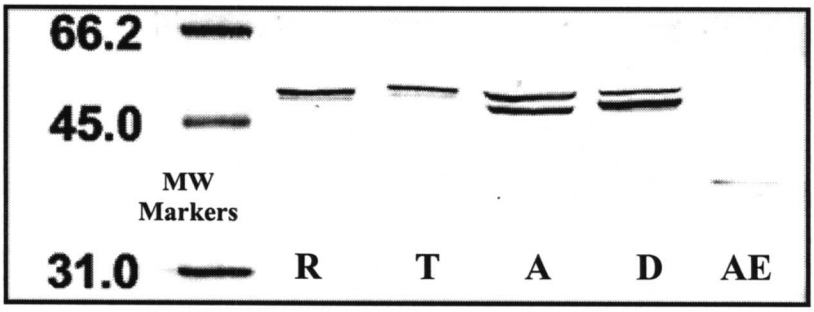


Figure 1.2. Comparison of Immunoblots of Microsomes from Five Representative Phyla Probed with Anti-CYP2K1.

Figure 1.2 underscores the uniqueness of the protein recognized by anti-CYP2K1 in sea anemone microsomes (shown therein for *A. elegantissima)* as compared to that of various vertebrate species. Avian (duck) and reptilian (alligator) microsomes appear to contain two proteins that are strongly recognized by anti CYP2K1, whereas pisces (trout) and mammalian (rat) liver microsomes contain one major immunoreactive protein band to this antibody. This species variability in immunoreactivity of protein bands with antibodies against known CYP proteins indicates the need to reevaluate data presented in previous studies reporting a lack of induction of MFO characteristics. Proper study of biomarker research must begin with the elucidation of first principles.

Studies with anti-trout LMC5 (CYP3A) IgG. The trout LMC5 (CYP3A) antibody cross-reacted with a protein band of variable intensity and of approximately 31 KDa molecular weight in both *A. elegantissima* and *B. cavernata* (Fig. 1.2).

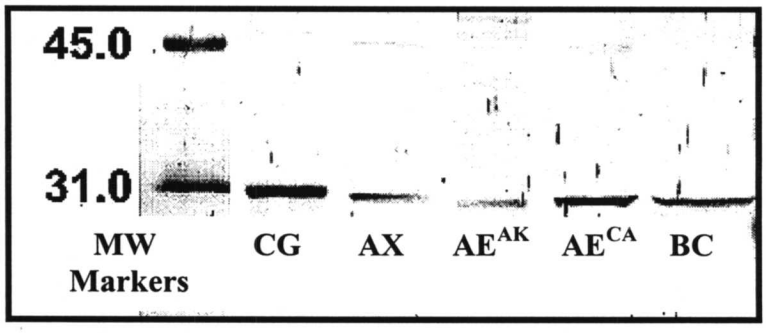


Figure 1.3. Immunoblots of Columnar Microsomes from Three Sea Anemone Species Probed with Anti-CYP3A.

This variability at constant microsomal protein concentration is indicative of variable expression of the immunoreactive protein in the different sea anemone species. Interestingly, *A. elegantissima* from Alaska appear to express a lower content of anti-CYP3A immunoreactive

protein than its California counterpart. In order to resolve the protein at the intensity level shown it was necessary to load 30 μg protein per lane on the gel. In the same manner as with anti-CYP2K1, a band of relatively weaker intensity and about 70 KDa was also seen with anti-CYP3A in *A. elegantissima* and *B. cavernata* and a 54 KDa band was recognized in *A. elegantissima* (not shown). In *A. pallida,* anti-CYP3A revealed several faint bands with molecular weights corresponding to 72, 68, 60 and 56 KDa at 30 μg microsomal protein per lane; these bands appeared often with only secondary antibody and might reflect non-specific recognition of the protein (data not shown). The lower intensity of the bands recognized by the anti-trout CYP3A IgG as compared to that recognized by anti-CYP2K1 despite the loading of three times the amount of protein per lane as was used for anti-CYP2K1 probing illustrates the relative enrichment of protein with common epitopes common to anti-CYP2K1 in sea anemone microsomal fractions. To the best of our knowledge no proteins have been reported in marine invertebrate species that cross react with the magnitude of intensity shown by the sea anemones described herein. Nevertheless, the 31 KDa protein that cross reacts with the anti CYP3A antibody has not been reported in a marine invertebrate, thus its identity herein suggests that further study of this protein is of heuristic value.

Studies with anti-scup CYP1A1 IgG. To further investigate the characteristics of proteins that immunoreact with antibodies to cytochromes P450 in sea anemones, cross-reactivity with monoclonal scup anti-CYP1A1 antibody was examined (Fig. 1.4).

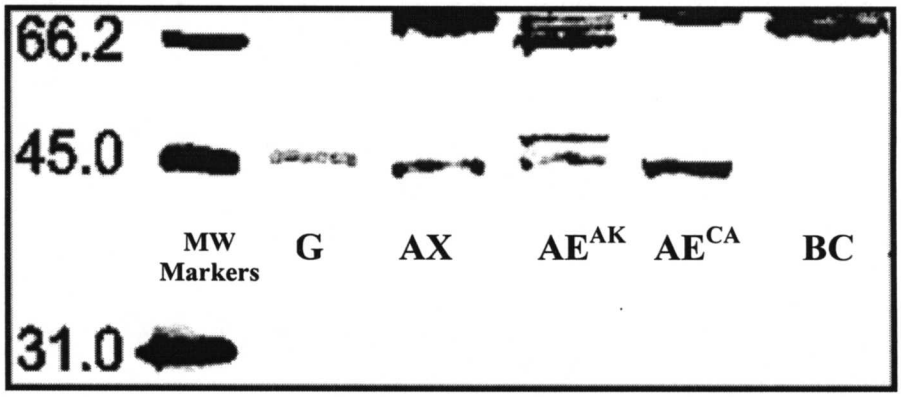


Figure 1.4. Immunoblots of Columnar Microsomes from Three Sea Anemone Species Probed with Anti-CYP1A1.

The major band recognized by scup anti CYP1A1 was approximately 69 KDa and appeared in each of the sea anemones except in *Condylactus* (lane 2). Relatively lower intensity bands of about 48-50 KDa were also recognized by anti-CYP1A1 in all species with the exception of *B. cavernata* (lane 6). Anti-scup CYP1A1 recognized a darker band of about 65 KDa and lighter bands of about 62, 56 and 47 KDa in *A. pallida*. These higher molecular weight bands however were seen in some instances in western blots with the secondary antibody alone indicating nonspecificity of these bands (data not shown). As noted above for anti-CYP3A, interesting differences were observed in the immunoblots of microsomes from A. elegantissima from Alaska and California. The latter has an immunoblot pattern essential identical to the other sea anemone species depicted in Figure 1.3. The blot of the former, reveals a doublet in the 48-50 KDa range that is not seen in the other sea anemones depicted. Also, the higher molecular

weight band of the California sea anemone appears to be about 2 KDa lower in molecular weight than its Alaskan counterpart. CYP1A is an inducible, nonconstitutive protein of vertebrates.

Studies with anti-scup CYP2E1 IgG. Of significant interest is the immunoblot obtained when anti-CYP2E1 IgG was used to probe microsomes (20 - 40 μg per lane) from *A. Elegantissima,* (Fig. 1.4) as this antibody is derived from a rabbit liver CYP2E1. Availability of this antibody precluded study of each of the sea anemone species with which we have worked. The fact that cross-reactivity of proteins in the molecular weight range of 50 KDa, i.e., the range corresponding to typical mammalian cytochromes P450, was observed suggests that this protein is highly conserved. The antibody also appears to recognize a doublet, indicating the possible existance of 2 isoforms of CYP2E1-like protein. Higher molecular weight bands of varying intensity appear in the blots at about 90 Kda; such high molecular weight protein is not recognized by this antibody in vertebrates (Schlenk *et al.*, 1993), which underscores again the uniqueness of the invertebrate CYP-like immunoreactive proteins.

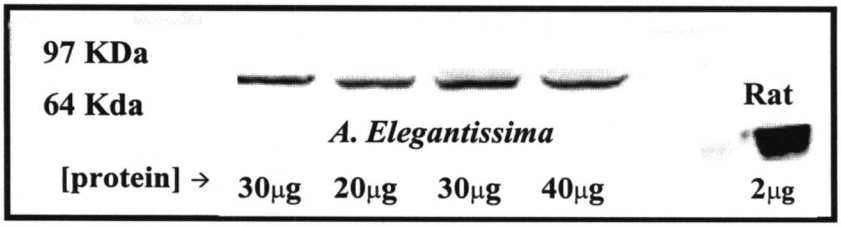


Figure 1.5. Immunoblots of Columnar Microsomes from Three Sea Anemone Species Probed with Anti-CYP2E1.

Studies with anti-scup CYP2C7 IgG. Cytochrome P450 2C7 is associated with drug metabolism in mammals and in some cases steroid hydroxylation. In the sea anemone anti-CYP2C7 recognized only a few very faint bands of high molecular weight (> 80 KDa) at high protein concentration on western blots (data not shown). Again, the appearance of these bands in western blots with secondary antibody alone suggests nonspecificity.

Studies with anti-scup CYP2C11 IgG. Anti-CYP2C11 cross-reacted moderately with a single protein band of approximately 50 KDa in microsomal fractions from the sea anemones *B. cavernata* and *A. elegantissima* (Fig. 1.5).

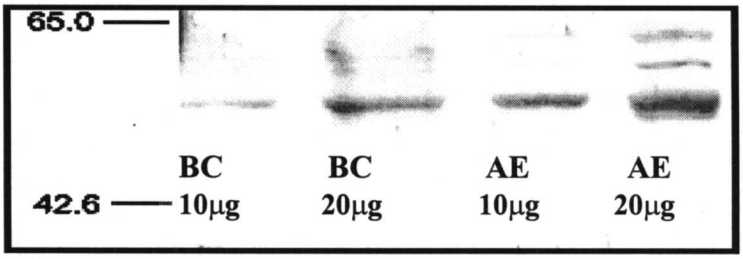


Figure 1.6. Immunoblots of Columnar Microsomes from Three Sea Anemone Species Probed with Anti-CYP2C11.

The immunoblot was of 10 and 20 mg of microsomal protein per lane and the concentration dependence of the immunoreactive band is indicated in the blots. It is also noted that at equivalent concentrations of blotted protein that the immunoreactive band is more highly concentrated in *A. elegantissima.*

DISCUSSION

Western blot analyses of sea anemone microsomes probed with the various antibodies support the presence of cytochromes P450 or proteins with P450-like epitopes in these organisms. The proteins which were recognized in sea anemones have unique molecular weights relative to cytochromes P450 reported for vertebrates and invertebrates in the literature. For instance, the CYP2K1 (LMC2) and 3A1 (LMC5) antibodies cross-react with proteins of about 50 and 59 KDa, respectively in trout liver microsomes (Miranda *et al.*, 1989) and at 50 and 53 KDa in channel catfish liver microsomes (Schlenk *et al.*, 1993). A 53 KDa protein has been detected by the LMC5 antibody in human liver microsomes (Miranda *et al.*, 1990).

We have detected anti-CYP2E1-reactive proteins in sea anemone microsomes. CYP2E1/2 is associated in mammals with the metabolism of ethanol, isoniazid, acetone, carbon tetrachloride, trichloroethylene, alachlor, glycol ethers and various other xenobitic agents. Moreover, it is inducible by each of these chemicals; thus, these proteins have potential for biomarker research and mechanistic understanding of the effects of exposures to such chemicals. It is also a protein associated with oxidative stress caused by chemicals and may be associated with the greater capacity to absorb oxygen radicals that we have discussed in Chapter 6 of this document.

Some sea anemones contain unicellular algal symbionts, which are commonly called zooxanthellae or zoochlorellae. These symbionts contribute fixed carbon and oxygen, recycle limited nutrients (i.e. nitrogen), and provide energy to the sea anemone (Shick, 1990). It is possible that they might also contribute P450, but the following information suggests that is not the case here. *A. elegantissima* typically contains algal symbionts while *B. cavernata* does not, however, both species show similar patterns of immunoreactivity with antibodies to cytochromes P450. Further, when microsomes of aposymbiotic *A. pallida* were compared to those of symbiotic *A. pallida* for CYP2K and CYP3A antibody recognition, the cross-reactivity patterns with these antibodies were essentially identical (data not shown).

Finally, the monoclonal anti scup CYP1A1 antibody cross-reacts with a band approximately 54 KDa in scup liver microsomes (Stegeman, 1989). In contrast to the LMC2 and LMC5 antibodies, in sea anemones, the scup antibody recognizes a protein of approximately 54 KDa. However, this 54 KDa protein, which is the major band in rat and fish microsomes, appears as a minor band in the sea anemones that we have probed. In invertebrates, the nature of anti-CYP1A-reactive protein has not been characterized, albeit, if it is a nonconstitutive, inducible protein in the sea anemones, the variability between species and the same species from different geographical locations may reflect different stress factors and natural dietary factors in the regulation and expression of these immunoreactive proteins to CYP1A antibody.

In conclusion, these results suggest the presence in sea anemones of cytochromes P450 homologues, or proteins with similar antigenic determinants or epitope regions as cytochromes P450 of some fish and mammals. Interestingly, these proteins do not appear to be within the 50-60 KDa range of most reported cytochromes P450. The presence of these proteins and of an active microsomal reductase suggests the presence of a microsomal mixed function oxygenase (MFO) system in the sea anemones, and thus, a capability to oxidize and depurate organic xenobiotic chemicals that might bioaccumulate in these marine organisms. Nevertheless, we cannot rigorously exclude the possibility of antibody recognition of epitopes not related to cytochromes P450. Further characterization with respect to MFO activities, possible induction by various chemicals and sequence analysis will be required before these immunoreactive proteins can be assigned as expressed products of the cytochromes P450 supergene family.

CHAPTER 2

MICROSOMAL METABOLISM OF BENZO[A]PYRENE *IN VITRO* BY THE INTERTIDAL SEA ANEMONE, *BUNODOSOMA CAVERNATA*

G.W. Winston, M. Mayeaux and L. M. Heffernan

INTRODUCTION

Cytochrome P450 is the terminal oxidase of the microsomal mixed-function oxygenase (MFO) system and contains a redox-active Fe-porphyrin (heme) center in the active site. An NAD(P)H-dependent cytochrome P450 reductase and molecular oxygen are additionally required for aryl hydrocarbon hydroxylase (AHH) activity.

Utilizing the classical polycyclic aromatic hydrocarbon, benzo[a]pyrene (B[a]P) as a substrate, earlier investigations have used the stereoselective formation of specific B[a]P oxidative metabolites by the MFO system to help elucidate the specific cytochrome P450 isoforms involved (Stegeman, 1981; Payne *et al.*, 1987). Benzo[a]pyrene is a procarcinogenic and promutagenic by-product of incomplete combustion of petroleum products. Enzymatic activation by the MFO system to highly electrophilic oxidative intermediates such as B[a]P-diolepoxides or B[a]P quinones are thought to be important in the formation of DNA adducts and therefore, have long been associated with the initiation phase of chemical carcinogenesis (Sims *et al.*, 1974; Buening *et al.*, 1978).

Patterns and proportions of metabolic by-products formed from B[a]P differ between species and among phyla. Many cytochromes P450 enzymes are unique to one species or phylogenetic group; consequently, specific activities, such as regiospecific oxidations, i.e. hydroxylation at selective positions of the PAH ring structure, on any substrate, vary accordingly. Although the majority of the work regarding MFO activities in marine species has been with fishes (Stegeman and Binder, 1979; Stegeman and Chevion, 1980; James and Bend, 1980), mollusks (Anderson, 1978; Livingstone,1988), and crustaceans (Payne, 1976; Singer and Lee, 1977; James, 1984), there have been a few reports of a functioning MFO system in other marine invertebrates including annelids (Fries and Lee, 1984), coelenterates (Khan *et al.*, 1972; Gassman and Kennedy, 1992), and echinoderms (den Besten *et al.*, 1990; 1991; 1994).

Several studies have provided evidence for the presence of an active cytochromes P450-dependent MFO system in coelenterates. Gassman and Kennedy (1992) found MFO activity in the scleractinian coral, *Favia fragrum*. They found differences in benzo[a]pyrene hydroxylase activities between corals collected from different sites in Florida. Corals from contaminated sites exhibited significantly higher B[a]P hydroxylase activity than corals collected from less-polluted sites. Nevertheless, the inducibility of cytochromes P450 antibodies is still a subject of intense investigation in the marine biochemistry field We have reported, based on western blot analysis, that microsomal fractions of several species of sea anemone contained proteins which cross-reacted with antibodies raised to both fish and mammalian P450 isoforms (Heffernan *et al.*,

1996). Interestingly, proteins with similar epitopes to trout cytochromes P450 were observed but these proteins displayed unique molecular weights (see Chapter 1).

In this chapter, we demonstrate the presence of a functioning MFO system in the intertidal sea anemone, *Bunodosoma cavernata*, by identifying several oxidative metabolites of benzo[a]pyrene *in vitro*. Furthermore, we show the first evidence of organic hydroperoxide-dependent MFO in cnidarians suggesting that these marine organisms have the potential to oxidize xenobiotics via a peroxidatic pathway involving cytochromes P450. The data in this chapter will serve to supplement previous studies that provide evidence of a functioning cytochromes P450-dependent MFO system in cnidarians.

MATERIALS AND METHODS

Collection of animals. Intertidal sea anemones, *Bunodosoma cavernata*, were collected from rock jetties at the mouth of Belle Pass, south of Port Fourchon, Lafourche Parish, Louisiana. This pass is heavily-utilized by support vessels for commercial oil exploration and by commercial and recreational fishing vessels. The sea anemones were transported to the campus of Louisiana State University at Baton Rouge and held in a recirculating-water culture system. Salinity in the culture system was maintained at 25 ppt with artificial sea salts (Fritz Chemical Co., Dallas, Texas). Temperature in the culture system was maintained between 26 and 28°C. Photoperiod was maintained on a 14:10 light:dark cycle with actinic fluorescent lighting. The animals were fed freshly-hatched *Artemia* sp. nauplii (Bay Brand, San Francisco, California). Ammonia and nitrite levels were monitored every other day by a colorimetric assay (Hach Chemical Co., Loveland, Colorado) and remained below detectable limits. The animals appeared healthy and feeding activity was evident when freshly-hatched artemia were introduced into the aquaria.

Microsomal preparation and determination of protein and P450 content. The columnar region of the sea anemones were homogenized with a Tekmar Ultra-Turrax® in four volumes of the following buffer system: 100 mM K_2PO_4, pH 7.4; 0.125 M sucrose; 1 mM dithiothreitol; 1 mg/ml aprotinin; 0.7 mg/ml leupeptin; 0.7 mg/ml pepstatin; 1.0 mM phenylmethylsulphonyl fluoride; 1 mg/ml soybean trypsin inhibitor; 0.1 mM phenanthroline; and 1 mM EDTA. The homogenate and resultant supernatants were serially centrifuged at: 500 x g for 5 min; 8,500 x g for 15 min; 14,000 x g for 15 min; and 100,000 x g for 1 h. The final microsomal pellet was resuspended in 100 mM K_2PO_4, pH 7.4 and 0.25 M sucrose in a Dounce homogenizer, aliquoted into 1 ml eppendorf tubes, and stored at -80° C. Microsomal protein content was determined by the fluorescamine method (Böhlen *et al*, 1973) as modified for microplate analysis by Lorenzen and Kennedy (1993). Total microsomal cytochromes P450 content was determined by difference spectroscopy of dithionite-reduced, CO-bound microsomes as described by Omura and Sato (1964) using an extinction coefficient of 91 $mM^{-1}cm^{-1}$.

Identification of B[a]P metabolites. Sea anemone microsomes (0.7 mg microsomal protein) were incubated in 2 ml reaction mixtures containing: 100 mM K_2PO_4, pH 7.4; 10 mM $MgCl_2$; 0.1 mM EDTA; and 80 mM ^{3}H- B[a]P (40 mCi/mmol) at 30° C for time periods of 30 min to 4 h in the presence of 2 mM NAD(P)H or for 15 min in the presence of 0.5 mM *tert*-butyl

hydroperoxide (*t*-BHP). The reactions were terminated with 1 ml of ice-cold acetone. B[a]P metabolites were extracted with ethyl acetate, dried with Na_2SO_4, and evaporated to dryness under nitrogen. Metabolite residue was redissolved in 100 ml methanol.

Benzo[a]pyrene and its metabolites were separated by high performance liquid chromatography (HPLC) with a C18 reverse-phase column and a linear solvent gradient of the following profile: 60-100% acetonitrile:water in 40 min at 1 ml/min flow rate and monitored at 254 nm. Retention times of these metabolites were compared to authentic standards obtained from the National Cancer Institute's Chemical Repository. Half-ml fractions were taken of the HPLC eluants, placed into a scintillation cocktail, and disintegrations per min counted on a LKB model scintillation counter equipped with automatic quench control. Quantification of metabolites was achieved by conversion of radioactivity units to molar equivalents.

RESULTS

CO-binding spectrum and P450 content. Carbon monoxide-binding spectra obtained with dithionite-reduced microsomes from *B. cavernata* result in an absorbance maximum at 418 nm with a smaller peak at 450 nm (Data not shown). Despite rigorous use of protease inhibitors and protective anti-oxidants in our homogenization buffer, we have obtained this spectrum consistently with *B. cavernata,* microsomes. Microsomal cytochromes P450 content from *B. cavernata* was determined to be 40-52 pmol cytochrome P450/mg microsomal protein.

Oxidation of benzo[a]pyrene. Initially, we studied the effect of microsomal protein concentration on B[a]P oxidation. The range of concentrations studied was from 50-350 mg microsomal protein/ml. Tetrols, 9,10-B[a]P-diol, 1-6-B[a]P-quinone, and 3-OH-B[a]P metabolites increased linearly from 50 to approximately 350 mg of microsomal protein/ml.

Based on the protein concentration curves, a microsomal protein concentration of 350 mg/ml was used in time course studies. After 30 min and 1 h of incubation, only trace levels of 3-OH-B[a]P could be detected by HPLC analysis; more polar metabolites that coelute with B[a]P 7,8-diol-9,10-epoxides and the corresponding tetrol were not detected. A 2 h incubation period was sufficient to reproducibily detect phenolic metabolites, however, quinones, epoxides, and tetrols were barely detectable. An incubation period of 4-8 h resulted in reproducible levels of all of the metabolites described herein, albeit the yields plateaued by 4 h. All subsequent studies employed a 4 h incubation period.

Incubation times for the *in vitro* NAD(P)H-dependent reactions in the present study are relatively long because of the low microsomal cytochromes P450 content and the apparently low B[a]P hydroxylase activity in *B. cavernata* columnar microsomes. Lower AHH activity has also been shown to correlate with lower levels and activities of microsomal electron transport components in other aquatic species (Stegeman, 1981). James (1989) suggested that more-highly evolved invertebrates such as molluscs and crustaceans possess a greater ability to metabolize PAHs than more-primitive invertebrates such as the cnidarians.

Three classes of products are formed by cytochrome P450-mediated oxidative metabolism of B[a]P. Thes include phenols [1-OH-, 3-OH-, 6-OH- (a transient intermediate), 7-OH-, and 9-OH-B[a]P]; dihydrodiols (4,5-, 7,8-, and 9,10-B[a]P-diol); and quinones (1,6-, 3,6-, and 6,12-B[a]P-dione). The predominate phenol produced is 3-OH-B[a]P (Casarett and Doull, 1986; Cavelieri *et al.*, 1991). T-Butyl hydroperoxide-, NADH-, and NADPH-dependent B[a]P metabolite HPLC profiles can be seen in Figures 2.1-2.3, respectively.

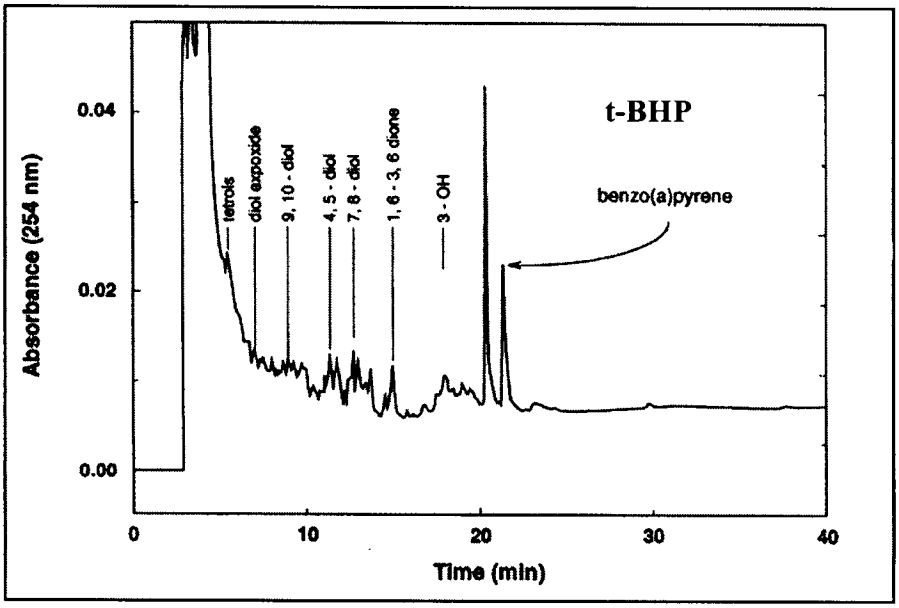


Figure 2.1. HPLC Chromatogram of B[a]P Metabolites Formed During Incubation of *B. cavernata* Microsomes In Vitro: t-Butyl Hydroperoxide-dependent.

Percentages of total NAD(P)H- and *t*-BHP-dependent metabolites of B[a]P catalyzed by microsomes from *B. cavernata* as determined from elution times of authentic standards and quantified by radiometry are given in Table 2.1.

Phenols: The most abundant metabolite produced during NADPH- and NADH-dependent B[a]P metabolism by *B. cavernata* microsomal fractions was 3-OH-B[a]P (60.7% and 66.3% of total B[a]P metabolites, respectively; Table 2.1). The predominance of this metabolite is consistent with what has been reported in several other aquatic invertebrates (Lee, 1986; Singer *et al.*, 1980; den Besten *et al.*, 1990; 1991, 1994; Winston *et al.*, 1991) and marine vertebrates (Stegeman, 1981; Klotz *et al.*, 1983; Stegeman and James, 1985; Michel *et al.*, 1991).

Diols: Microsomes from *B. cavernata* incubated with NAD(P)H and *t*-BHP also produced B[a]P-diols including 4,5-, 7,8-, and 9,10-B[a]P-diols (Table 2.1). B[a]P-diols are formed enzymatically from B[a]P epoxides which can be hydrolyzed by epoxide hydrolase to the corresponding dihydrodiol (Cavelieri *et al.*, 1991). The presence of dihydrodiols additionally

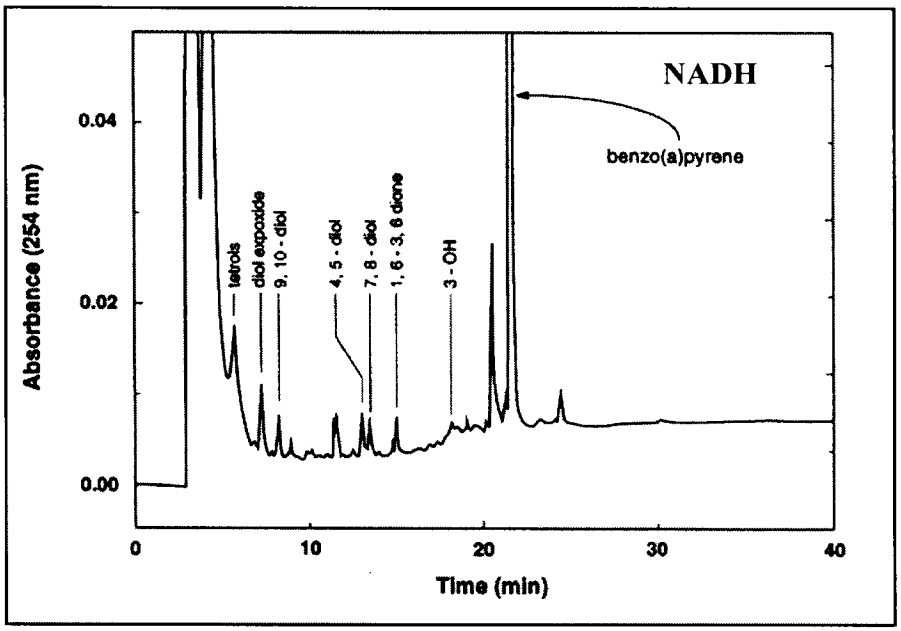


Figure 2.2. HPLC Chromatogram of B[a]P Metabolites Formed During Incubation of *B. cavernata* Microsomes In Vitro: NADH-dependent.

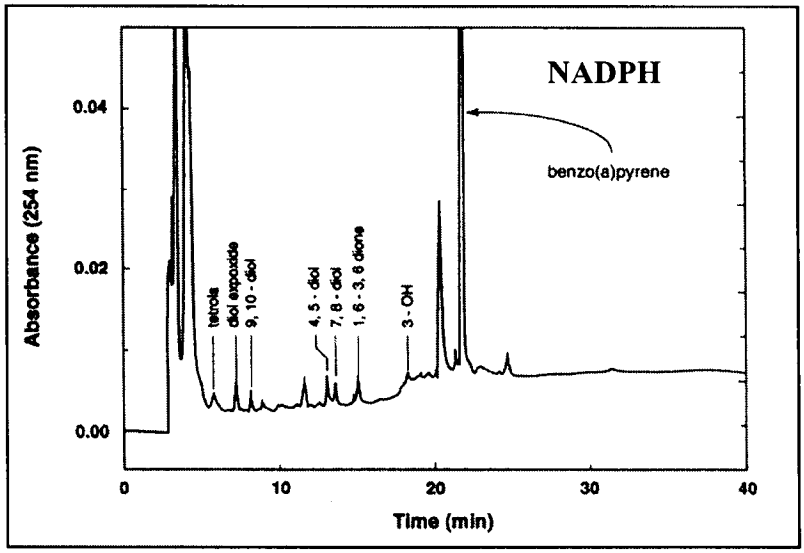


Figure 2.3. HPLC Chromatogram of B[a]P Metabolites Formed During Incubation of *B. cavernata* Microsomes In Vitro: NADPH-dependent.

infers epoxide hydrolase activity in *B. cavernata* and will be discussed below. The relative proportions of B[a]P-diol derivatives also vary relative to the specific P450 isoform and electron transport system involved. NADPH-dependent diol production in *B. cavernata* produced primarily the 9,10-B[a]P-diol (9.0%) with lesser amounts of the 7,8-B[a]P- diol (6.2%) and 4,5-

B[a]P-diol (5.8%). NADH produced the same pattern of diol formation but resulted in smaller percentages of total diols: 9,10-B[a]P-diol (6.3%); 7,8-B[a]P-diol (5.9%); and 4,5-B[a]P-diol (5.5%). *t*-BHP produced increases in 9,10-B[a]P-diol (22.6%) and 7,8-B[a]P-diol (20.1%) with the relative percentage of the 4,5-B[a]P-diol remaining relatively unchanged (7.8%).

Quinones: Microsomes from *B. cavernata* incubated with NAD(P)H and t-BHP also produced quinones including 1,6-, 3,6-, and 6,12-B[a]P-diones (Table 2.1). The relative proportion of quinone production to other metabolites produced by P450-mediated oxidation of B[a]P has been used as an index of one-electron oxidation vs oxygenation mechanisms catalyzed by cytochromes P450 (Cavelieri *et al*, 1991).

Tetrols and diol epoxides: *B. cavernata* microsomes supported the formation of B[a]P-tetrols and diol epoxides (Table 2.1). The presence of these two metabolites infers an active epoxide hydrolase in *B. cavernata* microsomal preparations. Epoxide hydrolase readily catalyzes hydration of B[a]P-epoxides to their corresponding dihydrodiols, with the exception of 7,8-dihydrodiol-9,10-epoxide, which is relatively resistant to this hydrolase.

Organic hydroperoxide-dependent metabolism of B[a]P: From the relative size of the remaining B[a]P peak in Figures 7-9 and from the radiometric profiles of HPLC eluates it was determined that the specific activity of the *t*- BHP-dependent reaction (15 min incubation time) was approximately 67-fold greater than NAD(P)H-dependent reactions (data not shown). This is consistent with other organic hydroperoxide-dependent P450-catalyzed oxidations of B[a]P by aquatic organisms (Jewell and Winston, 1989; Livingstone, 1991).

Table 2.1.
Benzo[a]pyrene Metabolite Profile of *B. cavernata* Microsomes

	NADPH	NADH	*t*-BHP
Tetrols	8.1	3.9	6.4
Diol epoxides	3.1	3.1	5.8
9,10-diol	9.0	6.3	22.6
4,5- diol	5.8	5.5	7.8
7,8- diol	6.2	5.9	20.1
B[a]P quinones	7.1	9.0	17.4
3-OH	60.7	66.3	20.1

Values are expressed as percentages of total B[a]P metabolites.

DISCUSSION

As discussed in Chapter 1, relatively little is known about MFO activity in cnidarians. *In vivo* B[a]P monooxygenase activity was not detected in jellyfish (Lee, 1981) and Payne (1977) did not detect *in vitro* B[a]P hydroxylase activity in the cold-water sea anemone, *Metridium* sp.

Conversely, Khan *et al.* (1972) measured *in vivo* aldrin epoxidation by *Hydra littoralis*. Gassman and Kennedy (1992) found that total cytochromes P450 levels were not different in corals taken from polluted sites relative to reference sites. However, B[a]P hydroxylase activity was higher from coral located in areas exposed to PAHs indicating inducible isoforms. Results from these and other studies (Heffernan *et al.*, 1996) and data presented in the present study provide considerable evidence for the presence of an active cytochromes P450-dependent MFO system in *B. cavernata.* The rate-determining step in many P450-catalyzed oxidations is the redox cycling of the associated NAD(P)H-dependent cytochrome P450 reductase. Catalytic rates of NAD(P)H-dependent P450 metabolism depend upon rates of electron transfer via the associated reductase from the corresponding reduced pyridine nucleotide. The organic hydroperoxide-dependent P450-mediated oxidation of B[a]P by-passes the reductase component by providing both a source of oxygen and reducing equivalents (Rahimtula and O'Brien, 1974). By by-passing the reductase component of the MFO system, one can estimate the catalytic rates of P450 under conditions in which the active P450 peroxygenase complex is not limiting. The fact that the peroxidatic activity of P450 with respect to B[a]P hydroxylation indicates the reductase to be rate-limiting in the activities reported herein.

The P450-dependent MFO system requires reducing equivalents for oxygen activation. Electrons are transferred to the terminal P450 oxygenase via a closely-allied, membrane-bound flavoprotein, NADPH-cytochrome P450 reductase. NADH has also been shown to support cytochromes P450 activity in aquatic species via cytochrome b_5 (Stegeman, 1981) but at rates generally 10% of NADPH-dependent metabolism. Conversely, Jewell and Winston (1989) found increased B[a]P hydroxylase activities with NADH as the electron donor in *P. clarkii* hepatopancreas microsomes while *P. clarkii* green gland microsomes utilized NADPH more effectively. The preference for NADH- as compared to NADPH-dependent B[a]P metabolism metabolism by *P. clarkii* hepatopancreas microsomes was also noted for *A. rubens* pyloric caeca microsomes (den Besten *et al.*, 1994). Livingstone *et al.* (1988) found that NAD(P)H is not required for B[a]P metabolism in *M. edulis*. In the present study, NADPH-dependent rates of B[a]P metabolism were approximately 70% greater than with NADH in *B. cavernata* (data not shown).

In contrast to organic hydroperoxide-dependent activities in most other organisms studied in which quinones were the predominant metabolites, *B. cavernata* P450-mediated peroxygenase activity produced primarily diols (50.5%) (Table 2.1). The metabolite profile produced by *t*-BHP may be related to the concentration or type of hydroperoxide used in this experiment much like that seen by Cavelieri *et al.* (1987) or may be related to differences seen in the unique molecular weight proteins (P450 isoforms) which cross-reacted with antibodies to fish P450 isoforms detected by Western blot analysis in the study by Heffernan *et al.* (1996). It has been suggested that the organic hydroperoxide-mediated preference for phenol and quinone production by the sea anemone and other marine invertebrates is a consequence of habitation in hypoxic intertidal zones, which may have been a driving force in the evolution of the MFO systems of these animals (den Besten *et al.*, 1994).

Oxidation of B[a]P at the C-6 position of the aromatic ring structure yields the 6-OH B[a]P metabolite. Cavelieri *et al* . (1991). presented evidence indicating that both 6-OH- and 3-OH-B[a]P are formed catalytically by the P450-mediated one-electron oxidation of B[a]P.

Aromatic hydrocarbon epoxides, such as B[a]P epoxides which are formed enzymatically by P450, are relatively unstable and generally rearrange to the corresponding phenol (Casarett and Doull, 1986). The 6-OH derivative is not detected in chromatograms; it is thought to be rapidly auto-oxidized to the corresponding 1,6- 3,6-, or 6,12-quinone. Phenolic B[a]P derivatives are typical metabolic by-products of P450-mediated, NAD(P)H-dependent B[a]P oxidative metabolism and has been found to be the primary metabolite among most phyletic lines (Table 2.2); however, other oxidative metabolites are produced by NAD(P)H- and organic hydroperoxide-dependent pathways.

Table 2.2.
Relative Percentages of NAD(P)H- and Organic Hydroperoxide-dependent Metabolites of B[a]P from Different Marine Species

	B[a]P metabolite[a]			
	Tetrols, Diol epoxides	Phenols	Diols	Quinones
B. cavernata[b]				
NADPH	11.2	60.7	21.0	7.1
NADH	7.0	66.3	17.7	9.0
t-BHP	12.2	20.1	50.5	17.4
A. rubens[c]				
NADPH	---	36	25	19
NADH	---	18	10	61
CHP	---	17	21	39
M. edulis[d]				
NADPH	10.9	17.1	28.3	47.3
NADH	0.0	12.3	26.7	61.0
CHP	10.6	17.1	35.2	36.6
C. meanas[d]				
NADPH	8.0	42.8	22.4	27.0
NADH	3.3	74.9	18.0	3.9
CHP	20.4	9.0	26.7	43.9
P. clarkii[e] (male)				
NADPH	---	84.0	16.0	---
NADH	---	85.0	15.0	---
CHP	trace	trace	1.0	99.0
P. argus[e]				
NADPH	---	30.0	38.7	31.3
NADH	---	---	---	---
CHP	---	4.7	33.6	72.5

[a] percentage of total B[a]P metabolites; [b] present study; [c] den Besten *et al.*, 1994;
[d] Lemaire *et al.*, 1991; [e] Winston *et al.*, 1991.

We report the presence of diol metabolites from B[a]P catalyzed by sea anemone microsomes. Table 2.2 is a summary of the relative percentages of NAD(P)H- and organic hydroperoxide-dependent metabolites of B[a]P from different marine species. Jewell and Winston (1989) reported differences in diol formation between NAD(P)H- and cumene hydroperoxide (CHP)-dependent MFO activity in the freshwater crayfish *P. clarkii*. Although all three diols were detected in incubations with *B. cavernata* microsomes, only the 7,8-B[a]P-diol (17 and 15% of total B[a]P metabolites in the NADPH- and NADH-dependent reactions, respectively) was detected with *P. clarkii* hepatopancreas microsomes. Only 1% of the total B[a]P metabolites formed in the CHP-dependent reaction with *P. clarkii* microsomes was the 7,8-B[a]P-diol. Differences in NADPH-dependent diol formation was observed in other decapod crustaceans including the spiny lobster, *Panulirus argus* (James and Little, 1980), the American lobster, *Homarus americanus* (James, 1989) sea star, *A. rubens* (den Besten *et al.*, 1993; 1994), and the blue crab, *Callinectes sapidus* (Lee, 1986) (Table 2.2).

Cavelieri *et al.* (1987) found differences in relative proportions of metabolites produced when incubated with different concentrations of CHP and rat hepatic microsomes. Low CHP concentrations ($\leq$ 0.15 mM) produced primarily 3-OH-B[a]P with lesser amounts of quinones and 9,10-B[a]P-diol. At CHP concentrations > 0.15 mM, quinone production increased to > 85% of total B[a]P metabolites. They suggest that CHP may interfere with the binding site involved in 3-OH production favoring quinone production and concluded that the different P450 isozyme binding sites determined the specific patterns of B[a]P metabolites formed. Livingstone *et al.* (1988) and Michel *et al.* (1991) found that the primary metabolite of NAD(P)H-dependent B[a]P metabolism in *M. edulis* were quinones. NADH-dependent microsomal B[a]P metabolism of sea star, *A. rubens*, like that of *M. galloprovincalis* (Michel *et al.*, 1991, 1993a and b) favored production of quinones (61% of total B[a]P metabolites) (den Besten *et al.*, 1993; 1994). Additionally, Livingstone detected oxidative metabolites of B[a]P without the addition of reduced pyridine nucleotides indicating a NAD(P)H-independent MFO pathway in *M. edulis*.

The presence of B[a]P 7,8,9,10-tetrol is indicative of the catalytic formation of B[a]P 7,8-dihydrodiol-9,10-epoxide, which is believed to be responsible, at least in part, for the initiation phase of carcinogenesis (Sims *et al.*, 1974; Buening *et al.*, 1978). However, this view of initiation has been challenged by Li *et al.*, (1995) who have presented cogent evidence that depurinating adducts of B[a]P oxidation and resulting formation of unrepaired apurinic sites in DNA are significantly more important in initiating carcinogenesis than stable adducts such as those formed from B[a]P 7,8-dihydrodiol-9,10-epoxide.

In conclusion, incubation of benzo[a]pyrene with microsomes from the intertidal sea anemone, *Bunodosoma cavernata*, results in the production of oxidative metabolites of benzo[a]pyrene consistent with an active P450-mediated MFO system. These results are consistent with other studies describing cytochromes P450-catalyzed oxidations of B[a]P by marine invertebrates. Although the metabolic profiles observed in this study are likely unique to this species, these data strongly indicate a functioning cytochromes P450-dependent MFO system in *B. cavernata*. Studies are now underway in our laboratory to assess the metabolism of B[a]P by sea anemones *in vivo*.

CHAPTER 3

SPECTRAL PROPERTIES AND CATALYTIC FUNCTION OF THE COLUMNAR MICROSOMAL FRACTION OF THE SEA ANEMONE

L.M. Heffernan and G.W. Winston

INTRODUCTION

Cnidarians are primarily marine organisms that live in a wide diversity of habitats from tropical to arctic regions and from intertidal to pelagic zones. The phylum contains over 10,000 living species and is composed of three classes: Hydrozoa (e.g., hydra); Scyphozoa (e.g., jellyfish); and, Anthozoa (e.g., sea anemone, sea fan, coral, sea pansy). These organisms play important roles in marine environments, particularly in the coral reef communities. Cnidarians are among the most primitive eumetazoa, and lack discrete organs because they have only evolved to the tissue level of development. Further, they exhibit radial symmetry, a simple nerve network, and a thin body wall that surrounds a sac-like digestive cavity. The medusa (free-swimming) and the polyp (sedentary) are the two basic body designs of cnidarians. Finally, cnidarians are unique in containing within their tentacles stinging cells called nematocysts, which are used to immobilize prey (Campbell, 1993).

A general characteristic of the organisms belonging to the phylum cnidaria as compared to other marine invertebrates is their rapid uptake and slow elimination rates of organic xenobiotic chemicals (Khan et al., 1972; Knap *et al.*, 1982, Lee, 1975; Solbakken *et al.*, 1982, 1983, 1984, 1985). The detrimental effects of anthropogenic compounds, such as pesticides and petroleum products, have been clearly documented in corals, which belong to the same phylum as do the sea anemones, through both field and laboratory studies (Burns and Knap, 1989; Jackson *et al.*, 1989; Loya and Rinkevich, 1980; Rinkevich and Loya, 1977; Rinkevich and Loya; 1979; Scott, 1990). However, very little is known about their ability to metabolize these compounds to more hydrophilic substances that can be eliminated.

The presence of a cytochrome P450-dependent mixed function oxidase (MFO) system has been clearly demonstrated in several other marine invertebrate phyla (Lvingstone, 1991); however, its presence has been far less rigorously documented in the cnidarian (Gassman and Kennedy, 1992, Heffernan, *et al.*, 1996; Khan *et al.*, 1972; Winston *et al.*, 1998). In fact, most early cnidarian studies reported an absence of cytochrome P450 monooxygenase activity (Lee, 1975, Lee, 1981, Payne, 1977). For instance, Lee (1975) did not detect *in vivo* metabolism of naphthalene, benzo[a]pyrene (B[a]P), or 3-methylcholanthrene (3MC) in either the jellyfish (genus unknown) or in the ctenophore, *Pleurobrachia pileus*. In the sea anemone, *Metridium* sp., Payne (1977) reported an absence of *in vitro* metabolism of B[a]P (fluorometric assay), and Lee (1981) found no *in vivo* metabolism of unidentified polyaromatic hydrocarbons (PAH). More recently, Firman (1995) failed to detect the presence of cytochrome P450 in the reef-building coral, *Montastraea faveolata*, by either the carbon monoxide (CO)-binding spectra or by

metabolism of P450 substrates (i.e., chlordane [*in vivo*] and ethoxyresorufin [*in vitro*]). However, early molluscan studies also failed to discover an MFO system (Lee et al., 1972; Payne, 1977; Vandermeuleun and Penrose, 1978), even though molluscs are known to contain an active cytochrome P450-dependent microsomal mixed function oxygenase system (Livingstone, 1991).

In contrast to the afore mentioned studies of cnidarians, which reported the inability to detect either cytochrome P450-dependent activity or its characteristic spectral properties, several recent studies indicated the presence of a functional cytochrome P450-dependent MFO system in two cnidarian classes. The freshwater hydrozoan, *Hydra littoralis*, can perform *in vivo* epoxidation of aldrin, presumably by MFO activity (Khan *et al.*, 1972). In the anthozoa, *in vitro* metabolism of B[a]P was detected in the sea anemone, *Bunodosoma cavernata* (Winston *et al.*, 1998), and in the scleractinian coral, *Favia fragum* (Khan *et al.*, 1972). Based on the CO-liganded, sodium dithionite (DTN)-reduced microsomes, *F. fragum*, contained a cytochrome P450 content between 60 to 350 pmol per mg microsomal protein (Gassman and Kennedy, 1972). Finally, based on western blot analysis several isoforms of cytochrome P450 exist in the sea anemone (Heffernan *et al.*, 1996). In each of these species, a microsomal protein between 50-60 KDa molecular weight (MW), the typical MW ascribed to vertebrate cytochromes P450, was only weakly recognized at 30 mg protein in western blots probed with monoclonal mouse anti-scup CYP1A1, polyclonal rabbit anti-rat CYP2E1, polyspecific rabbit anti-rat CYP2C11, polyclonal rabbit anti-trout CYP2K1, and polyclonal rabbit anti-trout CYP 3A1. In addition to the 50-60 KDa proteins, there were proteins with uncharacteristic molecular weights that were strongly recognized by anti-trout CYP2K and CYP3A in several species of sea anemone.

In view of the equivocal nature of cytochrome P450 in cnidarians; the purpose of this study was to further investigate its presence in the sea anemone. Specifically, we examined the spectral characteristics of the CO-liganded, DTN-reduced microsomes and the presence of MFO components (i.e., reductases) and ethoxyresorufin *O*-dealkylase (EROD) activity in several species of sea anemone.

MATERIALS AND METHODS

Animals. *Bunodosoma cavernata* were collected from the Gulf of Mexico at Fourchon, Louisiana. *B. cavernata* is sometimes confused with the morphologically-similar species, *Bunodactis texaensis*, but the blue stripe and reddish coloration as opposed to gray streaks on the tentacles of the organisms collected for this study is consistent with *B. cavernata* (Escartin and Porte, 1996). *Anthopleura elegantissima* and *A. xanthogrammica* were obtained from Pacific Bio-Marine Laboratories and North Coast Invertebrate Collectors in California. All sea anemones were maintained in a recirculating system with Instant Ocean^{tm} sea water. *A. elegantissima* and *A. xanthogrammica* were kept at approximately 34°/oo salinity and 12°C, while *B. cavernata* were kept at approximately 25°/oo salinity and 23°C.

Microsomal Preparation. Each sea anemone microsomal preparation consisted of approximately 50 animals for the smaller *A. elegantissima*, 30 animals for *B. cavernata*, and only 1-2 animals for the larger *A. xanthogrammica*. The tentacles were discarded and the columnar

region of the sea anemone was immediately submerged in homogenization buffer. Microsomes were prepared by homogenizing the tissue in four volumes homogenization buffer with a hand-cranked plastic meat grinder, a Tekmar Tissumizer^{tm}, and then a Potter-Elvehjem tissue homogenizer. The homogenization buffer was 100 mM potassium phosphate pH 7.6, containing 125 mM sucrose, 1 mg/mL aprotinin, 1 mM ethylene diaminetetraacetic acid (EDTA), 1 mg/mL leupeptin, 1 mg/mL pepstatin, 0.1 mM phenanthroline, 1 mM *para*-methylsulfonylfluoride (PMSF), 1 mg/mL soybean trypsin inhibitor, and 1 mM dithiothreitol (DTT). Homogenates were serially centrifuged for 20 min at 8,500 x g and 15 min at 14,000 x g. The resulting pellets were discarded. The 14,000 x g supernatant was then centrifuged for 1 h at 105,000 x g to pellet the microsomal fraction. The microsomal pellet was washed once in the homogenization buffer and centrifuged again at 105,000 x g for 1 h. Microsomes were resuspended and stored at -80°C in 100 mM potassium phosphate pH 7.6, containing 250 mM sucrose at a protein concentration of approximately 10 mg/mL. Approximately 10 mg of microsomal protein was produced for every 4 g of tissue. Protein concentrations were determined by the 96-well microplate reader fluorescamine assay as described by Lorenzen and Kennedy (1993), with two exceptions. The fluorescamine was prepared in HPLC-grade dioxane. The assay was conducted in 250 mM sodium phosphate buffer, pH 8.5.

Spectral Properties. Spectral analysis was performed with a Perkin Elmer lambda 5 dual beam spectrophotometer. Sea anemone microsomal cytochrome P450 spectra were examined in terms of both the carbon monoxide (CO)-difference spectrum of sodium dithionite (DTN)-reduced samples and the DTN-difference spectrum of CO-liganded samples (Omura and Sato, 1964). These spectra can be performed by either (1) adding the DTN and background-correcting prior to the addition of the CO, or by (2) adding the CO and background-correcting prior to the addition of the DTN. The CO-difference and DTN-difference spectra were examined both ways. Prior to the addition of either DTN or CO, microsomal samples (200mL) were solubilized with 100 mL of a 10 mM potassium phosphate, pH 7.4 (containing 1 mM EDTA, 1 mM DTT, 150 mM NaCl, and 1.5% Triton N-101) and diluted immediately with 900 mL 100 mM potassium phosphate (pH 7.4) to a final protein concentration of approximately 2 mg/mL. This sample was split between the reference and test cuvettes for analysis. After the addition of the CO and DTN, the solubilized microsomes were scanned immediately and then at 5 min intervals while maintaining the sample at room temperature. The extinction coefficient used for determining the cytochrome P450 content (450-490 nm) was 91 $cm^{-1}mM^{-1}$. The actual extinction coefficient for the sea anemone may be different.

Reductase Activities. The NAD(P)H cytochrome *c* (P450) reductase activities were measured as an increase in cytochrome *c* absorbance over time at 550 nm (Lake, 1987). The NAD(P)H-cytochrome *c* reductase reactions contained a final concentration of 0.048 mM cytochrome *c*, 1 mM potassium cyanide, approximately 200 mg microsomal protein, and either 0.1 mM NADH or 1 mM NADPH in 100 mM potassium phosphate, pH 7.7. The NADPH-dependent reactions were performed in tandem cuvettes in order to correct for the rate of non-enzymatic activity and noise contribution from the microsomes. There was no NADH-dependent non-enzymatic activity. Thus, these reactions were performed in standard cuvettes; samples were corrected for the noise contribution of the microsomes. Activities were calculated based on an extinction coefficient of 21 $cm^{-1}mM^{-1}$.

The NADH potassium ferricyanide (b_5) reductase activity was measured as a decrease in ferricyanide absorbance over time at 420 nm (Ichikawa *et al.*, 1969). The NADH-ferricyanide activity reactions contained a final concentration of 0.97 mM potassium ferricyanide, 1 mM potassium cyanide, approximately 200 mg microsomal protein, and 0.4 mM NADH in 1 mL 100 mM potassium phosphate, pH 7.7. The NADH-dependent reactions were performed in standard cuvettes; samples were corrected for non-enzymatic activity. There was only a minor contribution from the microsomes; thus, the microsomal contribution was not corrected. Activities were calculated based on an extinction coefficient of 1.02 $cm^{-1}mM^{-1}$. All reductase activities were initiated by the addition of the cofactor.

Ethoxyresorufin O-Dealkylation (EROD) Analysis. The ethoxyresorufin *O*-dealkylation activity was measured as a continuous fluorimetric assay performed with a 96-well microplate reader (Eggens and Galgani, 1992). The optimized reaction and standards contained 50 mM Tris pH 7.2, 4 mM NAD(P)H, and 4.5 mM ethoxyresorufin in a final volume of 110 mL. The microsomal protein, 150 - 300 mg per well, was included in the reactions, but not in the standards. Enzymatic reactions were initiated by the addition of the ethoxyresorufin, and plates were incubated at 37°C in an orbital shaker set at 220 rpm. Plates were read every 15 min with the Cytofluor 2300 system at the excitation wavelength 530 nm and emmission wavelength 590 nm. The ethoxyresorufin and resorufin were freshly diluted in 50 mM Tris, pH 7.2 from stocks (in DMSO), based on an extinction coefficient for ethoxyresorufin of 22.83 $cm^{-1}mM^{-1}$ at 464.0 nm and for resorufin of 40.0 $cm^{-1}mM^{-1}$ at 571.3 nm. Each sample contained less than 2% DMSO. When microsomes were incubated with the ethoxyresorufin without the presence of cofactor, there was a linear loss in fluorescence over time that interfered with the ability to calculate the EROD activity. Therefore, the fluorescence from the microsomes incubated without the cofactor was subtracted from the fluorescence produced when the microsomes were incubated with the cofactor. Optimal conditions for the EROD assay were established in *A. xanthogrammica* microsomes.

RESULTS

Spectral properties. The spectral properties of the CO-liganded, DTN-reduced microsomes show a low amplitude chromophore at 450 nm indicative of cytochrome P450 in the sea anemone (Table 3.1; Fig. 3.1, panel A). The presence of this 450 nm chromophore was observed consistently in the CO-difference spectra in three species of sea anemones, *A. elegantissima*; *A. xanthogrammica*; and, *B. cavernata*. Quantification of cytochrome P450 from its putative absortivity requires that the spectrum be free of interference at 490 nm, which is the isobestic wavelength between CO-liganded and unliganded reduced microsomes. A positive absorbance around 490 nm was often observed in the sea anemone microsomes; thus, the quantification of cytochrome P450 content in the majority of our microsomal preparations is dubious by this method. Based on the microsomal preparations that did not have positive absorbance at 490 nm, the columnar region of the sea anemone, *B. cavernata*, had a microsomal cytochrome P450 content of about 52 pmol per mg protein, a value consistent with numerous other values reported for marine invertebrates by the same method. In addition to the 450 nm chromophore, a predominant peak was consistently observed in the spectra of sea anemone

microsomal fractions with a wavelength maximum between 417-420 nm in the DTN-reduced CO-liganded microsomes.

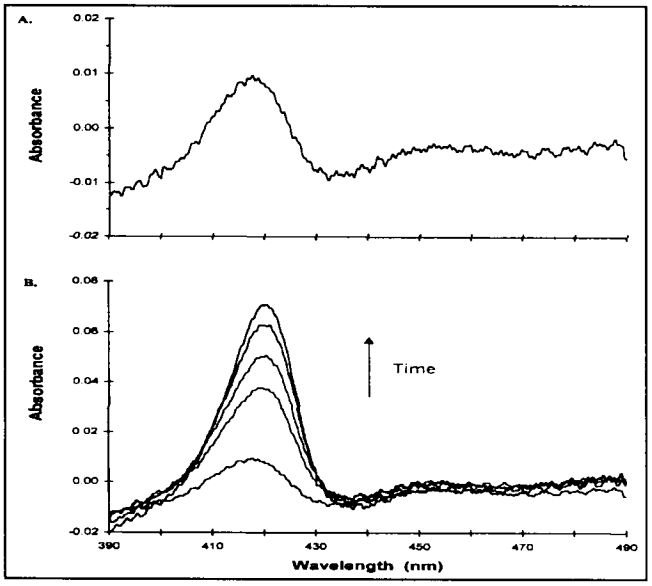


Figure 3.1. Spectral Properties of CO-Liganded, DTN-Reduced Sea Anemone *(B. cavernata)* Microsomes.

A 420 nm peak in mammalian microsomal preparations is indicative of denatured cytochrome P450. However, the appearance of the 418 nm chromophore was not altered by the inclusion of antioxidants (DTT), chelators (EDTA, phenanthroline), and proteolytic inhibitors (aprotinin, leupeptin, pepstatin, PMSF, soybean trypsin inhibitor) in a sucrose (or glycerol) homogenization medium (data not shown). A 418 ± 2 nm chromophore has been observed in many aquatic invertebrates; albeit, its identity or their identities has yet to be established.

Table 3.1
Experimental Protocols for Determination of Cytochrome P450-binding Spectra

	CO-difference		DTN-difference	
Spectra Type	sample cuvette	reference cuvette	sample cuvette	reference cuvette
DTN prior to CO	DTN	DTN	DTN	----
	BC [a]		BC[a]	
	CO	----	CO CO	
CO prior to DTN	CO	----	CO CO	
	BC [a]		BC[a]	
	DTN	DTN	DTN	----

[a] background correct (BC); equal quantities of solubilized microsomes were added to sample and reference cuvettes prior to background correcting.

Sea anemone microsomal cytochrome P450 spectra were examined by both CO-difference and DTN-difference spectra. A relatively large amplitude 418 nm chromophore was detected by both the CO-difference and DTN-difference spectra regardless of order of addition of DTN and CO; its amplitude was not altered by the method. Whereas, the ability to detect the 450 nm chromophore was clearly altered by the spectral method used. In the DTN-difference spectra, the 450 nm peak was detected albeit, it was not well resolved from the 418 nm peak. The ability to detect the 450 nm peak was particularly difficult in the DTN-difference spectra when the CO was added prior to the DTN, thus a property of the chromophore is refractory reducibility of CO-liganded as compared to unliganded metalloproteins by DTN.

In the CO-difference spectra, the 418 and 450 nm peaks were well resolved regardless of the order of addition of DTN and CO. Consistent with the above discussion of reducibility by DTN od CO-liganded proteins, a larger 450 nm peak was usually observed when DTN was added prior to the CO. Regardless of the CO-difference method used, the maximum 450 nm chromophore for the sea anemone was attained within five min and it typically was stable for the entire assay (90 min). Further, the 418 nm peak slowly increased over 10 min (CO added prior to DTN) or 20 min (DTN added prior to CO), (Fig. 3.1, panel b) and then began to decrease in amplitude at about 40-60 min (not shown). No corresponding increase in the 450 nm peak was observed in concomitance with the decrease in the 418 nm peak indicating that the latter chromophore is not reflecting denatured cytochrome P450 in these n the sea anemone microsomal preparations.

NAD(P)H-Reductase activities. *A. elegantissima*, *A. xanthogrammica* and *B. cavernata*, microsomal fractions all showed NAD(P)H-dependent cytochrome *c* reductase activity and NADH-dependent ferricyanide (b_5) reductase activity (Table. 3.2).

Table 3.2
Microsomal mixed-function oxidase components of
sea anemone columnar tissue

Species	NADPH[a,b] cyto c R	NADH[a] cyto c R	NADH[a] ferri R	418[c]	P450[d]
A. elegantissima	3.4 ± 0.5	12 - 17	175 - 240	45.1	----
A. xanthogrammica	2.0 ± 0.2	15 - 22	73 - 232	30.9	----
B. cavernata	2.1 ± 0.3	9 - 25	114 - 165	114.0	52

Mean ± standard deviation.

[a] nmol/min/mg protein; cyto c R = cytochrome c reductase; ferri R = ferricyanide reductase; KCN was included in each reductase assay.

[b] NADPH cytochrome c reductase assays were performed in tandem cuvettes to eliminate non-enzymatic activity.

[c] (490-418nm) x 1000 x mg^{-1}.

[d] pmol/mg microsomal protein.

Both of these flavoprotein reductases are integral components of the microsomal mixed function oxygenase system. Each of these reactions was linear with respect to protein concentration and time. The NADPH-dependent cytochrome *c* (P450) reductase activity was 1.8-3.9 nmol/min/mg protein for each species of sea anemone at 1 mM NADPH. In *B. cavernata*, the NADPH-dependent cytochrome *c* reductase activity increased significantly as the concentration of NADPH was increased. The reaction initially plateaued between 10 to 15 mM NADPH, and then continued to increase to at least 25 mM NADPH. Thus, the reductase activity reported here is rate-limited by NADPH. Further, these data indicate the presence of both a low and high K_m NADPH-dependent cytochrome *c* reductase activity. In contrast, the NADH-dependent reductase reactions contained excess cofactor. The NADH-ferricyanide reductase activity ranged from 73-232 nmol/min/mg protein, while the NADH-cytochrome *c* reductase activity was 9-25 nmol/min/mg protein for each species of sea anemone.

Ethoxyresorufin O-deethylation (EROD). Both *A. elegantissima* and *A. xanthogrammica* microsomal fractions catalyzed microsomal NAD(P)H-dependent EROD (Table 3.3). As will be discussed below in more detail, EROD activities were not always linear over the entire time course of the reaction; therefore, all samples were read at 15 min intervals between 0 to 90 min. In order to conserve microsomal samples and yet examine the reaction over time, a continuous assay (i.e., without stopping the assay with methanol) was employed.

Table 3.3
Microsomal NAD(P)H-dependent EROD Activities in the
Sea Anemone Columnar Tissue

Species	NADPH[a]	NADH[a]
A. elegantissima	2.28 ± 0.23 (4)	1.24 ± 0.30 (3)
A. xanthogrammica	0.80 ± 0.17 (7)	0.37 ± 0.04 (7)
B. cavernata	ND (6)	ND (6)

Mean ± std. dev. (number of microsomal preparations examined in triplicate).
[a] pmol/min/mg protein. ND = not detected

The microsomal EROD activity required the presence of either NADH or NADPH as a cofactor. Cofactor-independent activity was observed in the common marine mussel, *Mytilus edulis* L. (e.g., Livingstone *et al.*, 1989). In contrast to the higher NADH-dependent cytochrome *c* and ferricyanide reductase activities observed in the sea anemone, NADPH appeared to be the preferred cofactor with respect to EROD activity in each of the three sea anemone species studied. In both *A. elegantissima* and *A. xanthogrammica* microsomal reactions, the NAD(P)H-EROD activity was linear from approximately 30 to 90 min and often began to decrease between 90 to 120 min (not shown). Curiously, the initial 15 or 30 min often showed a decrease in fluorescence. This "lag period" was primarily observed in reactions with lower EROD activities, while more active samples tended to be linear over the whole reaction time. Consistent with this finding, the length of the "lag period" decreased as the protein concentration in the reaction increased indicating an interfering reaction with a microsomal constituent.

All calculations for the rate of activity were determined from only the linear region. If measured over the entire assay (vs. only the linear region), NAD(P)H-EROD activity was often not detectable in the sea anemone. This feature might explain why Firman (1995) reported a lack of EROD activity in the reef-building coral, *M. faveolata*. Finally, the NAD(P)H-EROD activity increased linearly with respect to protein concentration (NADPH, 150 to 300 mg protein per well; NADH, 150 to 400 mg protein per well) (Fig. 3.3). Below this protein range, EROD activity could be detected; however, the results were less reproducible because the values were too close to the detection limit of the assay.

In both *A. elegantissima* (approximately 50 animals were pooled/sample owing to the very small size of this species) and *A. xanthogrammica* (1-2 animals/sample were used as this species is about 60 times larger than the former), NAD(P)H-dependent EROD activity was detected in most of the microsomal preparations (Table 3.2). In *A. elegantissima*, the NAD(P)H-dependent activities were almost three-times higher than the typical values for *A. xanthogrammica*. In *A. xanthogrammica*, the typical EROD activity was 0.80 ± 0.17 pmol/min/mg protein with NADPH and 0.37 ± 0.04 with NADH; however, in addition to these seven preparations there was one microsomal preparation that did not contain EROD activity and one preparation that contained activity comparable to that observed in *A. elegantissima*.

For *B. cavernata*, any increase in fluorescence observed in the presence of 4 mM NADPH was so gradual that it could not be clearly discerned as activity. These microsomes were examined between 0 to 450 mg protein per well over 0 to 120 min. Regardless of the reaction conditions, the EROD values were too close to the assay's detection limit. In the presence of NADH, there was definitely no increase in fluorescence.

DISCUSSION

In this chapter it is shown that sea anemone columnar microsomes contain the major components characteristic of a cytochrome P450-dependent MFO system. These characteristics include a CO-binding spectra similar to that observed in other marine invertebrates and NAD(P)H-dependent cytochrome *c* and ferricyanide reductase activities. They also exhibit NAD(P)H-dependent dealkylation of a benchmark P450 substrate, ethoxyresorufin.

Based on the accepted molar extinction coefficient of vertebrate cytochrome P450, from the CO-difference spectra of DTN-reduced microsomes, the columnar region of the sea anemone, *B. cavernata*, has a cytochrome P450 specific content of about 52 pmol per mg protein. The P450 content for the other species of sea anemone could not be determined by the standard method because of a positive absorbance at 490 nm. An increase in absorbance at 490 nm has also been observed in the octopus, *Octopus pallidus* (Cheah et al., 1995), and in the mussel, *Anodonta cygnea* (Koivusaari et al., 1980). However, Gassman & Kennedy (1992) did not report any interference at 490 nm in their coral microsomal CO-binding spectra.

The presence of cytochrome P450 was not detected in the reef-building coral, *M. faveolata* (Firman, 1995). However, in the scleractinian coral, *F. fragum*, Gassman & Kennedy (1992) detected microsomal cytochrome P450 content throughout the coral's reproductive cycle,

which coincides with a lunar cycle. During the October lunar (reproductive) cycle, the level of cytochrome P450 fluctuated randomly, varying between 60 to 350 pmol per mg microsomal protein. They suggested that any pattern of cytochrome P450 content over the reproductive cycle was masked by the small sample size, high individual variation, or effects of pollutants. The cytochrome P450 content typically observed in this coral was approximately 150 pmol per mg protein. The data from the coral study indicates the heuristic value of studying cytochromes P450 in sea anemones over a circannular cycle.

The cytochromes P450 content observed in the anthozoa are consistent with results found in annelids, echinoderms, molluscs, and in some crustaceans (Berghout et al., 1991; den Besten et al., 1994; Fries and Lee, 1984; James, 1989; Jewell and Winston, 1989; Livingstone, 1991; Livingstone et al., 1989). For instance, most aquatic invertebrates have cytochrome P450 levels between 20 to 140 pmol P450 per mg microsomal protein. However, the hepatopancreas of the freshwater crayfish, *Astacus astacus* L., had about 310 pmol P450 per mg microsomal protein (Lindstrom-Seppa et al., 1983), while the microsomal P450 content [577 pmol/mg protein (Escartin and Porte, 1996); 720 pmol/mg protein, (Jewell and Winston, 1989)] of the hepatopancreas of the red swamp crayfish, *Procambarus clarkii*, was comparable to that of the rat liver.

We also observed a chromophore near 418 nm in the CO-difference spectrum of the microsomal fractions from the three species of sea anemones that we studied; similar observations have been made for the mollusc (Burns and Knap, 1989; Kirchin *et al.*, 1992; Koivusaari *et al.*, 1980; Livingstone and Farrar, 1984; Livingstone, 1988, Livingstone *et al.*, 1989; Sole *et al.*, 1994; Sole *et al.*, 1995; Weinstein, 1995), echinoderm (den Besten *et al.*, 1990), crustacean (James, 1990; Koivusaari *et al.*, 1980; Lindstrom-Seppa *et al.*, 1983, Quattrochi and Lee, 1984a; Quattrochi and Lee, 1984b; Singer *et al.*, 1980), and annelid (Berghout *et al.*, 1991; Nelson *et al.*, 1976). The presence of the 418 nm chromophore is consistent with results seen in the coral, *F. fragum* (Gassman and Kennedy, 1992); however, the amplitude of this chromophore in the coral appears to be lower than that typically seen in the sea anemone. Some investigators have suggested the 418 nm chromophore of invertebrates is denatured cytochrome P450 (Gilewicz *et al.*, 1984; Wilbrink *et al.*, 1991). In contrast, other investigators have suggested that it is another heme-centered protein that interferes with the detection of the 450 nm peak (Berghout *et al.*, 1991; Livingstone *et al.*, 1989; Nelson *et al.*, 1976; Singer *et al.*, 1980). Unless invertebrate P420 has a different extinction coefficient than that of vertebrates (the chromophore classically ascribed to denatured P450 in various vertebrate studies), it is unlikely that the 418 nm chromophore that we have observed in our spectral analyses is entirely denatured P450. If it were, the cytochromes P450 specific content in the sea anemone would be much higher than that of marine invertebrates, given that the sea anemone has a particularly high amplitude 418 nm absorbance. Further, the sea anemone P450 content would be roughly equivalent to that of mammalian microsomes. It is more likely that this chromophore is another heme protein [e.g., a cytochrome or a peroxidase (Applebly, 1969; Lindenmeyer and Smith, 1964)] that may interfere with the observation of the 450 nm peak. This hypothesis is further supported by the large amplitude of the 418 nm chromophore, even when antioxidants, proteolytic inhibitors, and glycerol were included in the homogenization media (e.g., Koivusaari *et al.*, 1980).

Despite the presence of a large 418 nm peak in the sea anemone microsomes, the 418 and 450 nm peaks were well resolved in the CO-difference spectra, regardless of the order of addition of DTN and CO (Fig. 2). However, the order of addition of the DTN and CO was important in resolving this chromophore in microsomal fractions of the mussel (*Mytilus edulis*) digestive gland, which also has a high amplitude chromophore at 418 nm. In the CO-difference spectra (DTN was added prior to CO), particularly abundant 418 nm chromophore interfered with the development of the 450 nm chromophore over time and resulted in more inconsistent estimates of P450 concentrations.

In the sea anemone, a maximum 450 nm peak was obtained by a CO-difference spectra as compared to a DTN-difference spectra. The larger 450 nm peak was more consistently observed in the CO-difference spectra when DTN was added prior to CO. This is consistent with the natural state of cytochromes P450 catalytic cycle in which reduction of the heme protein is required before binding of molecular oxygen; CO binds to the oxygen-binding locus. Further, in digestive gland microsomes of the pond snail, *Lymnaea stagnalis*, the CO-difference spectrum resulted in a larger and better resolved 450 nm peak than did the DTN-difference spectrum (Wilbrink *et al.*, 1991). In contrast, James *et al.* (1979) demonstrated that the DTN-difference spectra was necessary to obtain the maximum cytochrome P450 content in the spiny lobster, *Panulirus argus*, hepatopancreas microsomes. The reason for this difference in the crustacean spectra has not been elucidated.

Similar to the sea anemone, Livingstone and Farrar (1984) observed that the spectral pattern obtained in the microsomal CO-difference spectra (CO added before DTN) of the mussel attained a maximum amplitude within 5-10 min after the addition of DTN. In contrast, the mussel CO-difference spectra (DTN added before CO) required more than 20 min to fully develop; during which time, the 418 nm peak slowly decreased as the 450 nm peak developed. This coinciding increase in the 450 nm peak and decrease in the 418 nm peak was also reported in the pond snail, *L. stagnalis*, and in additional studies in the mussel, *M. edulis* (Wilbrink *et al.*, 1991), but reported as not present in the octopus, *O. pallidus* (Burns and Knap, 1989). Sole *et al.*, (1995) suggested that the increase in the 450 nm chromophore which coincided with a decrease in the 418 nm chromophore is indicative of a conversion process. Such a conversion process does not appear to occur in the sea anemone. In the earthworm, *Lumbricus terrestris*, Berghout *et al.* (1991) detected a significant increase in the 450 nm peak with time, especially in their semi-purified P450 fractions. However, these authors did not mention any coinciding change in the 418 peak even though it was a major chromophore in their crude microsomal preparations. They suggested that the increase in the 450 nm peak over time was a result of the invertebrate cytochromes P450 being reduced at a very slow rate.

The sea anemones, *A. elegantissima*, *A. xanthogrammica*, and *B. cavernata*, each contained NAD(P)H-dependent cytochrome *c* reductase activity and NADH-dependent ferricyanide (b_5) reductase activity in the microsomal fraction (Table 3.3). There are no other reports of these MFO components in cnidarians. NADPH-dependent cytochrome *c* (P450) reductase activity was 1.8-3.9 nmol/min/mg protein for each species of sea anemone at 1 mM NADPH. The range of NADPH-dependent cytochrome *c* reductase activity in most invertebrates is approximately 2-12 nmol/min/mg protein; thus, the reductase activity in the sea anemone is at the lower end of the range observed in most invertebrates studied (Escartin and

Porte, 1996; Kirchin *et al.*, 1992; Livingstone, 1991; Sole *et al.*, 1994; Yawetz *et al.*, 1992). The range of reductase activities reported in the earthworm (*L. terrestris*), barnacle (*Balanus eburneus*), and several molluscs (Berghout *et al.*, 1991; Michel *et al.*, 1993a&b; Sole *et al.*, 1994; Stegeman and Kaplan, 1981; Vrolijk and Targett, 1992) is much higher than that which we report for sea anemones herein.

For each of the three species of sea anemones studied, NADH-ferricyanide reductase activity ranged from 73-232 nmol/min/mg protein, while the NADH-cytochrome *c* reductase activity was 9-25 nmol/min/mg protein. These sea anemone reductase activities were similar to activities observed in the sea urchin, *Echinus esculintus*, but significantly lower than that observed in other invertebrates (den Besten *et al.*, 1991). The NADH-ferricyanide reductase activities in the molluscs and other echinoderms were between 360-2300 nmol/min/mg protein, while the NADH-cytochrome *c* reductase activities in the molluscs, crustaceans, and other echinoderms were between 32-400 pmol/min/mg protein. Even though most invertebrates fall in the lower end of these ranges, their activities were still significantly higher than the activity observed in the sea anemone (den Besten *et al.*, 1990; Jewell and Winston, 1989; Kirchin *et al.*, 1992; Lindstrom-Seppa *et al.*, 1982; Lindstrom-Seppa *et al.*, 1983, Livingstone, 1985; Livingstone, 1991; Livingstone *et al.*, 1985; Sole *et al.*, 1995; Stegeman and Kaplan, 1981).

Despite the relatively low reductase activities compared to other aquatic invertebrates, the sea anemone, *A. elegantissima* and *A. xanthogrammica*, catalyzed NADPH-dependent microsomal EROD activities relatively similar to the levels observed in most aquatic invertebrates (e.g., annelids, crustaceans, various molluscs) (Table 3.3 and 3.4). The levels for these invertebrates ranged between 0 to 6 pmol/min/mg protein. In contrast, the sea anemone EROD activities were 10-20 times lower than levels reported in *Patella caerulea* and *Avicularia gibbosula* (gastropods), in *Brachidontes variabilis* and *Donax trunculus* (bivalves) (Yawetz *et al.*, 1992), and in the sand worm, *Nereis virens* (Reily *et al.*, 1992). In these molluscs, the activity varied with the collection site from 0-60 pmol/min/mg protein; thus, the particularly high EROD activities may have been due to exposure to xenobiotics (Yawetz *et al.*, 1992). As expected, the EROD activity of crustacean microsomes fortified with mammalian cytochrome P450 reductase were even higher (James, 1990).

The relatively low level of activity reported for crustacean hepatopancreas microsomal MFO has been ascribed to the presence of an endogenous inhibitor in the microsomal preparations (Bend *et al.*, 1981, James *et al.*, 1979; Lindstrom-Seppa *et al.*, 1982; Lindstrom-Seppa *et al.*, 1983; Payne, 1977). For example, when spiny lobster hepatopancreas microsomes were incubated with sheepshead liver microsomes, the fish liver microsomes showed a decrease in NADPH-dependent EROD and B[a]P hydroxylase activity (James *et al.*, 1979). In contrast, when the sea anemone microsomes were incubated with rat liver microsomes, NADPH-dependent EROD activity was actually greater than the expected value based on an additive effect (data not shown). Thus, unlike in the spiny lobster, the presence of endogenous inhibitors in the sea anemone was not apparent.

A. elegantissima and *A. xanthogrammica* microsomes catalyze NAD(P)H-dependent EROD activity; however, these activities were higher in *A. elegantissima*. In *A. xanthogrammica*, two of the microsomal preparations differed substantially with respect to

typical EROD activity. In one sample the activity was variable and so near the detection limit of the assay as to preclude quantifiable accuracy, while the other sample had activity comparable to the level observed in *A. elegantissima*. These differences between the two samples may be the result the numbers of individuals used to prepare the microsomal batch; microsomes were prepared from 1-2 individuals instead of approximately 50 individuals of the smaller species, *A. elegantissima*. Large individual variation in MFO activity is common in studies of marine invertebrates (e.g., Kreiger *et al.*, 1979; Michel *et al.*, 1991). In both of these species, NADPH-dependent EROD activity was about two times higher than the NADH-dependent EROD activity (Table 3.3). The preference for NADPH as compared to NADH is consistent with the NAD(P)H-dependent hydroxylation of B[a]P activity in the sea anemone microsomes (Winston et. al, 1998). Despite the particularly strong preference for NADPH as compared to NADH as an electron donor in the MFO system in the vertebrates, several aquatic invertebrate MFO systems are capable of effectively utilizing NADH as an electron donor in the metabolism of P450-substrates (e.g., den Besten *et al.*, 1994; Jewell and Winston, 1989; Lindstrom-Seppa *et al.*, 1983; Livingstone, 1991; Wilbrink *et al.*, 1991). In fact under certain conditions NADH has been shown to be the preferred cofactor. For instance, in the crayfish, *P.clarkii*, and sea star, *Asterias rubens*, NADH was shown to be the preferred cofactor for B[a]P hydroxylase activity (den Besten *et al.*, 1994; Jewell and Winston, 1989). The preference for NADH in aquatic invertebrates appears to vary between substrates, species, and tissues. Why this variation in cofactor preference occurs in marine invertebrates represents an exciting challenge to the marine biochemistry field.

B. cavernata were collected from a site heavily contaminated with petroleum products; whereas, both *A. elegantissima* and *A. xanthogrammica* were collected from relatively pristine sites. Currently, it is unclear if the marine invertebrate cytochromes P450 are inducible because induction studies in those organisms have yielded conflicting results (Livingstone, 1991). However, based on western blots probed with a fish CYP1A antibody, the putative 1A isoform is not induced in the sea anemone, *B. cavernata*, after exposure for 3 days to 3-methylcholanthrene (20 mg/kg) (Heffernan *et al.*, 1996). In contrast, B[a]P hydroxylation activity reported for coral, *F. fragum*, collected from the polluted Kemphouse Reef was over three times greater than for coral collected from the relatively pristine South Caryfort Reef. The more inshore site, Kemphouse Reef, contained 3-15 times more PAH's and pesticides than did the South Caryfort Reef; thus, these authors suggested that the increase in activity was associated with an induction in MFO activity in the coral. However, there was no coinciding increase in P450 content with the increase in B[a]P activity (Gassman and Kennedy, 1992).

The CO-binding spectral properties, reductase activities, and NAD(P)H-dependent metabolism of classical cytochrome P450 substrates (i.e., aldrin epoxidation, B[a]P hydroxylation, ethoxyresorufin *O*-dealkylation) of earlier sea anemone microsomal studies (Gassman and Kennedy, 1992; Heffernan *et al.*, 1996; Khan *et al.*, 1972; Winston et. al, 1998) and this chapter indicate the presence of a functional cytochrome P450-dependent mixed function oxygenase system in cnidarians. Accurate detection of the spectral properties and MFO activity often required homogenization buffers with proteolytic inhibitors and anti-oxidants, sensitive assays, and long incubation periods; thus, the failure of some studies to detect the presence of an MFO system may have been due to problems with the assay conditions used rather than to a true lack of activity. A similar situation occurred when Payne (1977) did not

detect B[a]P activity in the mussel (*M. edulis*), while several more recent studies with this mussel have reported B[a]P activity (e.g. Lemaire *et al.*, 1991; Livingstone, 1991; Livingstone *et al.*, 1989).

Table 3.4
Comparison of NAD(P)H-dependent EROD Activities of Marine Invertebrates.

Species[a]	pmol/min/mg protein	Reference
Cnidarian		
coral (*Montastraea faveolata*)	below detection	Firman, 1995 (dissertation)
Mollusc		
Octopus (*Octopus pallidus*)	1.4 - 4.82	Burns & Knap, 1989
Octopus (*O. pallidus*)	0.18 - 0.32	Butty & Holdway, 1997
Octopus (*O. pallidus*)	1.4 ± 0.5	Cheah et al., 1995
Mussel (*M. edulis*)	5	Stegeman, 1985
Calico clam (*M. maculata*)	below detection	Stegeman, 1985
Bermuda mussel (*A. zebra*)	below detection	Stegeman, 1985
Pond snail (*L. stagnalis*)	below detection	Wilbrinket al., 1991
Gastropod (*C. gibbosum*)	below detection	Vrolijk & Targett, 1992
Chiton (*Crytochiton stelleri*)	below detection	Schlenk & Buhler, 1989
Gastropod (*L. stagnalis*)	95 ± 33	Meimberg et al., 1997
Gastropod (*P. caerulea*)	53 ± 25	Yawetz et al., 1992
Gastropod (*A. gibbosula*)	35 ± 39	Yawetz et al., 1992
Bivalve (*B. variabilis*)	48 ± 22	Yawetz et al., 1992
Bivalve (*D. trunculus*)	24 ± 7	Yawetz et al., 1992
Crustacean		
Crayfish (*P. clarkii*)	0.7 ± 0.6	Escartin & Porte, 1996
Crayfish (*P. clarkii*)	0.51 ± 0.24	Porte & Escartin, 1998
Crayfish (*A. astacus*)	below detection	Lindstrom-Seppa et al., 1983
crab (*Carcinus aestuarii*)	0-50 (exposed)	Fossi et al., 1998
Blue crab (*C. sapidus*)[b]		
stomach	2.6	Singer et al., 1980
hepatopancreas	below detection	Singer et al., 1980
Spiny lobster (*P. argus*)[b]	28 - 98	James, 1984; 1989; 1990; James & Little, 1984
Annelid		
Earthworm (*L. terrestris*)	below detection	Berghout et al., 1991; Liimatainen & Hanninen, 1982
Sand worm (*N. virens*)	30	Reily et al., 1992 (abstract)
Tiger worm (*Eisenia f. fetida*)	traces levels	Achazi et al., 1998
Pot worm (*Enchytraeus crypticus*)	3.98 ± 0.55	Achazi et al., 1998
Terrestrial annelid	15.41 ± 9.48	Hellwig[c]

[a] microsomes were prepared from mollusc digestive gland, crustacean hepatopancreas (except Blue crab), earthworm and sand worm midgut, tiger worm and pot worm whole body.
[b] Microsomes were sodium cholate-solubilized and recentrifuged, then fortified with mammalian reductase.
[c] data as given in Achazi et al., 1998

Several potential explanations exist for why early studies did not detect the MFO system in the cnidarian. Firstly, in most metazoans, cytochrome P450 tends to concentrate in particular organs. As previously discussed, it is difficult to isolate P450 from discrete regions of the cnidarians. Secondly, in experiments that tested many organisms from several different phyla, enzymatic assays and tissue preparation were typically optimized for one organism and all other animals were analyzed under the same conditions. Particularly in organisms with low MFO activity, establishing optimal conditions can be very critical. For instance, our research indicates that the detection of MFO activity in the cnidarians requires longer incubation times, during which the MFO activity remained linear. Thirdly, because invertebrate cytochromes P450 tend to concentrate in the digestive and reproductive system, the lack of optimal buffer conditions (i.e., protease inhibitors and reducing agents) could have prevented isolation of a functional protein (e.g., Livingstone, 1991). This problem is further compounded in the cnidarian because it is essentially a large digestive sac. Finally, Lee (1981) suggested that sea anemones may contain endogenous inhibitors of cytochrome P450 that are released upon homogenization. The presence of endogenous inhibitors of the MFO system has been reported in the hepatopancreas of several crustaceans (Bend *et al.*, 1981; James *et al.*, 1979; Lindstrom-Seppa *et al.*, 1982; Lindstrom-Seppa *et al.*, 1983; Pohl *et al.*, 1974). However, the EROD data reported herein indicates that the sea anemone columnar region microsomes do not contain such endogenous MFO inhibitors.

In conclusion, despite the lack of MFO activity reported in anthozoans and scyphozoans by several investigators who have studied cnidarians (Firman, 1995; Lee, 1975; Lee, 1981; Payne, 1977), other studies indicate that anthozoans and hydrozoans do contain a functional cytochrome P450-dependent MFO system (Gassman and Kennedy, 1992; Heffernan *et al.*, 1996; Khan *et al.*, 1972; Winston et. al, 1998, present study). The evidence is based on the presence of proteins that immunoreact with cytochrome P450 antibodies, a characteristic CO-difference spectra in DTN-reduced coral and sea anemone microsomes, active compliments of MFO components (i.e., P450 and b_5 reductases), and metabolism of classical cytochrome P450-catalyzed reactions (i.e., ethoxyresorufin *O*-dealkylation, B[a]P hydroxylation, and aldrin epoxidation). Preliminary on going studies in our laboratory also indicate that *B. cavernata* can catalyze NAD(P)H-dependent aldrin epoxidation. It is interesting that the P450 content and activity does not differ substantially from most other invertebrates, despite the fact that cnidarian microsomes are composed of either the whole animal (i.e., hydra, coral) or the entire columnar region (i.e., sea anemone).

CHAPTER 4

DISTRIBUTION OF MICROSOMAL CO-BINDING CHROMAPHORES, IMMUNOREACTIVITY OF ANTI-CYTOCHROME P450 2K1 AND EROD ACTIVITY IN SEA ANEMONE TISSUES

L.M. Heffernan and G.W. Winston

INTRODUCTION

The presence of an active cytochromes P450-dependent MFO system (CYP) in several species of sea anemones and a scleractinian coral (Phylum: cnidarian; Class anthozoan) was indicated in the previous chapter. This conclusion was based on spectral properties characteristic of P450, NAD(P)H-dependent cytochrome c reductase activities, and metabolism of substrates classically employed as indicators of cytochrome P450-dependent mixed-function oxidase activity, which included ethoxyresorufin O-dealkylation, benzo[a]pyrene hydroxylation, and aldrin epoxidation (Gasssman & Kennedy, 1992; Heffernan & Winston, 1998; Heffernan *et al.*, 1996; Winston *et al.*, 1998; unpublished data). Cnidarians have evolved only to the tissue level of development and contain numerous tissue types of diverse function. The microsomal mixed function oxygenase (MFO) system of marine invertebrates has not been as intensely studied as vertebrates and the cnidarians are among the least studied. Where MFO has been studied in cnidarians the focus has been principally on microsomal fractions prepared from the whole animal (coral) or entire columnar region (sea anemone) rather than on microsomes prepared from specific tissues that might concentrate CYP enzymes.

Cytochromes P450 have been shown to have a wide tissue distribution in aquatic invertebrates; however, they tend to be most concentrated in tissues associated with processing of food or pollutants. This has been demonstrated in molluscs (Livingstone & Farrar, 1984; Schlenk & Buhler, 1989; Stegeman, 1985), crustaceans (Burns, 1976; Jewell & Winston, 1989; Khan *et al.*, 1972; Lindstrom-Seppa *et al.*, 1983; Stegeman & Kaplan, 1981; James, 1989), annelids (Fries & Lee, 1984; McElroy, 1990; Nelson *et al.*, 1976), and echinoderms (denBesten *et al.*, 1990, 1991). In fact, the CYP content and MFO activities are often 2 to 10 times lower or even below the detection limits of the assay in tissues that tend not to concentrate CYP. In contrast to these studies, there are a few studies of aquatic invertebrates that have reported that CYP content and P450-dependent activities are not concentrated in any particular organ (Schlenk & Buhler, 1989; Khan *et al.*, 1972; denBesten *et al.*, 1990).

Studies of the P450-dependent MFO that used whole or mainly whole cnidarians may have underestimated or misrepresented various parameters due to interferences caused by introduction of nonspecific tissues that might not concentrate P450 in the microsomal preparations. Some of the MFO properties reported for cnidarians were at the lower end of the range of values expected, while other MFO properties were within the expected range of values. For instance, EROD activity in the sea anemone, *Bunodosoma cavernata*, was similar to levels observed in most aquatic invertebrates, while it was lacking in the reef-building coral, *Montastraea faveolata* (Firman, 1995; Heffernan & Winston, 1998). Further, B[a]P activity in

the scleractinian coral, *Favia fragum*, is relatively low compared to most aquatic invertebrates (Gassman & Kennedy, 1992). The B[a]P activity was more comparable to activities observed in invertebrate tissues that do not concentrate CYP enzymes (denBesten *et al.*, 1990; Stegeman, 1985; Stegeman & Kaplan, 1981).

Isolation of particular tissues from cnidarians is complicated by the amorphic and non-rigid structure of these organisms, which are comprised essentially of a large digestive sac surrounded by a nerve net work, a gonadal region, a thick muscular region, and a tough outer wall. The purpose of this study was to determine the relative distribution of CYP specific content and a corresponding MFO activity in various tissue regions of the sea anemone. *Anthopleura xanthogrammica* was chosen for this study because it is one of the largest of the sea anemones and therefore more amenable to dissection and separation of the various regions. Two individuals were divided into four tissue regions referred to as the soft, inner, outer, and tentacle region. In view of only two animals available for this study we recognize that it is very preliminary, nevertheless, to the best of our knowledge it is the first study of tissue type distribution of CYP and MFO in cnidarians.

MATERIAL AND METHODS

Animals. *Anthopleura xanthogrammica* were obtained from North Coast Invertebrate Collectors in Bodega Bay, California. The sea anemones were maintained in a recirculating system with Instant Ocean^{tm} sea water at approximately 34 ‰ salinity and 12°C. The amorphic and non-rigid structure complicates the ability to separate distinct tissues; thus, these regions do not consist of one tissue. Instead, they consist of four tissue regions referred to as the tentacle, soft, inner, and outer region. In order to collect these regions the animal was cut down the center. The tentacles, which include the nematocyts and most of the algal/diatom symbiont, were removed as the first region. The second region consisted of the softest tissues, which is highly amorphous (soft region: digestive sac, gonads, & mesentery filaments). After the removal of as much of the soft tissues as possible, the fibrous inner region (perfect and imperfect mesentery, retractor muscle) was separated from the tough outer layer (tough outer muscle, epidermal tissue, basal disk and oral disk). These regions were isolated from two sea anemones, with the exception that the tentacular region was not saved from the second animal. The various tissue regions of the sea anemone were immediately submerged in homogenization buffer.

Microsomal Preparation. Microsomes were prepared by homogenizing each of these tissue regions in four volumes homogenization buffer with a hand-cranked plastic meat grinder, 3 - 4 passes with Tekmar Tissumizer^{tm} , and then 3-4 passes with a Potter-Elvehjem tissue homogenizer. The homogenization buffer was 100 mM potassium phosphate pH 7.6, containing 125 mM sucrose, 1 ìg/mL aprotinin, 1 mM ethylene diaminetetraacetic acid (EDTA), 1 ìg/mL leupeptin, 1 ìg/mL pepstatin, 0.1 mM phenanthroline, 1 mM *para*-metylsulfonylfluoride (PMSF), 1 mg/mL soybean trypsin inhibitor, and 1 mM dithiothreitol (DTT). Homogenates were serially centrifuged for 20 min at 8,500 x g and 15 min at 14,000 x g. The resulting pellets were discarded. The 14,000 x g supernatant was then centrifuged for 1 h at 105,000 x g to pellet the microsomal fraction. The microsomal pellets were washed once in the homogenization

buffer and centrifuged again at 105,000 x g for 1 h. Microsomes were resuspended and stored at -80°C in 100 mM potassium phosphate pH 7.6, containing 250 mM sucrose at a protein concentration of approximately 10 mg/mL. The typical yield of microsomes was between 4 - 10 mg of microsomal protein per g^{-1} of tissue wet weight. Protein concentrations were determined by the 96-well microplate reader fluorescamine assay as described by Lorenzen and Kennedy (1993), with two exceptions. The fluorescamine was prepared in HPLC-grade dioxane and the buffer used for the assay was 250 mM sodium phosphate, pH 8.5.

Western blots. Proteins were separated on 10% SDS-polyacrylamide gels (Laemmli, 1970) and transblotted for 50 min onto nitrocellulose with 10 mM Tris, 100 mM Glycine, and 10% methanol transfer buffer (Towbin *et al.*, 1979). The blot was blocked overnight with 5% Carnation powdered milk in 50 mM Tris with 20 mM sodium chloride (TBS), incubated with the rabbit anti-trout CYP 2K for 1 h, anti-rabbit containing a biotinylated conjugate for 1 h, and Sigma ExtrAvidin for 30 min. The blot was washed between each of these incubation steps with TBS for 4 times for 5 min intervals. Prior to developing the color of the bands, the blot was rinsed well with distilled water. The color was developed with 100 mM sodium bicarbonate (pH 9.8) containing 1 mM magnesium chloride plus 5-bromo 6-chloro 3-indolyl phosphate (15 mg BCIP per 500uL DMF) and nitroblue tertrazoleum (30 mg NBT per 500 uL 70% DMF). The developer was prepared just prior to staining. The color development was stopped by rinsing and then soaking the blot in distilled water for 10 min.

Spectral Properties. Sea anemone microsomal cytochrome P450 spectra were obtained with a Perkin Elmer lambda 5 dual beam spectrophotometer. Both a carbon monoxide (CO)-difference spectrum of sodium dithionite (DTN)-reduced samples and the DTN-difference spectrum of CO-liganded samples (Omura & Sato, 1964) were obtained as described in the previous chapter.

Ethoxyresorufin *O*-Dealkylation (EROD) Analysis. The ethoxyresorufin *O*-dealkylation activity was measured as a continuous fluorimetric assay performed with a 96-well microplate reader (Eggens & Galgani, 1992). The optimized reaction and standards contained 50 mM Tris pH 7.2, 4 mM NAD(P)H, and 4.5 μM ethoxyresorufin in a final volume of 110 μL. The microsomal protein, 150 - 300 μg per well, was included in the reactions, but not in the standards. Enzymatic reactions were initiated by the addition of the ethoxyresorufin, and plates were incubated at 37°C in an orbital shaker set at 220 rpm. Plates were read every 15 min with the Cytofluor 2300 system at the excitation wavelength 530 nm and emmission wavelength 590 nm. The ethoxyresorufin and resorufin were freshly diluted in 50 mM Tris, pH 7.2 from stocks (in DMSO), based on an extinction coefficient for ethoxyresorufin of 22.8 $cm^{-1}mM^{-1}$ at 464 nm and for resorufin of 40.0 $cm^{-1}mM^{-1}$ at 571 nm. Each sample contained less than 2% DMSO. When microsomes were incubated with the ethoxyresorufin without the presence of cofactor, there was a linear loss in fluorescence over time that interfered with the ability to calculate the EROD activity. Therefore, the fluorescence from the microsomes incubated without the cofactor was subtracted from the fluorescence produced when the microsomes were incubated with the cofactor. Optimal conditions for the EROD assay were examined in *A. xanthogrammica* microsomes.

RESULTS

The absorbing chromaphore at approximately 450 nm in CO-liganded, DTN-reduced microsomes suggests the presence of cytochrome P450 in each of the tissue regions (Fig. 4.1). Panel B of Figure 4.1 is an enlarged view of the T,S and I spectra of panel A. In addition to this absorbance at 450 nm, each tissue region displayed a large amplitude chromophore at 418 nm. These peaks appear in both the DTN-difference (CO added prior to DTN) and CO-difference spectra (DTN added prior to CO) and were well separated. For each of these methods, the 450 nm peak was of approximately the same amplitude and this was not altered over time. In contrast, the 418 nm peak did not reach its maximum for 10-20 min. The two methods used to achieve the spectra resulted in peaks of different amplitude. The peak at 418 nm was of lower amplitude in the CO-difference spectrum. The amplitude of the 418 nm peak did not change over time in the CO-difference spectrum as it did in the DTN-difference spectrum. All spectra described below refer to CO-difference spectra.

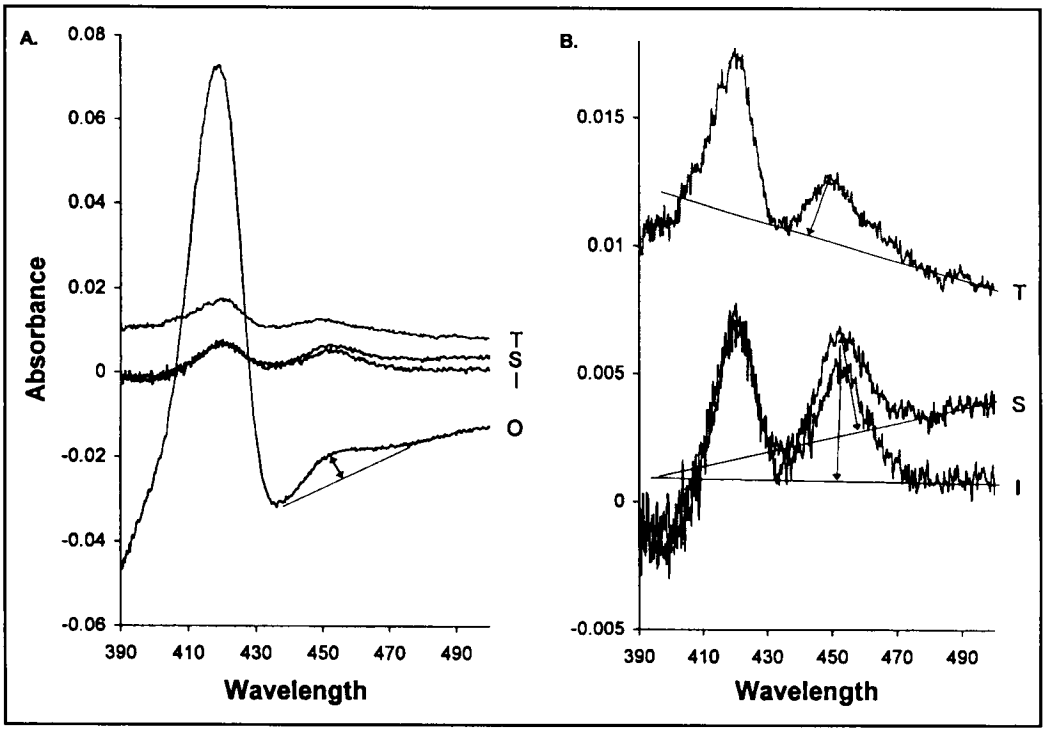


Figure 4.1. CO-binding Spectrum (390-495 nm) of Microsomal Fractions of Outer, Inner and Soft Tissue Regions of *A. xanthogrammica.*

In some of the tissue regions broad, positive absorbance that overlapped with the 490 nm region of the spectrum was observed (Fig. 4.1, panel A). This had also been noted in microsomal fractions prepared from whole columnar region of the animal (Chapter 1). This 490 nm absorbance was more profound in the outer region of the sea anemone. Quantification of P450 from the classical extinction coefficient derived from mammalian cytochrome P450 ($A_{450-490}$ = 91 cm^{-1} mM^{-1}) requires that the spectrum be free of interference at 490 nm, the isobestic wavelength between CO-liganded and unliganded reduced microsomes. In addition to the

interference caused by the 490 nm absorbance in the quantification of cytochrome P450, the baseline of the spectrum tended to slope, thereby introducing perturbations in the true absorbance value at 490 nm. Therefore, the P450 content was not based on the difference between 450 and 490, but on the difference between the 450 and a tangential drawn along the slope of the spectra (Fig. 4.1, panel B). as reported by Cheah *et al.*, (1995). Based on these calculations, the P450 content in each of these tissues was between 16 to 31 pmol/mg protein (Table 4.1). According to these calculations and the visual appearance of the spectrum, the P450 content was slightly higher in the soft and inner region. The wavelength maximum was approximately 451 nm for each of the tissues. In each tissue region, the size of the 450 nm peak appeared to reach its maximum amplitude immediately and did change within 40 min.

In each tissue region, there was a predominant peak at 418 nm. The wavelength maximum (λ_{max}) was approximately 418 nm for each tissue region. In contrast to the 450 nm peak, the 418 nm chromaphore was significantly more concentrated in the tough outer region of the sea anemone than in any of the other regions. Further, the 418 nm peak reached a maximum amplitude in 40 min in tentacle microsomes and 20 min in microsomes of each of the other tissue regions. The final size of the 418 nm peak was about 2 times larger than the initial peak (data not shown).

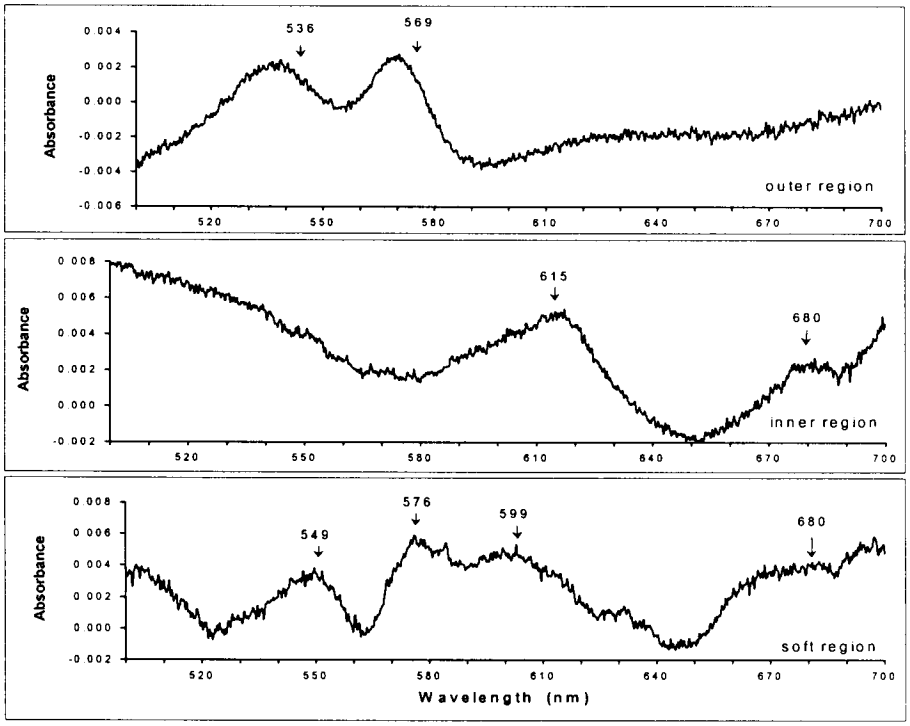


Figure 4.2. CO-binding Spectrum (500-700 nm) of Microsomal Fractions of Outer, Inner and Soft Tissue Regions of *A. xanthogrammica.*

The 500-700 nm region of the microsomal spectra is diagnostic of heme proteins as absorbance patterns in this region are indicative of d-d transitions associated with the metal porphyrin structure (Huheey *et al.*, 1993). The absorbance patterns in this region varied between the tissues (Fig. 4.2). The outer region contained two distinct chromophores at 535 and 569 nm with

a trough at 555 nm. The inner region only contained one well-defined peak with a λ_{max} at 548 nm. There were also at least two overlapping peaks between 570-640 nm (λ_{max} at 576 and 599 nm) and above 660 nm (no clear λ_{max}). Finally, the inner region contained one distinct chromophore at 615 nm. There were also chromaphores above 660 nm. The tentacle region was not analyzed.

Sodium azide- (NaN_3) and potassium cyanide- (KCN) binding spectra of reduced and oxidized sea anemone microsomes of the inner region.were generated and are shown in Figure 4.3 in comparison with the CO-binding spectra classically employed for cytochromes P450 and other heme-proteins detection. NaN_3 and KCN are benchmark ligands used to probe transition metal centers of proteins.

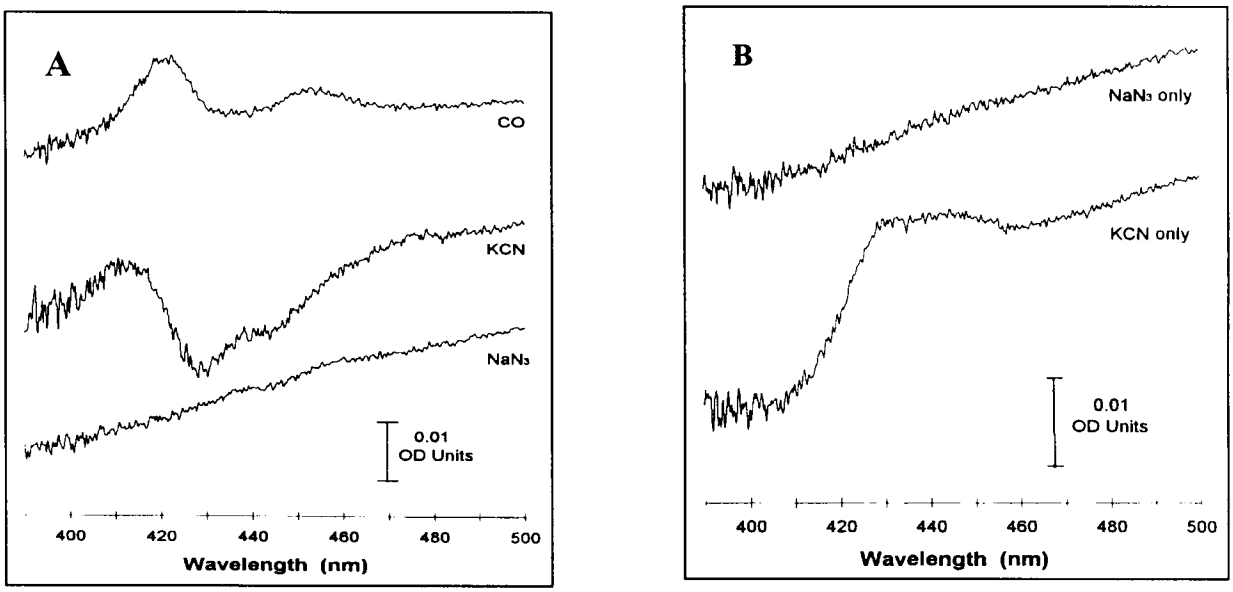


Figure 4.3. CO, KCN and NaN_3 Binding Spectrum of (A) DTN-reduced and (B) Oxidized Microsomes from Inner Region Tissue of *A. xanthogrammica.*

When oxidized microsomes were incubated with KCN the difference spectrum resembled that of an inverse type I or a type II binding spectra obtained when certain ligands interact with the heme center of cytochromes P450 (Jewell and Winston, 1989). The spectrum was characterized by a broad maximum at approximately 440 nm and a minimum at about 410 nm (Fig. 4.3, panel B). Incubation of inner region microsomes with either CO or NaN_3 did not produce this spectrum. A linearly rising absorbance was observed with both of these ligands (Fig. 4.3, panel B; shown for NaN_3 only) indicating a time-dependent interaction of the ligands with the heme center. . When these microsomes were reduced with DTN prior to the addition of KCN the spectrum generated contained a maximum at approximately 410 nm and a minimum at about 430 nm. This pattern is reminiscent of the spectrum generated when cytochromes P450 interact with a hydrophobic pocket distal to the heme center. When NaN_3 is incubated with DTN-reduced microsomal spectrum a rising flat line like that obtained with oxidized microsomes was observed. Finally, the CO-liganded, DTN-reduced microsomal spectrum contained the 418 and 450 nm peaks anticipated for a cytochrome P450 heme center.

Immunoblot Analysis and EROD Activity:

A rabbit anti-trout CYP2K antibody cross-reacted with a protein band in each tissue region (Fig. 4.4). Any differences between the intensity of recognition by the CYP2K antibody in the different tissue regions appeared to be minor.

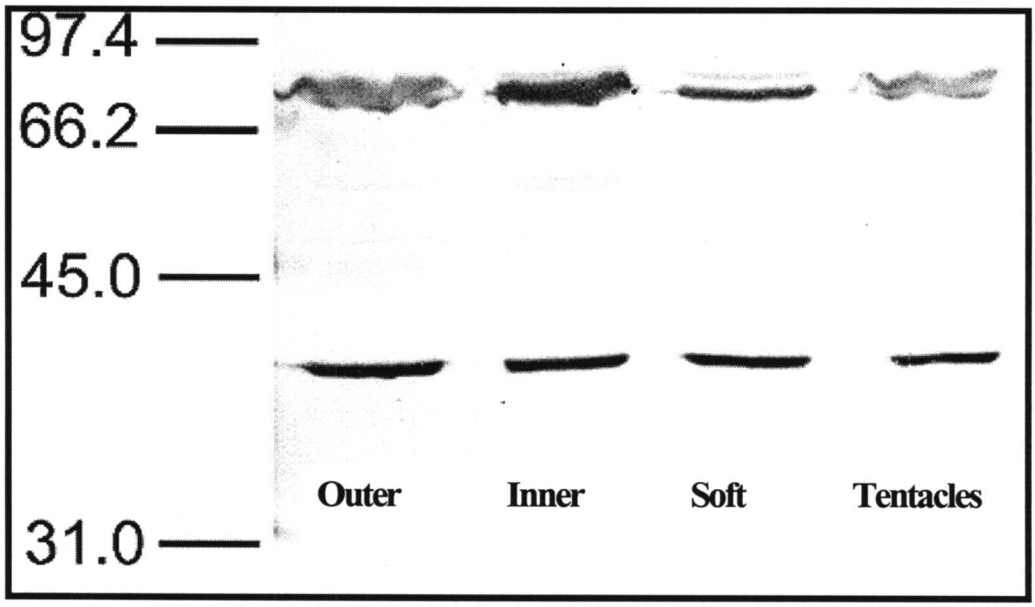


Figure 4.4. Western Blots of CYP2K1 Antibody-Reactive Protein of Microsomes from Four Tissue Regions of the Sea Anemone *A. elegantissima.*

Microsomes from each of the tissue regions catalyzed NAD(P)H-dependent EROD activity (Table 4.1). EROD activity required the presence of either NADH or NADPH. Further, activity was higher in the presence of NADPH than NADH for each tissue region. NADH-EROD activity per mg protein was highest in the tentacles. The activity in the inner and soft regions was slightly less than the tentacular activity, while the outer region contained about a third as much activity. NADPH-EROD activity was not as evenly distributed between the tissue regions. In one animal, the pattern was similar to the NADH-dependent activity, except there was a bigger difference in activity between the soft and inner region. While in the other animal, the tissue regions contained approximately the same rate of NADPH-EROD activity. EROD activity was linear for up to 20 min for each tissue. The duration of linearity of EROD activty catalyzed by microsomal fractions of the outer region tended to be shorter, particularly in the AX 5/24/97 animal. Unlike in the columnar microsomes a drop in the initial fluorescense between 0-15 min was not evident; the reaction increased linearly for the entire time course.

Table 4.1
Quantitation of Microsomal Cytochrome P450, 418 nm chromaphore, and NAD(P)H-EROD Activitiesin *A. xanthogrammica* Tissue Regions

Tissue Regions	P450[a]	418[b]	NADPH[c] EROD	NADH[c] EROD
1st individual				
outer muscle	21.6	64.0	0.17	0.09
inner region	28.8	3.7	0.66	0.27
gonad region	22.0	2.8	1.09	0.26
tentacles	16.2	3.8	2.61	0.36
2nd individual				
outer muscle	16.0	89.3	1.69	0.27
inner region	31.2	8.7	1.48	0.47
gonad region	30.9	15.2	1.46	0.48

[a] pmol/mg protein; calculation based on slope of a baseline, instead of absorbance at 490 nm. [b] (490-418) x 1000 x mg^{-1}. [c] pmol/min/mg protein; performed in triplicate. [d] EROD activity of entire column was 0.80 ± 0.17 (NADPH) and 0.37 ± 0.04 (NADH).

DISCUSSION

The CO-liganded, DTN-reduced binding spectra of microsomes from each of the sea anemone tissue regions contained a 418 and 450 nm peak (Fig. 4.1). Spectral analysis of the soft region microsomes indicated that the 418 and 450 nm peak could be detected in both the DTN-difference and CO-difference spectra despite the order of addition of the CO and DTN. Similar to the microsomal fraction of the sea anemone columnar region (Heffernan & Winston, 1998), the use of DTN-difference spectra resulted in poorer resolution of the 450 and 419 nm peaks; while both of the CO-difference spectra and the DTN-difference spectra demonstrated good resolution of these peaks and the 450 nm peak. While the amplitude of the 450 nm peak was similar regardless of which of these three methods was used, the CO-difference (DTN added prior to CO) spectra resulted in the smallest 418 nm peak whether read immediately or after 20 min. Thus, each of the CO-liganded, DTN-reduced spectra were performed as CO-difference spectra with the DTN added prior to CO. The spectral characteristics obtained by each of these methods varies between different marine invertebrates. In the pond snail *Lymnaea stagnalis* digestive gland microsomes, the CO-difference spectra resulted in a larger and more resolved 450 nm peak (Wilbrink *et al.*, 1991). In contrast, the DTN-difference spectra was necessary to obtain a 450 nm peak of maximum amplitude in microsomal fractions of the spiny lobster, *Panulirus argus*, hepatopancreas (James and Little, 1984) and mussel, *Mytilus edulis*, digestive gland (Livingstone & Farrar, 1984; Livingstone *et al.*, 1989).

Despite the method used, quantification of P450 specific content was complicated by the tendency for the overall baseline of the spectra to slope and the presence of a 490 nm chromaphore in some of the tissue regions, which confounds the ability to exploit the isosbestic point commonly used to quantify the change in absorbance of the 450 nm peak upon liganding of CO in classical spectral analysis of cytochromes P450 (Fig. 4.1). These features, which confound quantification of cytochromes P450 have also been observed in other marine organisms (Cheah *et al.*, 1995; Koivusaari *et al.*, 1980). In the octopus, *Octopus pallidus*, the P450 was quantified from the difference between the absorbance of the 450 nm peak and an artificial baseline that followed the slope of the spectra (Cheah *et al.*, 1995). This same procedure was used to quantify the P450 content in the different tissue regions of the sea anemone microsomes (Fig. 4.1) reported herein.

The CO-liganded, DTN-reduced binding spectra of the tissue regions studied suggest that P450 does not concentrate in a particular region of the sea anemone (Fig. 4.1; Table 4.1). There was a little more P450 in the inner and soft region, but the differences did not appear to be significant. Based on most aquatic invertebrate studies, cytochrome P450 should have concentrated in tissues associated with processing of food and pollutants. The soft region was composed predominantly of the gonads and the mesentery filaments. The mesentery filaments are the primary location of both extracellular and intracellular digestion. The inner region contained the perfect mesenteries, which are also important in processing of food. The perfect mesenteries play an important role in both absorption of nutrients and storage of lipids. The particularly high concentration of lipids would tend to concentrate pollutants; thus, the perfect mesenteries would be expected to play a role in metabolism of these pollutants.

In each of the tissue regions studied, a protein band of about 40 KDa was strongly recognized by a trout CYP2K1 antibody. The intensity of this recognition was enormously greater than with any other antibody used from mammalian or fish sources. There was no clear difference in the intensity of the antibody recognition between the different tissues. The structure and function of this protein in sea anemone tissues remains to be established. A 40 KDa protein has also been detected in microsomes from the columnar region of at least five species of sea anemone (Heffernan and Winston, 1998). It has also been detected in several other marine invertebrates; however, no such protein is recognized by this antibody in trout liver, the source of the CYP antigen used to create the antibody. In trout liver, this antibody recognizes a protein of about 59 KDa (Miranda *et al.*, 1990). To the best of our knowledge, this CYP2K1 antibody does not recognize a protein of 40 KDa in any vertebrate studied (Heffernan *et al.*, 1996; unpublished data). Currently, all cytochromes P450 reported in other invertebrates (non-insect) are between 48-56 KDa proteins (Batel *et al.*, 1986; Berger & Fairlamb, 1993; Briand *et al.*, 1993; Conner & Singer, 1981; Kirchin, 1988; Lee, 1986; Livingstone *et al.*, 1989; Quattrochi & Lee, 1984a, 1984b; Schlenk & Buhler, 1989; Yawetz *et al.*, 1992); however, recent studies have reported additional proteins of about 40 KDa in other marine invertebrates that strongly cross-react with CYP antibodies (Peters *et al.*, 1998; James & Boyle, 1998; Lee, 1986). If this antibody is recognizing a CYP isoform in the sea anemone, then the present data indicates that it does not tend to concentrate in any one particular tissue.

As suggested above, the soft region tissues would be expected to concentrate CYP because this region also contains the bulk of the digestive enzymes. These digestive enzymes could convert P450 to P420, which could be detected as an increase in the 418 nm peak. There was a large 418 nm peak associated with each of the tissue regions; however, the amplitude of the 418 peak was approximately 10 times greater in the tough outer region of the sea anemone than any of the other tissue regions. The 418 nm chromophore took 10-20 min to reach its maximum amplitude (data not shown); however, the 418 in each of the tissues increased proportionately within this time period. Whereas the slow rate of development of this chromophore suggests either CO- or DTN-binding as a rate limiting step in achieving an absorbance maximum the substantially larger 418 nm peak in the tough outer region suggests the presence of a different protein. Further, there was never any corresponding decrease in the 450 nm peak as the 418 increase in size. If the 418 nm peak reflected mainly a denatured form of P450, then that would suggest a concentration of CYP in the outer region which is contrary to the kind of tissues that typically concentrate P450, i.e., this tissue is not associated with food processing or xenobiotic accumulation.

A 418 nm chromophore has been observed in other aquatic invertebrates (denBesten, 1990; Livingstone & Farrar, 1984). While most of these studies focused on the internal organs not the outer surface of the organism, Livingstone & Farrar (1984) did report a 415 nm chromophore in tissues throughout the mussel, *Mytilus edulis*, albeit it was mainly associated with the digestive gland (i.e., gills, mantle, foot or rest of the organism). The coral, *Favia fragum.*, which does not have a tough outer surface, contains a 418 nm chromophore, but the amplitude of this is not nearly as great as the sea anemone in the microsomal preparation. A particularly high 420 nm peak has been observed in mouse skin (Pohl *et al.*, 1976), which these authors suggested is due in part to the presence of denatured P450, and partly to the presence of cytochrome oxidase. This (these) chromophore(s) interfered with the detection of P450 by the 420 nm peak.

We are able to report some of the physical characteristics of a 418 nm chromophore in sea anemone tissues, and polemically relate these characteristics to similar chromophores reported in the marine invertebrate literature, however, the actual identity of this chromophore stil remains as a major challenge of marine biochemistry. The wide distribution of a 418 nm chromophore that appears in the CO-binding spectrum in microsomal fractions of several aquatic or marine invertebrates suggests that it is a heme protein distinct from denatured CYP (den Besten, 1990; Livingstone & Farrar, 1984). The chromophore is consistently observed, despite the use of protease inhibitors, antioxidants, and glycerol in the microsomal preparations, a practice that has been routine in the last decade in studies of marine invertebrate proteins that appear in the peer-reviewed literature, including the biochemistry and physiology literature. Further, the extinction coefficient has not been determined for any of the chromophores described herein in an aquatic invertebrate, as purification to homogeneity of functional heme-proteins from such organisms has not been achieved. In the case of denatured CYP and from mammalian paradigms, which are empirically based on the fact that denatured P450 forms P420, unless the extinction coefficient is significantly different, many of the lower invertebrates would have P450 contents relatively equivalent to mammalian microsomes.

There is some evidence to suggest the 418 nm chromophore of invertebrates may be a reflection of the presence of peroxidase enzymes. For example, the 418 nm peak found in bean microsomes has been isolated and identified as containing two peroxidases (Ref). Nelson *et al.* (1976) found that earthworm (*Lumbricus terrestris*) microsomes, which shows a 418 nm chromophore in the CO-difference spectrum, contained peroxidase activity, while rat microsomes, which do not contain this chromophore, did not, suggesting that the 418 nm peak of the earthworm is associated with a peroxidase. Studies in our laboratory have shown that sea anemone columnar microsomes catalyse oxidation of vanillin, caffeic acid, ferulic acid and gaiacol, all classical activities of various peroxidase enzymes (Heffernan and Winston, unpublished results). However, salt extraction, which effectively separated the 418 nm peak from P450 in beans was not effective for this purpose in our sea anemone preparations.

The microsomal fraction of each of the sea anemone tissue regions studied contained peaks in the 500-700 nm range under CO-liganded, DTN-reduced conditions; however, the spectral pattern detected varied significantly in the different tissue regions. The detection of two peaks at 535 nm and 569 nm in the outer region was consistent with observations in mollusc digestive gland microsomes (Livingstone & Farrar, 1984). Two peaks were also detected in the soft region, but the peaks were at 548 and 576 nm. A similar chromophoric pattern with maxima at 548 and 573 nm was found in DTN-reduced, CO-liganded hepatopancreas microsomes of the crayfish, *P. clarkii*, (Jewell & Winston, 1989). However, this spectrum was devoid of P420 and contained only P450 in the 400 - 500 nm region (the Soret region). Two peaks within the 500-600 nm region are typically observed under reduced conditions in the presence of CO in most b-type cytochromes, including purified P420. Similar chromophores also are found in a peroxidase isolated from Halobacterium sp. (Fukumori *et al.*, 1985). In contrast, two peaks are observed in purified mammalian or fish (leaping mullet) P450 in the presence of CO under oxidized conditions, but not under reduced conditions (Schenkman & Kupfer, 1982; Omura & Sato, 1964; Sen & Arinc, 1998). Similarly, partially purified P450 from the marine crab, *Libinia emarginata*, contained two peaks at approximately 522 and 567 nm under oxidized conditions and only one peak under reduced conditions at about 558 nm. Thus, the complexity of spectral analysis of microsomal chromophores is noted, albeit, in the case of peroxidases, CYP and other cytochromes, spectrophotometric properties of the subcellular fractions that contain these proteins is quite consistent with those of the purified enzymes (numerous references).

At this time, it is impossible to identify the protein(s) from sea anemone microsomes that resulted in the peaks between 500-700 nm. However, their detection in the presence of DTN and CO does indicate that they are heme-proteins. The differences in the spectral characterisitics between each of the tissue regions indicate that there may be multiple heme-proteins that could be interfering with the detection of the P450 in the sea anemone. This may account for some of the difficulty in obtaining spectral characteristics, particularly in microsomes prepared from the entire columnar region.

Each of the tissue regions from *A. xanthogrammica* contained EROD activity. The highest activity was found in the tentacles, which was unexpected, since in the sea anemone, *Bundosoma* cytochrome c reductase activity was substantially lower in microsomes prepared with the tentacles (Heffernan and Winston, unpublished). Unfortunately, EROD activities could

not be compared because in *B. cavernata* EROD activity was not detected, whether microsomes were prepared from sea anemones with the tentacles intact or not. The primary difference between these two species is that *A. xanthogrammica* contains either an algal or diatom symbiont in the tentacles and oral disk, while *B. cavernata* is symbiont-free. It is possible that the higher activity observed in the *A. xanthogrammica* tentacles was due the symbiont, instead of the actual tentacles. Antioxidant enzyme activity was high in tissues containing algal symbionts (Dykens and Shick, 1982), and given the potential for exposure to phytochemicals present in symbionts, it is not unreasonable to postulate that significant MFO activity in sea anemones would be associated with the presence of symbionts.

The outer region in the one individual (AX 5/24/97) contained the lowest NAD(P)H-EROD activity, while in the second individual the NADH-EROD activity was not quite as low and the NADPH-EROD activity was actually relatively high. The difference in EROD activity in the outer region between these two individuals may be individual variability or variability in the amount of the algal symbiont from the oral disk region present during the preparation of microsomes. There was no corresponding difference in the P450 content.

Microsomes prepared from the soft and inner regions of the sea anemone, which do not contain symbiont, catalyze EROD activity. EROD activity and CYP content of these tissue regions were similar. In most aquatic invertebrates studied, both of these regions, particularly the soft region have higher concentrations of P450. However, there was no clear enhancement of EROD activity in either of these tissue regions. It would be worth reexamining this species after removing the algal symbiont. It is recognized that various contributions from the symbiont to the MFO parameters studied are feasible. Nevertheless, the presence of the 450 nm peak, the particularly large 418 nm peak, the EROD activity, and the cross-reactivity with the CYP2K antibody are properties of the sea anemone and not the symbiont, as evinced by the fact that each of these properties is observed in the symbiont-free sea anemone species, *B. cavernata* (Heffernan *et al.*, 1996; Heffernan & Winston, 1998).

Typically, large differences in MFO properties (cytochromes content, MFO activities, etc.) were observed between different tissues, while in a few studies the differences were very small. For instance, Stegeman (1985) found the CYP content in digestive gland to be four-fold greater than that of hepatopancreas in *M. edulis*, while there was less than a 16% difference in P450 content of the digestive gland and hepatopancreas in *Arca zebra*. Further, NADPH-B[a]P hydroxylation and NAD(P)H-cytochrome c reductase activities followed the same trend in these species. There is also a greater degree of variation in the species with the bigger difference in MFO properties between different tissues; the B[a]P activity appears to differ more than is apparent. Little variation was observed in the freshwater crayfish, *Cambarus* (Khan *et al.*, 1972), between tissue types, while significant variation was observed in the crayfish, *Procambarus clarkii* and *Astacus astacus* L. (Jewell & Winston, 1989; Linstrom-Seppa *et al.*, 1983). The possibility that differences in MFO properties would be more evident between the different tissue regions in another species of sea anemone cannot be rigorously excluded.

Based on the present data it does not appear that CYP is concentrated in any particular tissue in *A. xanthogrammica.* den Besten (1990) found that the echinoderm also did not concentrate CYP in a particular tissue as does the mollusc. He suggested that the presence of a

circulatory system may play a role in distribution of P450 in the invertebrates. In *M. edulis*, P450 was only found in the digestive gland and MFO associated activities were relatively low in other tissues (Livingstone & Farrar, 1984). In the sea star (*A. rubens*), MFO activity was also present in the gonads and stomach (den Besten, 1990). This author suggests that the mussel contains a circulatory system, so that one organ might have a more dominant role in detoxification and endogenous MFO system functions. The sea star and the sea anemone lacks a finite circulatory system which might explain the wider distribution of the MFO system among different tissue types studied.

We present evidence that small differences in CYP content might not be detected in the presence of other heme proteins, such as the 418 nm chromophore, which interferes with the detection of the 450 nm peak. Based on the spectral properties of the 500-700 nm region, additional heme-protein(s) appear to be present and variable between each tissue region. Until these heme-proteins are eliminated from the sample, spectral properties may be of minimal use in quantifying CYP content. As in sea anemones, a large 418 nm chromophore appears in the CO-binding spectrum of echinoderm microsomes, which may account for similar results reported in studies of microsomal MFO in these organisms. Addition of KCN to dithionite-reduced microsomes resulted in a difference spectrum with a maximum at 410 nm and a minimum at 430 nm. The iron center of the CO-liganded, DTN-reduced P450 is in a high spin state. When KCN is added in place of CO a low spin state results with a corresponding shift in the spectral properties contributed by heme (Huheey *et al.*, 1993). Omura & Sato (1964) found that KCN added to the CO-liganded, DTN-reduced rat liver microsomes did not alter the spectral properties, a finding that is corroborated in our studies with sea anemone microsomes. Taken as a whole our data with KCN indicates the presence of heme proteins in sea anemone microsomes with observable and alterable spectral properties upon bonding of KCN ligand.

Although the present studies were of only two animals and must be regarded as a preliminary analysis of the distribution of CYP and probably other CO-binding proteins in sea anemone tissue regions, a thorough literature review (Heffernan and Winston, 1998) indicates this study as the first analysis of this type in any cnidarian. The use immunohistochemical techniques clearly would compliment and advance such study as in the case of studies on the distribution of antioxidant enzymes in the coral and sea anemone (Hawkridge *et al.*, personal communication). Finally, we have employed biotinylated secondary antibodies in our immunoblot analyses. It has been suggested that sea anemone contain a high content of biotinylated proteins, which might lead to mendacious interpretation of our data.

CHAPTER 5

METABOLISM OF NITROARENES BY MICROSOMAL FRACTIONS OF THE THE SEA ANEMONE, *BUNODOSOMA CAVERNATA* AND THE COMMON MARINE MUSSEL, *MYTILUS EDULIS* AND GENERATION OF FREE RADICALS

Caroline A. Metosh-Dickey , Linda M. Heffernan and Gary W. Winston*

INTRODUCTION

Nitroarenes are indicated to be widespread throughout aquatic and marine ecosystems worldwide (Livingstone, 1991; Winston and Di Giulio, 1991) and readily accumulate in tissues of aquatic organisms (Winston and Di Giulio, 1991). The metabolic fate of nitroarenes is a function of various enzymes including those that can reduce the nitro group. Reduction of these compounds by mammalian enzymes has been well studied, while nitroaromatic reduction by submammalian species, especially invertebrates is not well characterized. These enzymes show preference for nitro group reduction by either by one- or two-electron transfer. Both pathways of nitro reduction have been suggested for the bivalve molluscs, *Mercenaria mercenaria* and *M. edulis*. (Carlson, 1972; Garcia-Martinez *et al.*, 1992, 1995). *p*-Nitrobenzoic acid reduction to its corresponding amine was catalyzed by the cytosolic fraction of *M.edulis* digestive gland and 1-nitropyrene was reduced to 1-aminopyrene by digestive gland cytosolic and microsomal fractions, all under anaerobic conditions (Hetherington et al, 1996). Hepatopancreas cytosol and microsomes of the crab *Carcinus maenus* also catalyzed these anaerobic reductions in the same study. These results of Hetherington *et al.* (1996) were interpreted to mean that 2-electron reduction of the nitro group had occured under anaerobic conditions. This is consistent with the idea that 2-electron metabolites remain stable in the absence of air as they can not autoxidize to free radical products.

Under aerobic conditions, the oxygen concentration within the tissues of organisms plays an important role in nitroarene metabolism. Univalent reduction of many nitroaromatic compounds generates nitro anion radicals (Mason, 1982; Metosh-Dickey *et al.*, 1999), which can act to directly damage membranes, proteins, or DNA (Biaglow, 1981; Mason, 1982; Metosh-Dickey *et al.*, 1998). Additionally, nitro anion radicals redox cycle with molecular oxygen (O_2) producing superoxide anion radicals ($O_2^{\cdot-}$) while regenerating the parent compound. Under aerobic conditions *M.edulis* digestive gland microsomal, mitochondrial and cytosolic fractions catalysed NAD(P)H-dependent, iron-mediated oxidation of the hydroxyl radical scavenging agent, α-keto-γ-methiolbutyric acid (Winston *et al.*, 1990; Hetherington *et al.*, 1996). Thus, in air 1-electron nitroreduction appears more widely distributed throughout digestive gland subcellular fractions.

Further evidence for the presence of 1-electron nitro group reduction in microsomal fractions of *M. edulis* is the enhancement of O_2 consumption by microsomes upon incubation with the nitroaromatic compounds 4-nitroquinoline N-oxide (4-NQO) and nitrofurantoin (Garcia-Martinez *et al.*, 1992, 1995). When catalase was added to the reaction cell after allowing oxygen to be consumed for a period of time, a liberation of oxygen was observed indicating that hydrogen peroxide was produced in the reaction, presumably through disproportionation of superoxide anion radical. *M. edulis* microsomal fractions have also been shown to activate 4NQO to more mutagenic products while reducing the mutagencity of nitrofurantoin (Garcia-Martinez *et al.*, 1992), and 1-nitropyrene was activated to bacterial mutagens by subcellular fractions of digestive gland of *M. edulis* (Marsh *et al.*, 1992). Whether mutagenic expression was the result of one- or two-electron reduction remains to be established. These organisms and other marine invertebrates contain antioxidant enzymes and compounds that can enhance or reduce the mutagenicity of xenobiotic compounds (Garcia-Martinez *et al.*, 1992; Jewell and Winston, 1989). In vivo metabolism of certain nitroarenes occurs in molluscs (Hansen *et al.*, 1972; Landrum and Crosby, 1981a), crustaceans (Foster and Crosby, 1987) and echinoderms (Landrum and Crosby, 1981b) as evinced by their abilities to excrete corresponding conjugated metabolites of nitroarenes. Also, by inference, the presence of antioxidants *in vivo*, NAD(P)H-dependent oxidation of KMBA by microsomal electron transport, detection of lipid peroxidation products and xenobiotic activation of dihydrorhodamine 123 in digestive gland and hemolymph lysosomes indicate the potential for free radical production and within the organism.

All of the above evidence for 1-electron nitro group reduction of nitroarenes by marine inveretebrate models is indirect (Garcia-Martinez *et al.*, 1992, 1995; Hetherington *et al.*, 1996). Based on the data presented in Chapter 1 of this report indicating the presence of active NAD(P)H-dependent flavoprotein reductases, we reasoned that these reductases could catalyze the univalent reduction of nitroaromatic compounds thereby generating free radical intermediates, which in turn could redox cycle and generate reactive oxygen species. The present study is the first to report direct evidence that microsomal electron transfer by marine invertebrates (common marine mussel and the sea anemone) catalyze univalent reduction of nitroaromatic compounds to a nitro anion radical. Mussel digestive gland microsomes and sea anemone columar microsomes were incubated in the presence of 4-nitropyridine N-oxide (4NPO) or 4-nitroquinoline N-oxide and scanned using electron spin resonance (ESR) spectroscopy. Because flavoproteins have been strongly implicated as catalysts of univalent nitro group reduction we also studied the potential of this class of proteins to participate in microsomal one-electron metabolism of 4NPO. Mussel microsomes catalyze the enhancement of O_2 consumption by nitroaromatic compounds (Garcia-Martinez *et al.*, 1995). Therefore, a spin trapping technique was used in conjunction with ESR spectroscopy to verify that enhancement of microsomal-catalyzed O_2 consumption by nitroarenes is indeed attributable to $O_2^{\cdot-}$ production. We compared sea anemone-dependent metabolism with mussel and rat as positive controls; the former because it has been reported to have enhanced microsomal oxygen consumption in the presence of nitroaromatic compounds and the latter because the ESR signal of the nitroanion radical and the spin trapped adduct signal of superoxide anion are well characterized in mammals.

MATERIALS AND METHODS

Materials. Tris-HCl, sucrose, ethylenediamminetetraacetic acid (EDTA), dithiothreitol (DTT), soybean typsin inhibitor, NADPH, NADH, nitrobenzene (NB), 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), Chelex-100 resin, 4-nitropyridine-N-oxide (4NPO), desferrioxamine mesylate (deferryl), p-methylsulfonylfluoride (PMSF), sodium azide (NaN3), p-chloromercuribenzoate (PCMB), glucose oxidase, xanthine oxidase (XO), potassium chloride (KCl)were purchased from Sigma Chemical Co., St. Louis, Missouri. Glucose, potassium phosphate, cupric sulfate ($CuSO_4$), were purchased from Fisher Scientific, Pittsburgh, Pennsylvania. DMSO was purchased from Mallenckrodt Specialty Chemicals Co., Chesterfield, MO.

Preparation of mussel microsomes. Microsomes were prepared from mussels as described by Garcia Martinez *et al.*, (1995). Digestive glands were removed, pooled and homogenized in homogenization buffer of 20 mM Tris-HCl, pH 7.6, containing 0.5 M sucrose, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 0.15 M KCL. All procedures were completed on ice and the fractions were obtained by successive centrifugations of 500g X 30 min, 8500g X 30 min, 14,500g X 30 min and 105,000g X 1 h. Microsomes were resuspended in buffer without PMSF and stored at -80°C. The microsomal protein concentration was determined by the flourescamine protein assay (Lorenzen and Kennedy, 1993).

Preparation of rat liver and sea anemone microsomes. Sea anemone microsomes were prepared as described by Heffernan *et al.*, (1996). Before homogenization tentacles were carefully removed. Animals were then pooled and homogenized in buffer of 50 mM Tris-HCl, pH 7.6 containing 250 mM sucrose, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 1 mg/ml soybean trypsin inhibitor. All procedures were completed at 4°C and fractions were obtained by successive centrifugations of 2000g x 5 min, 8500g x 15 min, 14,000g x 15 min and 105,000g x 1 h. The resulting pellet was resuspended in resuspension buffer of 50 mM Tris-HCL, pH 7.4 with 250 mM sucrose and no inhibitors. Microsomes were stored at -80°C. The microsomal protein concentration was determined by the flourescamine protein assay (Lorenzen and Kennedy, 1993). Rat liver was homogenized in 4 volumes of 0.25 M Sucrose, 10 mM TRIS-HCL, 1 mM EDTA (STE) buffer, pH 7.4. After initial centrifugation at 26,000 xg (4°C) for 20 min, the obtained supernatants were further centrifugated at 100,000 xg (4°C) for 1hour. The resulting pellet was resuspended in resuspension buffer of 50 mM Tris-HCL, pH 7.4 with 250 mM sucrose. Microsomes were stored at -80°C. The microsomal protein concentration was determined by the flourescamine protein assay (Lorenzen and Kennedy, 1993).

Electron Spin Resonance spectroscopy. ESR spectroscopy was conducted at room temperature using a quartz flat cell in a Bruker ESP 300 instrument fitted with a TM8490 cavity. The instrument was operated at 9.78 GHz, 10 mW microwave power unless otherwise stated and 100 kHz modulation frequency. Other instrumental conditions are given in the Figure legends. For anaerobic samples the 1.5 ml incubations were bubbled with nitrogen gas for 5 min before being initiated by the addition of 25 µl of appropriate cofactor and transferred to a nitrogen filled flat cell capped at both ends. Since nitro anion radicals are more stable at alkaline pH (12) a 100 mM potassium phosphate buffer, pH 9.6, was used in all anaerobic experiments. Final concentrations are listed in the Figure legends. For the aerobic experiments a 100 mM potassium

phosphate buffer, pH 7.4, which had been passed over a Chelex-100 resin to remove trace metals, was used. Final concentrations for all chemicals and proteins are stated in the Figure legends.

RESULTS

Nitroaromatic compounds are commonly found in terrestrial and aquatic environments (Winston and Di Giulio, 1991). Enzymes which reduce nitroaromatic compounds have been reported in various invertebrates (Garcia Martinez *et al.*, 1992, 1995; Hetherington, 1996). Digestive gland microsomes from the common marine mussel *Mytilus edulis* L. catalyze NAD(P)H-dependent reduction of 4NQO and various other nitroaromatic compounds with concomitant enhanced O_2 consumption (Garcia Martinez *et al.*, 1992, 1995). It can be inferred from these observations that a radical intermediate was involved, albeit this has not been directly demonstrated in an invertebrate model. Herein, we show that mussel digestive gland microsomes and sea anemone columnar microsomes univalently reduce 4NPO and presumably other nitrocompounds to produce a radical product (Fig. 5.1).

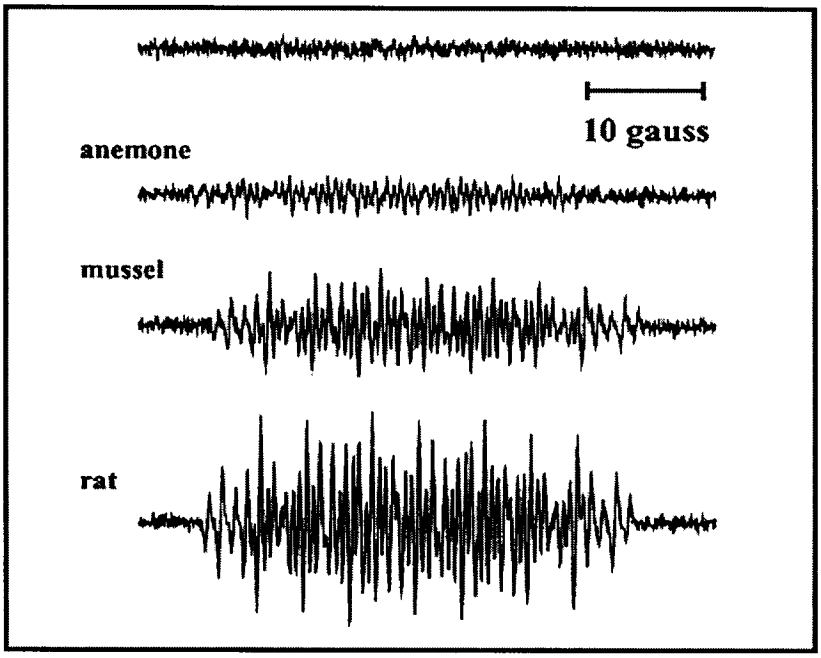


Figure 5.1. Electron Spin Resonance Spectra of Anaerobic NADH-dependent Reduction of 4-Nitropyridine N-Oxide and Formation of Nitroanion Radical by Sea Anemone Columnar Mussel Digestive Gland and Rat Liver Cytosolic Fractions. Instrument settings: Sweep width 50 G, gain 6.3 x 105, modulation 0.32 G, scan time 10.49 sec, Time constant 1.28 msec, scanned 48 times (8 min), 9.79 GHz, 10 mW power.

This nitroarene yields a distinct ESR signal which can be computer-simulated from known hyperfine splitting constants (Metosh-Dickey *et al.*, 1997a&b). As do mammalian rat microsomal systems, mussel microsomes use both NADH and NADPH to reduce 4NPO to its

nitro anion radical although NADH appears to be a preferred cofactor for mussel microsomes. Additionally columnar body microsomes from sea anemones catalyze one-electron NADH-dependent reduction of 4NPO at a much lower level per mg of protein than the other species shown here (Fig. 5.1). NADPH-dependent reduction of 4NPO to a free radical product was not evident under the conditions used (data not shown). The effect of flavoprotein inhibitors was studied to determine if a role for these enzymes exists in the production of the radical signal from the interaction between mussel microsomes and 4NPO. In support of this role for flavoprotein reduction of 4NPO, the addition of 240 μM $CuSO_4$ or PCMB completely suppressed the free radical signal produced by mussel microsomal reduction of 4NPO (Fig. 5.2).

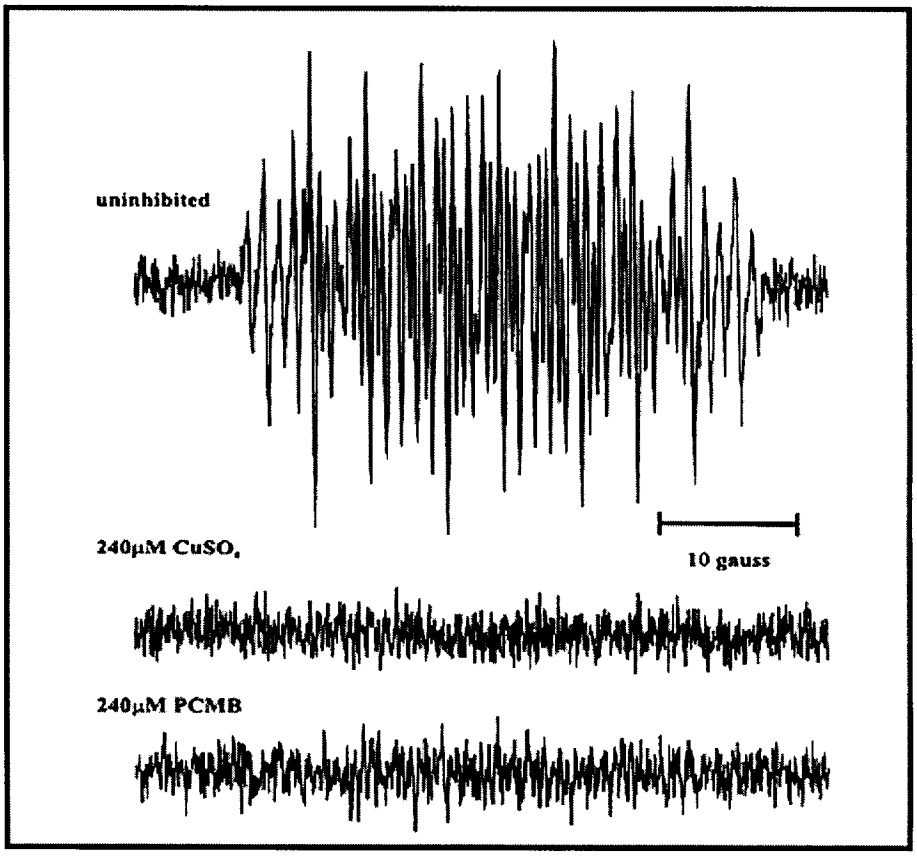


Figure 5.2. Electron Spin Resonance Spectra Showing the Effect of Flavoprotein Reductase Inhibitors on Nitroanion Radical Formation by Cytosolic Fractions of *M. Edulis*. Instrument settings were as in Fig. 5.1.

M. edulis digestive gland microsomes have been shown to enhance O_2 consumption in the presence of nitroaromatic compounds (Garcia Martinez *et al.*, 1992, 1995). It is assumed that this enhancement of O_2 consumption results from one-electron reduction of the nitro compound the product of which enters a redox cycle with O_2 producing $O_2^{\cdot-}$ at the expense of O_2. We detected $O_2^{\cdot-}$ when 4NPO was incubated with mussel microsomes and NADH (Fig 5.3, spectrum A).

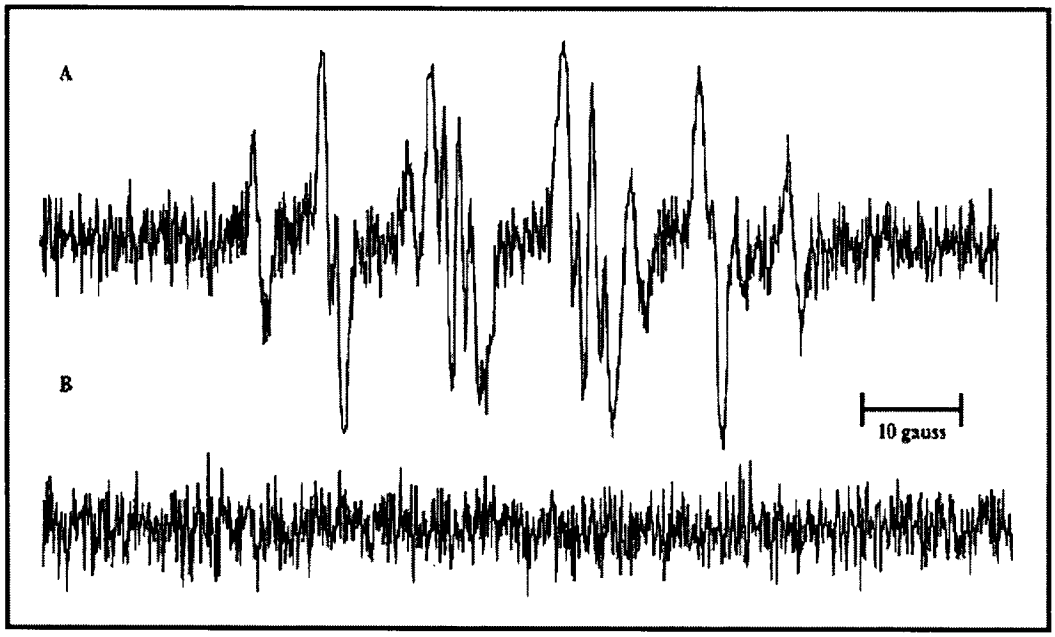


Figure 5.3. Electron Spin Resonance Spectra of Superoxide Anion Radical Formed During Aerobic Reduction. Instrument settings were as in Fig. 5.1.

If microsomes, 4NPO, DMPO or cofactor was left out of the reaction mixture the spectrum of Figure 5.3, spectrum B was obtained.

DISCUSSION

Nitroaromatic compounds found throughout the environment are consumed and metabolized by all animals. Nitro-reducing enzymes have been well characterized in mammals, especially rats while other species, particularly invertebrates, have not been as well studied. Microsomal fractions of mussel *(M. edulis)* digestive gland display enhance rates of O_2 consumption when incubated in the presence of nitro aromatic compounds and NAD(P)H, which indicates their ability to metabolize these compounds (Garcia Martinez *et al.*, 1995). This also implies the production of nitro anion radicals, which in turn can undergo redox cycling with molecular oxygen. There remains controversy over which enzymes are actually responsible for nitroarene reduction *in vivo* (Rosenkranz *et al.*, 1982; Kinouchi and Ohnishi, 1983; Bryant *et al.*, 1984; Metosh-Dickey *et al.*, 1999). Two-elctron reduction of nitro groups by invertebrates is also probable as indicated by the formation of aniline from nitrobenzene in the snail *Physa sp.* and crustacean *Daphnia magna* (Lu and Metcalf, 1975). The governing factors in whether a nitro group will be reduced to a radical or non-intermediate product are complex, involving, as indicated in the introduction to the present report, oxygen concentration, enzyme involvement, cofactor availability and the oxidation-reduction properties of the molecule in question

(Biaglow, 1981). Regarding the last possible factor it has been suggested, based upon electrochemical reduction measurements of DNP, that 1,3-DNP is a preferred one-electron acceptor, and 1,6- and 1,8-DNP preferred two-electron acceptors (Howard *et al.*, 1987; Klopman *et al.*, 1984).

In our species comparison of sea anemone and mussel microsomes, all were shown to produce 4NPO nitro anion radical indicating the presence of a single-electron transferring enzyme throughout the animal kingdom capable of reducing environmental pollutants to radical species. Both NADH and NADPH were capable of acting as electron donors for the reduction of 4NPO by mussel microsomes although NADH gave the more prominent signal. Sea anemone microsomes catalyzed a very low level radical yield detectable only with NADH. The higher level of NADH- than NADPH-flavoprotein reductase activities found in various tissues of *M. edulis* (Garcia Martinez *et al.*, 1995) and sea anemones (Heffernan and Winston, 1998) are likely responsible for the generally higher rates of 1-electron with NADH than NADPH. This indicates the presence in mussel microsomes of either an enzyme which can use both cofactors or the presence of more than one one-electron transferring enzyme. The suppression of the mussel microsomal mediated radical signal by $CuSO_4$ and PCMB is consistent with the presence of a flavoprotein catalyzing 4NPO reduction. A role for flavoprotein reductases in the redox cycling of nitroaromatics in 1-electron reduction of nitroarenes has been established for mammals (Mason, 1982) and studies of inhibitors of these enzymes indicate the same for flounder liver microsomal reduction of nitroarenes (LeMaire and Livingstone, 1994). Probable microsomal enzymes include cytochrome P450 and b_5 reductases and DT-diaphorase, which are present in specific tissues of mussel (Livingstone and Farrar, 1984; sea star (den Besten *et al.*, 1991) and and crustaceans (Livingstone, 1991). Further, dicoumarol-sensitive 4-nitroquinoline N-oxide reductase activity has been shown in digestive gland of *M. edulis in vitro.* Garcia-Martinez *et al.* (1992, 1995) showed that certain nitroaromatic compounds enhance mussel microsomal O_2 consumption. Here we show that $O_2^{\cdot -}$ is produced when mussel microsomes are incubated with 4NPO and NADH. This is consistent with O_2 consumption data of Garcia-Martinez *et al.* (1992; 1995) who showed that mussel digestive gland microsomes enhance O_2 consumption in the presence of nitrofurantoin and 4-nitroquinoline-N-oxide. Xanthine and aldehyde oxidases are flavoprotein reductases implicated in nitro group reduction; these enzymes are inhibitable by allopurinol and such inhibition has not been reported to occur in marine invertebrate systems. This is consistent with the findings of Dykens and Shick (1988) who did not detect xanthine oxidase in *M. edulis.*

Mussels and sea anemones periodically exist in hypoxic environments. Here, nitro anion radicals formed from one-electron reduction of nitro compounds are more stable and do not redox cycle. The consequences of anaerobic reduction of nitroarenes vs aerobic reduction with regard to their toxicity and mutagenicity is indicated to be quite different. In the former, oxyradicals are not generated and toxicity is a function of the xenobiotic radical or further reduced non-radical metabolites. In the latter, oxyradical production is proliferated via a redox cycle. Our data indicate that those compounds which are the best redox cyclers are not necessarily the more mutagenic (10). Interestingly however, cytotoxicity does appear to correlate with oxyradical production from this route. Metabolic activation of environmental pollutants to more toxic and mutagenic intermediates is of importance to all organisms. Although induction of various ideopathic lesions in organisms such as sea anemones and mussels

may not be of immediate concern to the human population at-large, it is a measure of pollution and may be used to detect and characterize the spread of pollution in the environment. Understanding how these organisms metabolize various compounds will help us to define the fate and effect of these compounds at the ecosystem level.

CHAPTER 6

A NEW ASSAY FOR MEASURING OXYRADICAL SCAVENGING BY SEA ANEMONE AND OTHER MARINE INVERTEBRATE TISSUES

Gary W. Winston and Francesco Regoli

INTRODUCTION

Formation of reactive oxygen species (ROS) in aerobic organisms is an unavoidable consequence of the coupling of oxidative phosphorylation of ADP with the reduction of molecular oxygen by four electrons to water. Other sources of ROS production include microsomal and photosynthetic electron transport chains, active phagocytosis and the activity of several enzymes, e.g. xanthine oxidase, tryptophan dioxygenase, diamine oxidase, prostaglandin synthase, guanyl cyclase and glucose oxidase, which produce different ROS as intermediates (Halliwell, 1978).

Xenobiotics and environmental pollutants may increase the intracellular formation of ROS through the Fenton reaction involving trace metals such as iron and copper (Halliwell and Gutteridge, 1984; Winston *et al.*, 1984) or redox cycling of several classes of organic compounds (Kappus, 1986; Winston *et al.*, 1990). During redox cycling certain molecules are reduced to their corresponding free radical, which rapidly donates its free electron to molecular oxygen producing the superoxide anion radical (O_2^-) and regenerating the parent compound to undergo another cycle (Kappus, 1986; Winston *et al.*, 1990). To counteract the biological formation of ROS, cells have evolved antioxidant defenses of specially adapted enzymes (superoxide dismutase, catalase and glutathione peroxidase) and smaller molecules such as vitamin E and β-carotene as free radical scavengers in membranes, and ascorbic acid, uric acid and glutathione for aqueous phases. Intracellular production of ROS does not necessarily imply cellular toxicity, but oxidative stress will occur when the balance between ROS formation and antioxidant defenses is exceeded. Oxidative stress has been implicated in damage to proteins, enzyme inactivation, peroxidation of lipid membranes, DNA alteration (Cohen and d'Arcy Doherty, 1987; Winston and Di Giulio, 1991; Di Giulio *et al.*, 1989) and pathologies including carcinogenesis, reperfusion injuries and inflammation (Cutler, 1991).

Studies on oxidative stress have classically been approached by analysis of specific, single antioxidants, their modes of action and responses to different stressors. Variations in the levels of antioxidant defenses have been used to indicate ROS-mediated toxicity (Winston and Di Giulio, 1991). This approach is useful in understanding specific relationships between stressors and antioxidants, albeit from such data it is difficult to quantify actual biological resistance to oxidative stress. For example, reduced levels of an antioxidant may not result in oxidative damage if a tissue is compensated for by the presence of other defenses. Antioxidants may act cooperatively to provide organisms with greater protection against ROS toxicity than expected by contributions of antioxidants acting alone (Wayner et al, 1986a; 1986b).

A measure of the total absorbance capacity of oxyradicals by a tissue can provide better understanding of an resistance to toxicity caused by ROS and methods have been proposed for this purpose. Wayner *et al.*(1985) measured the time required to obtain maximum oxygen consumption in a system containing plasma peroxidizable material, a free radical generator and plasma or specific antioxidants. By relating the results to the time of induction obtained with a known amount of the water-soluble vitamin E analogue, Trolox, a quantitative measure of the total radical-trapping antioxidant parameter (TRAP) was defined (Wayner *et al.*, 1985; Wayner et al, 1986a; 1986b). Glazer (1988) described a method to assess chemical damage caused by peroxyl and hydroxyl radicals to the protein phycoerythrin (PE) by measuring the decrease in its emission fluorescence as PE was oxidized. The inhibition of this loss of fluorescence by different molecules was used to screen for free radical scavengers (Glazer, 1988; De Lange and Glazer, 1989). Cao *et al.*, (1993) and Ghiselli *et al.*, (1995) proposed modifications of the Glazer appoach to quantify antioxidant behavior. The kinetics of PE fluorescence decay caused by peroxyl radicals is linear, but in the presence of an antioxidant there is a period of protection followed by rapid loss of fluorescence. Cao *et al.* (1993) used the difference between the area under the kinetic curve of the sample and that of the control (net protection area), assigning 1 oxygen radical absorbance capacity (ORAC) unit to the net protection area obtained with 1 μM Trolox. Ghiselli *et al.*, (1995) measured the lag-phase, i.e. the time of complete protection of PE provided by plasma antioxidants.

In this chapter, we describe a new, reliable approach based on the reaction between peroxyl radicals (or hydroxyl and alkoxyl radicals) and α-keto-γ-methiolbutyric acid (KMBA) which is oxidized to ethylene (Winston and Cederbaum, 1986) upon reaction with various ROS (Equation 1).

$$CH_3S\text{-}CH_2\text{-}CH_2\text{-}CO\text{-}COOH + {}^{\cdot}OOH(R) \text{ or } {}^{\cdot}OH(R) \rightarrow$$
$$1/2(CH_3S)_2 + H(R)OO^- \text{ or } H(R)O^- + CO_2 + CH_2{=}CH_2\uparrow \qquad (1)$$

Ethylene is easily measured by gas chromatographic analysis of aliquots removed from the head-space of the reaction cell. Partial inhibition of ethylene production is the basis of the Total Oxyradical Scavenging Capacity (TOSC) Assay for antioxidant solutions or biological tissues. Ethylene formation in the control reaction does not reach completion during the assay, which obviates against some of the problems reported by other investigators (Wayner *et al.*, 1985; Wayner et a., 1986a; 1886b; Glazer, 1988; De Lange and Glazer, 1989; Cao *et al.*, 1993; Ghiselli *et al.*, 1995), yet permits facile quantification of the oxyradical scavenging capacity of different samples. The gas chromatographic procedure described herein represents a simple alternative to the fluorometric and polarographic methods that have been developed and extends the capability of assessing antioxidant absorbance capacity to many investigators who may not have ready access to the other technologies. Analytical details of the procedure are described and validation of the method is provided with different antioxidant solutions and biological fluids.

MATERIAL AND METHODS

Chemicals. Ascorbic acid, bovine serum albumine (BSA), butylated hydroxyanisole (BHA), α-keto-γ-methiolbutyric acid (KMBA), melatonin, desferroxamine, reduced glutathione (GSH), oxidized glutathione (GSSG) and uric acid were puchased from Sigma Chemical Co. (St Louis, MO, USA); 2,2'-azobis-amidinopropane (ABAP) was obtained from Wako Chemicals (Richmond, VA, USA) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was from Aldrich Chemical Co. (Milwaukee, MI, USA).

TOSC-Assay. Peroxyl radicals were generated by the thermal homolysis of ABAP at 35 and 39°C. Preliminary experiments (see Results) indicated as appropriate assay conditions, 0.2 mM KMBA and 20 mM ABAP in 100 mM potassium phosphate buffer, pH 7.4. TOSC values were measured for different soluble antioxidants (GSH, ascorbic acid, uric acid, Trolox, BHA) and for oxidized glutathione, BSA and biological fluids. Although the rate of radical input is about 2-fold greater at 39 than at 35°C the TOSC values are not effected. Some combinations were also tested to detect possible synergistic interactions.

Reactions were carried out in 10 ml rubber septa-sealed vials in a final volume of 1 ml. Reactions were initiated by injection of 100 µl of 200 mM ABAP directly through the rubber septum. Ethylene production was measured by gas-chromatographic analysis of 1 ml aliquots taken directly from the head space of the reaction vials. By staggering the starting times for each vial 8-10 serial samples could readily be monitored in sequence at 12 min intervals. Analyses were performed with a Hach-Carle (Series 100 AGC) gas chromatograph equipped with a 6 foot Poropack N column (Supelco) and a flame ionization detector (FID). The oven, injection and FID temperatures were respectively, 60°, 280° and 190°C. Helium was used as the carrier gas at a flow rate of 30 ml/min.

Quantification of Total Oxyradical Scavenging Capacity (TOSC). The area under the kinetic curve was determined from the integrated areas under the least squares regression kinetic curves for both the control and sample reactions. TOSC is quantified according to the equation $TOSC = 100-(\int SA/\int CA \times 100)$ where $\int SA$ and $\int CA$ are respectively, the integrated areas from the curve defining the sample and control reactions. Thus, a sample that displays no oxyradical scavenging capacity would give an area equal to the control ($\int SA/\int CA=1$) and a resulting TOSC = 0. On the other hand, as $\int SA \rightarrow 0$ the hypothetical TOSC value approaches 100. Since the sample area is related to that of the control, the obtained TOSC values are not affected by small variations in instrument sensitivity, reagents or other assay conditions. A specific TOSC was calculated by dividing the experimental TOSC by the molar concentration of the antioxidant or by the µg of biological sample protein in the assay.

Tissue preparation. Rat liver were homogenized in 4 volumes of 0.25 M Sucrose, 10 mM TRIS-HCL, 1 mM EDTA (STE) buffer, pH 7.4. Sea anemone basal stalk, starfish pyloric caeca and mussel digestive gland were homogenized in 4 vol. of 50 mM potassium phosphate buffer, pH 7.5 containing 2.5% NaCl, aprotinin (1mg/mL), leupeptin (1 mg/ml), and pepstatin (0.5 mg/ml). Rat liver homogenization was in the absence of protease inhibitors. These homogenization buffers had no effect on the TOSC assay. After initial centrifugation at 26,000 xg (4°C) for 20 min, the obtained supernatants were further centrifugated at 100,000 xg (4°C) for

1hour. Cytosols were then aliquoted and stored at -80°C. Separation of soluble and proteic fractions was performed by precipitation with 80% saturated ammonium sulfate (Cao *et al.*, 1993) or by use of microconcentrators with a membrane cut-off of 3Kda (Microcon 3, Amicon Inc., Beverly, MA).

RESULTS

Time-dependence of antioxidant protection. Initial studies characterized the TOSC assay with respect to benchmark antioxidants. Time-courses for antioxidant activity of different concentrations of GSH, ascorbic acid, uric acid and Trolox are shown in Figure 6.1. Ethylene production from KMBA was inhibited in the presence of the antioxidants in a dose-dependent manner. Also, the higher the concentration of the antioxidant the longer was the period in which ethylene formation was totally inhibited relative to controls. Plotting the absolute value of the difference between the ethylene peak area obtained at each time point for the sample and control reactions permit visualization of the time in which the oxyradical scavenging capacity of the solution becomes exhausted.

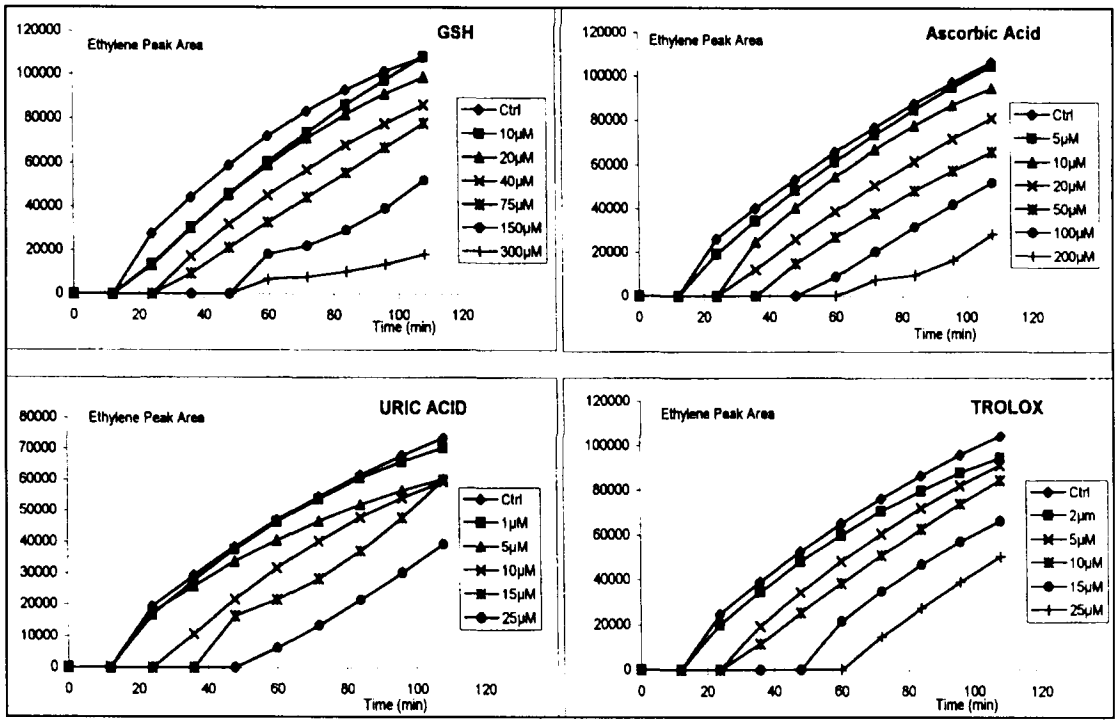


Figure 6.1. Time-courses of Antioxidant Activity of Different Concentrations of GSH, Ascorbic Acid, Uric Acid and Trolox. Y Axis Values Correspond to Relative Integrated Ethylene Peak Areas.

Effect of antioxidant concentration on TOSC. TOSC was determined for different concentrations of GSH, ascorbic acid, uric acid and Trolox are shown in Figure 6.2. A linear relationship was obtained between TOSC and GSH, ascorbic acid, uric acid and Trolox. From the linear range of TOSC values a specific TOSC is obtained by interpolation to a final equivalent of 1 μM. Thus, for these antioxidants the specific TOSC values are 0.84 ± 0.10 for GSH, 2.00 ± 0.11 for ascorbic acid, 3.05 ± 0.27 for uric acid and 4.37 ± 0.57 for Trolox.

TOSC of rat liver cytosol. Prior to characterizing TOSC of marine organism tissues, rat liver cytosol was analyzed as a model biological fluid (Fig. 6.2). The TOSC valuer of whole cytosol was compared to that of the separate soluble (deprotonated) and protein fractions (Fig. 6.3). It is noted that the soluble fraction accounts for the preponderance of the peroxyl radical scavenging capacity of whole cytosol. Under our conditions the TOSC of whole cytosol was 27.1 ± 3.6 (n = 6) corresponding to a specific TOSC extrapolated to 1 μg protein of 0.39 ± 0.02.

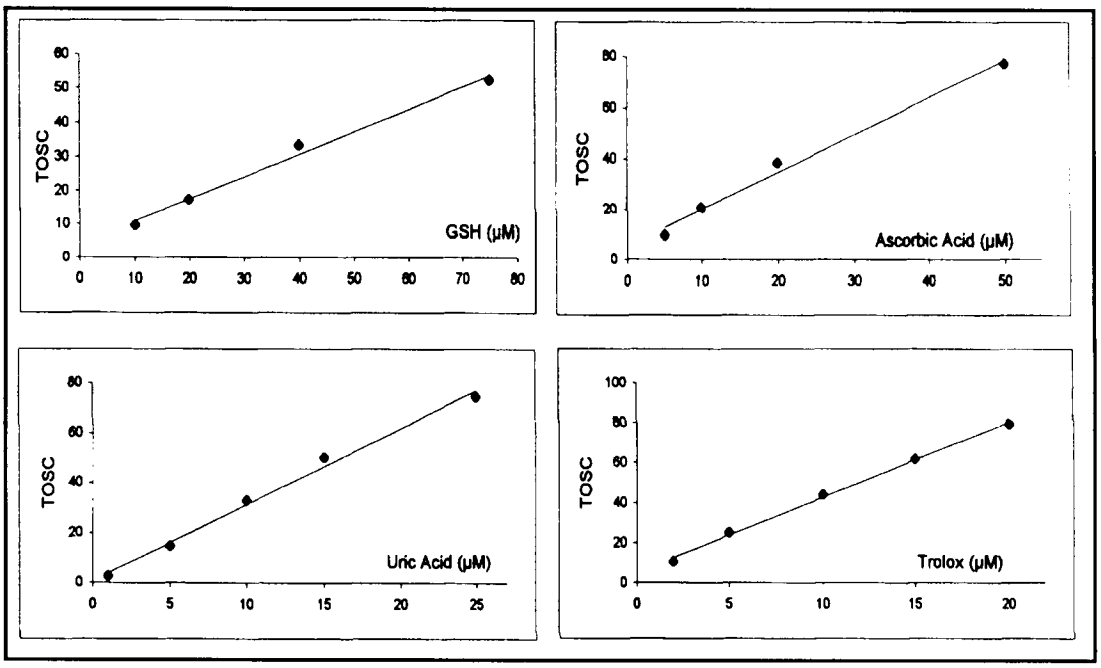


Figure 6.2. Total Oxyradical Scavenging Capacity (TOSC) Values vs. Concentration of Antioxidants.

TOSC of marine invertebrate cytosolic fractions. TOSC was measured in cytosolic fractions isolated from tissues of three marine invertebrates, i.e. digestive gland of the common mussel *Mytilus edulis*, pyloric caeca of the starfish *Leptasterias epichlora* and basal stalk of the sea anemone *Bunodosoma cavernata* (Table 6.1).

Table 6.1.
TOSC of Cytosolic Fractions from Rat Liver and Three Marine Invertebrates

Source of cytosol	TOSC/μg protein
Rat liver	0.39 ± 0.02 [a]
Mussel digestive gland	0.62 ± 0.03
Starfish pyloric caeca	0.74 ± 0.08
Sea anemone basal stalk	1.47 ± 0.06

n=6, [a] Mean ± SEM for 6 determinations

Rat liver was also analyzed for comparison with a mammalian system. Cytosols were diluted to obtain a concentration of 55 ± 8.7 mg of protein in the assay and the same final dilutions were made for the corresponding soluble and proteic fractions. Interspecies variations were observed for TOSC of cytosolic fractions; invertebrate species showed a consistently higher peroxyl radical absorbing capacity compared to rat liver (Table 6.1). From a value of 0.39 ± 0.02 μg protein for rat liver cytosol, TOSC values were 60 to 90% higher in digestive gland of mussel and pyloric caeca of starfish and a maximum value of 1.47 ± 0.076 μg protein was obtained in the basal stalk of the sea anemones. These data reflect the significant capacity of sea anemonies to be protected from oxidative stress caused by organic and metal contamination in their habitat. The relative contribution of soluble and proteic fractions to the TOSC of whole cytosol was quite similar in the three invertebrate tissues and rat liver; soluble antioxidants were responsible for the major protective potential generally accounting for 70-80% of the cytosolic TOSC.

DISCUSSION

Studies of the relationship between a given antioxidant and oxidative stress would be greatly improved by the ability to quantify the actual capacity of a tissue to counteract ROS toxicity. Herein, we report a simple, reliable gas chromatographic assay for measuring the total oxyradical scavenging capacity (TOSCA) of pure antioxidant solutions or biological fluids. Special consideration was given to marine invertebrates where anthoropogenic input of organic chemical and metals from petroleum exploration, harvesting and transporting is significant. The method is based on the oxidation of KMBA to ethylene upon reaction with certain oxyradicals (equation 1) and on the ability of various antioxidants to inhibit this reaction. The coefficient of variation was 2% (n=30) within a run and 6% (n=30) between runs.

Various other methods of merit have been published recently that are similar in concept to the one we present herein. The method described by Wayner *et al.* (1986a) is based on the time necessary to prevent maximum oxygen uptake as an indirect measure of lipid peroxidation

by plasma antioxidants. This approach requires a high dilution of sample to avoid rapid exhaustion of available oxygen; thus, self termination of lipid peroxidation is competitive with termination afforded by antioxidants (Wayner *et al.*, 1986a; 1986b). By sensitizing the reaction with oxidizable material these authors obtained TRAP values (see Introduction) comparable to those measured on neat plasma with a pressure transducer (Burton *et al.*, 1983; Burton and Ingold, 1981). The TOSC assay does not require sample dilution; good linearity was obtained between TOSC values and a wide range of final sample concentrations (data not shown). Also, the oxidation of KMBA to ethylene (and its quantitative inhibition in the presence of antioxidants) is easily measured without addition of sensitizer.

A fluorescence assay was proposed for rapid screening of molecules with potential ROS-scavenging activity (Glazer, 1988; DeLange and Glazer, 1989). This assay was based on the ability of molecules to inhibit damage to phycoerythrins caused by ROS. The measure of oxyradical-induced damage to phycoerythrins (Glazer, 1988; DeLange and Glazer, 1989) depends on the molecular structure of these proteins which contain 34 tetrapyrrole prosthetic groups, and their absorbance and fluorescence properties which are highly influenced by the chemical integrity of the protein (Klotz and Glazer, 1985). Because they have a high absorption coefficient and quantum yield (Glazer, 1984), fluorescence analysis of phycoerythrins is sensitive to very low levels (< than 10^{-12} M).

Compared with methods based on the decay of phycoerythrin fluorescence (Cao *et al.*, 1993), the oxidation of KMBA to ethylene is similarly sensitive to low concentrations of antioxidants. The specific TOSC for 1 μM solutions were calculated to be 0.84 ± 0.10 for GSH, 3.0 ± 0.3 for uric acid, 2.0 ± 0.1 for ascorbic acid and 4.4 ± 0.5 for Trolox. By relating the specific TOSC of various antioxidants to that of Trolox, a relative oxyradical scavenging capacity can be calculated. For soluble antioxidants, the total oxyradical scavenging capacity were of the relative order: Trolox > uric acid > ascorbic acid > GSH.

These present data with marine invertebrates represent the first baseline data sets from a new method with significant potential application in biomonitoring of aquatic pollution and its relationship to oxidative stress. Organic chemicals and metals are substantial sources of oxidative stress in the marine environment (Winston and Di Giulio, 1991). The higher oxyradical scavenging capacity of invertebrate tissues compared to the rat indicates for these species a greater protection against toxicity of peroxyl radicals and potentially other reactive oxygen species which can be assessed by a simple modification of the reaction. This may be due to the absence of respiratory pigments which regulate the oxygen flux to tissues and/or the lower activity of some antioxidant or xenobiotic metabolizing enzymes which in vertebrates more efficiently prevent or reduce oxyradical generation (Förlin *et al.*, 1995). The high TOSC values obtained in sea anemones might also reflect defenses against hyperoxia caused by endosymbiotic algae within the host (Dykens and Shick, 1982). Ethylene from KMBA was inhibited to a greater extent by homogenates of sea anemones with symbionts than those of corresponding apo sea anemones (Shick and Winston, unpublished results). A significant part of this inhibition was shown to be due to higher urate levels in the symbiont containing sea anemones. We have recently shown that urate is a potent scavenger of peroxyl radicals (Regoli and Winston, 1999). In all the considered species, soluble antioxidants always exhibited a greater oxyradical scavenging capacity than do protein fractions. These small molecules which better compete for

free radicals than DNA, lipids, and proteins provide the major protection against toxicity of reactive oxygen species whose production is greatly enhanced upon exposure to xenobiotic chemicals from petroleum producing operations.

The specific TOSC values for 1μM aqueous solutions of the main antioxidants have been reported to be 0.84 ± 0.1 for GSH, 2.00 ± 0.11 for ascorbic acid and 3.05 ± 0.27 for uric acid (Winston *et al.*, 1998). Based on these data and on the levels of antioxidant scavengers reported for digestive gland of *M. edulis* and rat liver (Ribera *et al.*, 1989; Viarengo *et al.*, 1990; Livingsone, 1991; Regoli and Principato, 1995; Rose and Bode, 1995), the expected contribution of GSH, ascorbic acid and uric acid on the obtained TOSC values has been calculated. Interestingly these antioxidants account for only 35% of the TOSC measured in soluble fractions from both rat liver and mussel digestive gland. Synergistic effects were not confirmed when GSH, ascorbic acid, and uric acid were measured in combination; the mixtures indicated additive effects of these antioxidants. The relatively low contribution of these major antioxidants to the total peroxyl radical absorbance capacity suggest that other small molecules play an important role as free radical scavengers, however, the influence of intracellular homeostatic mechanisms regenerating the reduced form of antioxidants cannot be rigorously excluded when interpreting the kind of results reported herein. Such considerations are presently under study in our laboratories with respect to the sea anemone.

In conclusion, the TOSC assay appears to be a simple and reliable method for rapid quantification of the relative biological resistance to oxyradical toxicity and may prove useful as a biomarker for detecting contaminant-mediated oxidative stress in marine organisms. Practical considerations such as ease of the analytical procedure, high reproducibility of the results and the ability to analyse 8-10 samples in less than 2h, (20-30 samples if a single time point is used) make this a useful procedure for investigations on the total oxyradical scavenging capacity and its relationship with oxidative stress resistance. We have presented our assay in terms of its peroxyl radical scavenging (absorbance) capacity as this radical is the basis of the other assays cited to which we compare the present. We name our assay total oxyradical scavenging capacity (TOSC) to reflect its utility in quantifying not only peroxyl radical scavenging but also other radicals (e.g., hydroxyl, trichloromethyl, alkoxyl, and alkyl) and non-radical oxidants such as peroxynitrite and hypohalous acids. Characterization of the interaction of KMBA with this spectrum of radicals is currently under study in our laboratory.

CHAPTER 7

INDUCTION OF IMMUNOREACTIVE PROTEIN WITH HEAT SHOCK PROTEIN AND CYTOCHROMES P450 ANTIBODIES

L.M. Heffernan, R.P. Ertl, M. Sole', W.A. Stickle and G.W. Winston

INTRODUCTION

The potential applications of biochemical markers of exposure and effect (biomarkers) that might benefit regulatory agencies has been discussed in detail in the Executive Summary of this report. Biomarkers can help to establish cause associated with injury in organisms, populations or communities, delineating the range and zones of exposure and the effects (reversible or irreversible) caused by contamination to organisms, populations or communities.

Two distinct advantages of monitoring biochemical changes for biomarker research programs are that they represent initial responses to organic and inorganic pollutants and biochemical responses reflect exposure and effect. In this chapter the responsiveness of two classes of proteins to organic and metal exposures are presented. These proteins are cytochromes P450, which are of crucial importance in detoxification and activation of organic xenobiotic chemicals and the stress proteins or heat shock proteins (HSP's). These proteins are inducible by such chemicals by chemical agents and by other stress factors including heat, salinity changes or oxidative stress. This inducibility of cytochromes P450 and HSP's by chemicals is the bases of their potential use in biomarker research and the induction of the former in fish have been useful in this application (Stegeman *et al.*, 1992). The latter are thought to protect certain proteins and nucleic acids from environmental stress including that caused by pollutants. Their usefulness as biomarkers therefore are as determinants of the degree to which the organism requires protection from environmental stress (Sanders, 1990).

In this chapter we present results of studies of the cytochrome P450 and heat shock protein response in certain species of sea anemones to the model polycyclic aromatic hydrocarbon, 3-methylcholanthrene, a representative polycyclic aromatic hydrocarbon, a crude oil preparation and to cadmium, a commonly occuring metal contaminant in drilling fluids. We show that antibodies raised to both of these proteins can be used to detect proteins that are induced by the above exposure protocols sea anemones thereby indicating their utility in biomarker research programs. Moreover, the data presented herein are to the best of our knowledge the first demonstration of CYP antibody recognition of proteins induced in marine invertebrates by xenobiotic chemical exposure.

METHODS AND MATERIALS

Preparation of sea anemone microsomes. Microsomes were prepared by homogenizing sea anemone columnar tissue, after removal of the tentacles, with a Tekmar Polytron Tissuemizer in 50 mM Tris-HCl buffer, pH 7.4, containing 0.125 M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreotol (DTT), 1 mM phenylmethylsulfonyl flouride (PMSF) and 1 mg/ml soybean trypsin inhibitor. Tentacular tissue contains adventitious proteases that can result in autogenous destruction of specific proteins under investigation. Homogenates were serially centrifuged for 10 min each at 500, 8,500, and 14,000 x g and the resulting pellets were discarded. The 14,000 x g supernatant was then centrifuged for 1 h at 105,000 x g to pellet the microsomal fraction. The microsomal pellet was washed once in 50 mM Tris-HCl/ 0.125 M sucrose, pH 7.4, centrifuged again at 105,000 x g for 1 h. The resulting supernatant was decanted and stored at -80°C until used for immunoblotting with anti-HSP antibody. The final pellet was resuspended and stored at -80°C in 0.25 mM sucrose/20% glycerol. Microsomal protein was estimated by the method of Lowry *et al.*, (1951).

Exposure of sea anemones to pollutants. The sea anemones *Condylactis gigantea* were continuously exposed to 0, 175 $mg \cdot L^{-1}$ and 350 $mg \cdot L^{-1}$ of crude oil per total gravel bed through which 50 ml of sea water was continuously flowed for 3 or 6 days. For the cadmium and 3-methylcholanthrene exposure studies Bunodosoma cavernata were used. Cadmium exposures were carried out in glass aquaria containing three sea anemones per aquarium. Static exposures of 96 h to two concentrations of cadmium chloride, 25 and 250 mg L^{-1} , were used. Exposure to 3-methylcholanthrene was accomplished by injecting 2 mg kg^{-1} body weight of this chemical in a corn oil vehicle directly into the oral cavity of the anemone for 3 consecutive days. Blue food coloring dissolved in corn oil was used in preliminary control experiments to ensure that the vehicle was taken up and distributed into the sea anemone tissues.

Immunoblotting. Western blot analysis was performed as described by Towbin *et al.*, (1979). Essentially, microsomes were reduced with β-mercaptoethanol, boiled for 4 min and protein bands separated by SDS/polyacrylamide (10%) gel electrophoresis. Gels were transferred to nitrocellulose filters in a Transblot apparatus (BioRad) for 50 min at 100V, filters were blocked with 5% powdered milk in 20mM Tris-buffered saline (TBS), pH 7.5, overnight, incubated with primary cytochrome P450 antibody in TBS with 1% gelatin for 2 h, incubated with an appropriate immunoglobulin biotin conjugate for 2 h, and again incubated with extraavidin alkaline phosphatase (Sigma) for 1 h, all at 37°C. Filters were washed 4 times with TBS between each step and rinsed with water after the final step. Blots were developed in 100mM $NaHCO_3$/1mM $MgCl_2$, pH 9.8, containing 3.0% NBT (v/v 70% N,N-dimethyl formamide (DMF) and 1.5% BCIP (v/v 100% DMF) and then soaked for 10 min in H_2O. Primary antibodies used were: mouse monoclonal IgG antibody to heat shock protein 70 (HSP 70) from StressGen, which recognizes the family of HSP 70, including HSP 70, HSP 72, HSC 70, GRP 75, HSP 78; monoclonal mouse anti-scup cytochrome P450 (CYP) 1A1; polyclonal rabbit anti-trout LMC2 (CYP2K1); and, polyclonal rabbit anti-trout LMC5 (CYP3A1).

RESULTS

HSP antibody studies. In light of the fact that HSP 70 protein is inducible by oxidative stress and in certain instances such stress has been correlated with PAH exposure, it was of interest to test whether the classical CYP1A inducer, 3-methylcholanthrene, could induce a HSP response. The western blot shown in Figure 7.1 was probed with a mouse monoclonal IgG antibody to HSP 70.

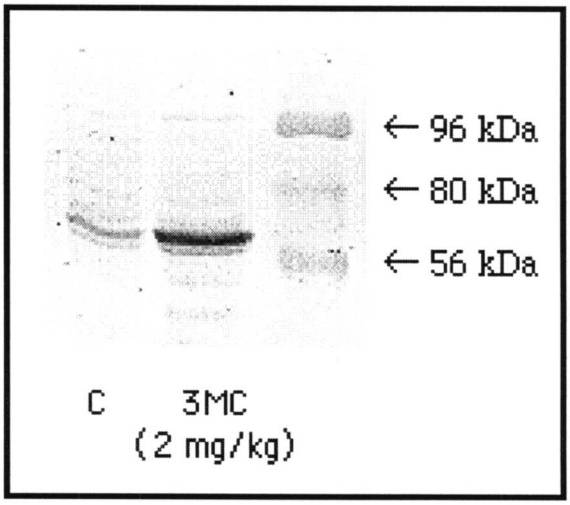


Figure 7.1. Western blot of 3-methylcholanthrene-treated and Untreated *B. cavernata* Cytosol Probed with Monoclonal IgM Antibody to HSP 70.

Recognition of a protein band at approximately 70 KDa was noted in control and 3-methylcholanthrene-treated sea anemone cytosols with monoclonal HSP 70 antibody when cytosolic protein was loaded at 30 or 100 μg per lane (shown for 30 μg per lane). This protein band is seen to be markedly induced in cytosol of the treated sea anemone (Fig. 7.1). A mouse monoclonal antibody to HSP 90 only faintly recognized cytosolic protein (loaded at 30 μg per lane) albeit, 3-methylcholanthrene exposure did not alter the antibody response. Exposure of the sea anemones to 25 and 250 mg L^{-1} of cadmium chloride caused similar induction of a 70 KDa protein as that observed with 3-methylcholanthrene treatment (not shown). No induction of proteins that cross-reacted with anti-HSP 70 or anti-HSP 90 IgG was caused by the oil exposure regimen.

Cytochromes P450 antibody studies. *Studies with anti-CYP1A antibody:* Western blots of sea anemone microsomal fractions were performed with various anti-cytochromes P450 antibodies. When probed with a mouse monoclonal anti-scup CYP1A1 antibody no proteins were recognized in 15 μg of microsomal protein from the sea anemone *A. elegantissima* per lane. However, protein bands of about 45 and 65 KDa were observed when the gel was loaded with 60 μg per lane (Fig. 7.2). Both of these protein bands appear to be well induced by exposure of the sea anemones to 25 mg L^{-1} of cadmium chloride, however when exposed to 250 mg L^{-1} of

cadmium chloride the induction response appears to have been attenuated, presumably owing to the toxicity of this cadmium concentration to the organisms, which impaired the biochemical sequelae required for induction. No induction of immunoreactive proteins to this antibody was noted with the oil exposure (not shown) or the 3-methylcholanthrene treatment regimens (Fig. 7.2).

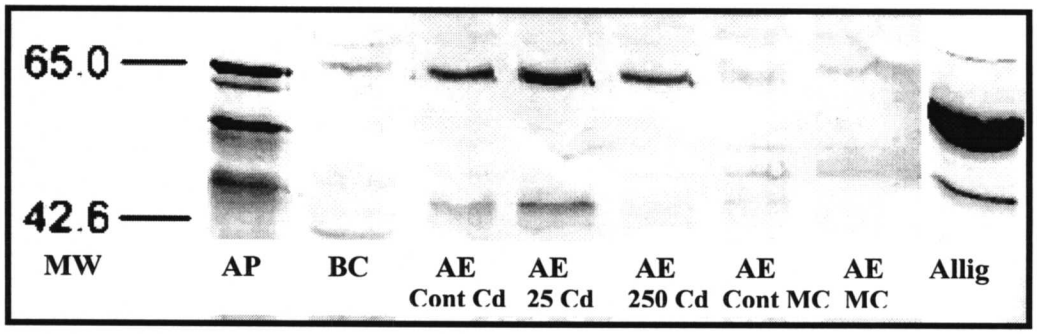


Figure 7.2. Western blot of 3-methylcholanthrene- and Cadmium-exposed *A. elegantissima* Microsomes Probed with Monoclonal IgG Antibody to CYP1A.

Studies with anti-CYP3A antibody: When microsomal fractions of *A. elegantissima* were probed with the polyclonal anti-CYP3A antibody similar results as those obtained with the monoclonal anti-scup CYP1A1 antibody were obtained. Immunoreactive proteins of about 45 and 70 KDa were observed in the western blots and these proteins were of significantly higher concentration following exposure to the lower cadmium level but not the higher level (Fig. 7.3).

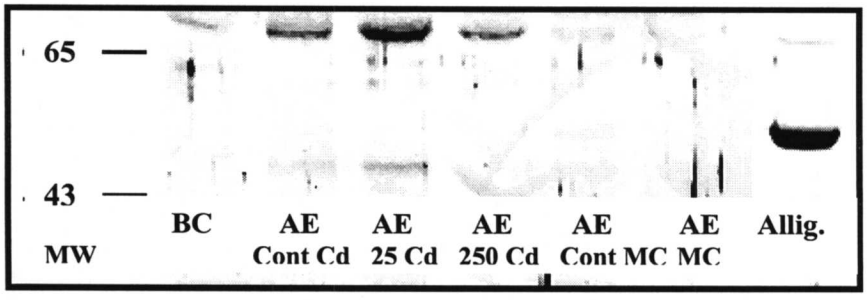


Figure 7.3. Western blot of 3-methylcholanthrene- and Cadmium-exposed *A. elegantissima* Microsomes Probed with Polyclonal IgG Antibody to CYP3A.

Neither 3-methylcholanthrene treatment (Fig. 7.3) nor oil exposure (not shown) appeared to cause induction of CYP3A-reactive protein in these microsomal fractions.

Studies with anti-CYP2K antibody: When *A. elegantissima* microsomes (shown for 30 μg per lane) were probed by immunoblotting with a polyclonal rabbit anti-trout CYP 2K antibody three protein bands were observed (Fig. 7.4). The very intense band of about 40 KDa described in chapters 1 and 4 of this report were present but this protein was not induced by any

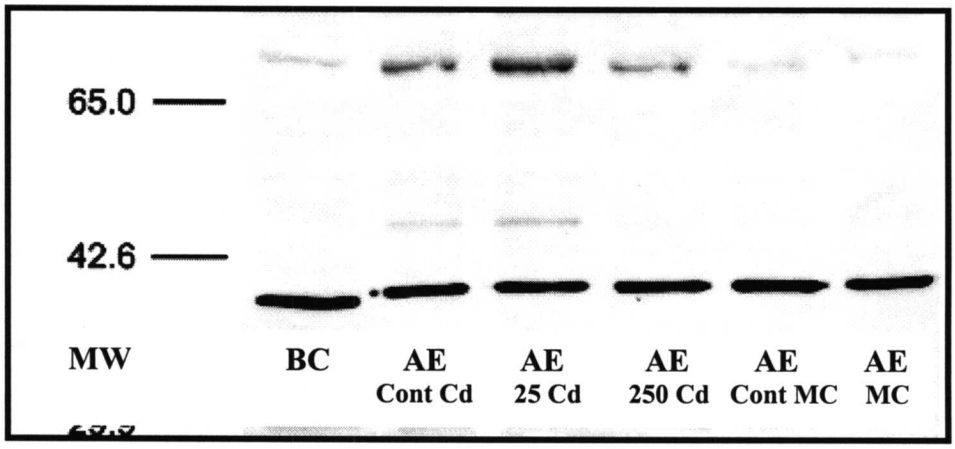


Figure 7.4. Western blot of 3-methylcholanthrene- and Cadmium-exposed *A. elegantissima* Microsomes Probed with Polyclonal IgG Antibody to CYP2K1.

of the exposure regimens employed. As described above for anti-CYP1A and anti-CYP3A two fainter bands of about 45 and 70 KDa were recognized by the anti-CYP2K antibody when microsomes were loaded at 30 μg per lane. These bands also appeared to be induced by the cadmium exposure regimen but not by 3-methylcholanthrene exposure.

DISCUSSION

All biomarkers of exposure and effect ultimately have to meet two important criteria. Firstly, the response must be of sufficient sensitivity to be detectable and quantifiable at environmentally relevant exposure levels. secondly, the biomarker response must be sustained over time. Before such criteria are met in field studies it is necessary to first identify whether certain chemicals can indeed elicit a biochemical response. To that end initial studies used acute exposures to concentrations of toxicant that may be higher than in specific environmental situations but well below that which causes noticeable stress at the organismic level.

Sea anenomes have several advantages as sentinels for pollution exposure; they actually inhabit oil platforms, are stationary (sessile), are ubiquitous and perhaps most importantly, can be easily cloned in the laboratory to generate genetically homogeneous populations thus, eliminating genetic variability in biomonitoring responses. Furthermore, because anenomes have evolved to only the tissue level of development, biochemical responses in this organism reflect that of only a few cell types. By analogy the response would be similar to an organism with one basic organ type. Thus, the whole organism is used for biochemical assays rather than having to dissect specific organs and characterizing each within several species. Sea anenomes in

particular, through their action of pumping water throughout their bodies, are continually replenishing the body burdon and thus, continually bioaccumulating pollutants.

Analyses of cytosolic fractions were conducted to assess the presence of possible stress or heat shock proteins in these organisms. Results of these studies will help to focus on target proteins as potential biomarkers of environmental contamination. The inducibility of P450 was examined by pre-exposing *A. elegantissima* to 3-methylcholanthrene (3MC), a polycyclic aromatic hydrocarbon known to induce CYP1A protein in mammals, fish, amphibians, reptiles and avians, (20 mg/kg) for three days. The amount of protein recognized by P450 antibodies to CYP1A, CYP2K, and CYP3A was not different in microsomes from control and oil or 3-methylcholanthrene exposed sea anemones. The lack of induction by 3-methylcholanthrene of a marine invertebrate cytochrome P450 1A isoform upon exposure to PAH is consistent with most other marine invertebrate literature (Livingstone, 1991). One study of CYP1A-like induction in the marine polychaete *Nereis virens* was reported by Reily *et al.* (1992). These investigators reported a time- and concentration-dependent increase in *Nereis* gut microsomal fractions of ethoxyresorufin O-deethylase (EROD) activity upon exposure of these sand worms in microcosm settings to petroleum-laden sediments collected in the field from a produced-water discharge site at Pass Fourchon in coastal Louisiana. EROD is a benchmark activity of CYP1A in mammals and submammalian vertebrates incuding numerous fish species. However, immunoblots of 50 μg of *Nereis* gut microsomal protein probed with a polyclonal antibody to fish CYP1A from β-naphthoflavone-pretreated trout did not indicate the presence of an inducible anti-CYP IgG-reactive protein, consistent with the present studies showing a lack of induction of CYP1A-like protein in sea anemone microsomes by exposure of the organism to 3-methylcholanthrene or crude oil.

Nevertheless, induction of proteins recognized by anti-HSP 70 IgM and anti-cytochromes P450 1A, 3A and 2K by exposure to cadmium were evident from the data presented herein. Furthermore, the polycyclic aromatic hydrocarbon 3-methylcholanthrene was shown to induce an immunoreactive protein to monoclonal anti-HSP 70 in sea anemone microsomes. In both instances, the proteins were recognized by these antibodies in the control organisms; thus, it remains to be established whether such proteins are truly constitutive or whether they reflect induction by natural exposures prior to the collection of these organisms for our studies. Clearly however, the laboratory exposures resulted in a higher content of these proteins in the sea anemone microsomes. A particulary striking result was the induction of an immunoreactive protein band of about 65 KDa by exposure to 25 μg/liter of $CdCl_2$ that was recognized by a monoclonal antibody raised against scup cytochrome P450 1A1. Cadmium also induced a protein of about 70 KDa that was recognized by a trout polyclonal antibody to cytochromes P450 3A1 and 2K1. The former isoform is steroid-inducible in mammals but the response to steroids has not been explored in marine invertebrates.

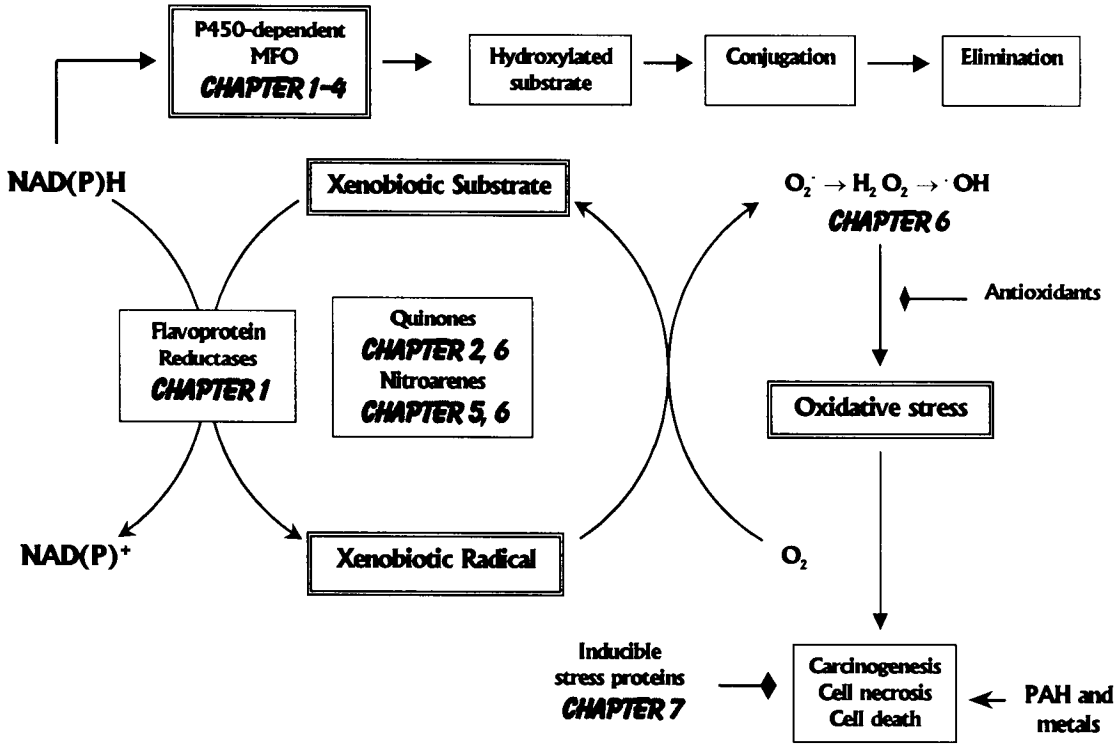
To the best of our knowledge this is the first demonstration of induction of cytochromes P450 antibody-reactive protein by cadmium or any metal in any organism. Furthermore, we know of no HSP-like proteins that are inducible in marine invertebrates by a polycyclic aromatic hydrocarbon other than that described herein in sea anemones by 3-methylcholanthrene. The potential of these inducible proteins in biomarker research is therefore of profound interest and further study is of the highest heuristic value. A protein of about 90KDa that cross-reacted with

a monoclonal anti-heat shock protein 90 IgM was also present in sea anemone cytosol (data not shown), albeit this protein did not appear to be induced by exposure to either 3-methylcholanthrene or cadmium. Further studies will explore the effects of metal exposure on these proteins. More specific quantification will be determined at a later date.

In conclusion sea anemones show significant potential as sentinel organisms with respect to biological endpoints. Although biochemical responses are the most rapid to obtain and the most sensitive it is recognized that they are of limited ecological relevance. Thus, a suite of biomarkers would promote the most comprehensive evaluation of the impact of environmental upsets, whether natural or inadvertant. This is important if regulatory agencies are to be able to assess the impact of ecological perturbations on the higher levels of ecosystem development such as the population or community level.

CONCLUSIONS

We have undertaken a comprehensive study of various parameters of sea anemone biochemistry and physiology. Historically, these parameters have been associated in the literature with biomarker responses to environmental contamination in various marine, aquatic and terrestrial organisms. We have introduced, presented findings and discussed these parameters in seven discrete but interrelated Chapters. The interrelationships between the various Chapters are shown schematically below and the concepts of the scheme are related to the specific Chapters in which they are discussed.



The left side portion of scheme indicates initial electron transfer from NAD(P)H to a xenobitic substrate through a flavoprotein reductase (cytochrome P450 reductase) to produce a xenobiotic radical (one-electron transfer) or a hydroxylated xenobiotic substrate. The latter undergoes subsequent conjugation reactions and elimination from the body, i.e., detoxification. Exposure to xenobiotic compounds such as chlorinated hydrocarbons (CH; e.g., PCB) and polycyclic aromatic hydrocarbons (PAH) can result in induction of P450 enzymes and flavoprotein reductases (Chapters 1-4). Chapter 1 presents immunochemical evidence for the existence of multiple forms of P450 in sea anemone species. These results suggest the presence in sea anemones of cytochromes P450 homologues, or proteins with similar epitope regions as P450 of some fish and mammals. The presence of these proteins and an active microsomal reductase suggests a functional MFO system in the sea anemones, and a capability to oxidize and depurate organic xenobiotic chemicals. Chapter 2 presents evidence that the P450 enzymes are catalytically functional in oxidizing the benchmark PAH benzo[a]pyrene (B[a]P). The intertidal

sea anemone *Bunodosoma cavernata,* which inhabits coastal Louisiana ecosystems was used in this study and compared to various other sea anemone species also found in the Gulf of Mexico and other coastal regions (California, Alaska). The species were selected because of their likelihood for exposure to oil spills, which have frequently impacted these regions. Incubation of B[a]P with *B. cavernata* microsomes produced oxidative metabolites of benzo[a]pyrene consistent with an active MFO system and were consistent with other studies of P450-catalyzed oxidations of B[a]P by marine invertebrates. The identity of quinone metabolites of B[a]P indicate the potential for oxidative stress caused by PAH exposure and provided rationale for the studies of Chapters 5-7.

Chapter 3 extends the findings of Chapters 1 and 2 to show the propensity of sea anemone P450 enzymes to oxidize ethoxyresorufin (ethoxy resorufin O-dealkylation; EROD) and display spectral characteristics that are critical in quantification of P450. EROD activity has been directly correlated with exposure to PCB and PAH in marine organisms. Despite the lack of MFO activity reported by several investigators who have studied cnidarians our studies, showing proteins that immunoreact with cytochrome P450 antibodies (Chapter 1 and 4), a characteristic CO-difference spectra in DTN-reduced sea anemone microsomes (Chapter 3), active compliments of MFO components (i.e., P450 and b_5 reductases), and metabolism of classical cytochrome P450-catalyzed reactions (i.e., EROD and B[a]P hydroxylation). indicate a functional MFO system. Preliminary studies in our laboratory also indicate that *B. cavernata* can catalyze NAD(P)H-dependent aldrin epoxidation. The P450 content and activity does not differ substantially from most other invertebrates. In Chapter 4 we show for the first time the tissue distribution of immunoreactive protein to P450 antibody, CO-binding chromophores and quantifiable P450 and EROD activity. We present evidence that small differences in CYP content might not be detected in the presence of other heme proteins, such as the 418 nm chromophore, which interferes with the detection of the 450 nm peak. Based on the spectral properties of the 500-700 nm region, additional heme-protein(s) appear to be present and variable between each tissue region. When KCN is added in place of CO a corresponding shift in the spectral properties contributed by heme was observed. Taken as a whole our data with KCN indicates the presence of heme proteins in sea anemone microsomes with observable and alterable spectral properties upon bonding of KCN ligand. Our spectral analysis compliments the immunochemical studies of Chapter 1.

Free radical intermediates have a different fate than the hydroxylated intermediates; radicals can bind to DNA and proteins and form adducts, peroxidize lipids and inactivate enzymes. Further, the radical intermediates react readily with molecular oxygen to generate superoxide anion radical, which can undergo enzymatic and nonenzymatic dismutation yielding potent oxidants, e.g., hydrogen peroxide and hydroxyl radical (right side portion of scheme). Interestingly, quinone metabolites of PAH oxidation (Chapter 2), nitro aromatic compounds and metal-chelates are classical substrates for this radical generating oxidation-reduction cycling, a cyclic pathway that proliferates free radical production and hence, oxidative damage (center portion of scheme). In Chapter 5 we show the first direct evidence for the production of free radicals via this mechanism in comparison with that of the common marine mussel *Mytilus edulis,* a benchmark sentinel species of environmental contamination in the marine environment. Comparing sea anemone and mussel microsomes, both were shown to produce 4NPO nitro anion radical indicating single-electron transferring enzymes are present in these species. Sea anemone

microsomes catalyzed very low level radical yield detectable only with NADH. The higher level of NADH- than NADPH-flavoprotein reductase activities found in various tissues of *M. edulis* and sea anemones are likely responsible for the generally higher rates of 1-electron with NADH than NADPH. The suppression of the mussel microsomal-mediated radical signal by $CuSO_4$ and PCMB is consistent with a flavoprotein catalyzing 4NPO reduction. The importance of this pathway has credence in light of its role in the induction of antioxidant stores (Chapter 6) and stress proteins (HSP), which we discuss in Chapter 7. PAH, CH and metal-chelates are all potent inducers of oxidant stress, which can result in carcinogenesis, cell necrosis and cell death. In Chapter 6 we describe a new method for measuring the total oxyradical scavenging capacity, a quantifiable index of antioxidant capacity, of the sea anemone in comparison with other marine invertebrates. The assay is simple and reliable for rapid quantification of relative biological resistance to oxyradical toxicity and indicates its usefulness as a biomarker of contaminant-mediated oxidative stress in sea anemones. Sea anemones had the highest oxyradical scavenging capacity of the marine organisms studied, which suggests that in a biomarker setting these organisms might be more resistant to oxidant challenge.

In Chapter 7 we establish further evidence of the uniqueness of the stress protein response to environmental contamination, thereby supporting the notion that sea anemones have significant potential as sentinel organisms with respect to biological endpoints. To the best of our knowledge this is the first demonstration of induction of cytochromes P450 antibody-reactive protein by cadmium or any metal in any organism. Furthermore, we know of no HSP-like proteins that are inducible in marine invertebrates by a polycyclic aromatic hydrocarbon other than that described herein in sea anemones by 3-methylcholanthrene. The potential of these inducible proteins in biomarker research is therefore of profound interest and further study is warranted.

Finally, although biochemical responses are the most rapid to obtain and the most sensitive it is recognized that they are of limited ecological relevance. Thus, a suite of biomarkers would promote the most comprehensive evaluation of the impact of environmental upsets, whether natural or inadvertant. This is important if regulatory agencies are to be able to assess the impact of ecological perturbations on the higher levels of ecosystem development such as the population or community level.

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The Department of the Interior Mission

As the Nation's principal conservation agency, the Department of the Interior has responsibility for most of our nationally owned public lands and natural resources. This includes fostering sound use of our land and water resources; protecting our fish, wildlife, and biological diversity; preserving the environmental and cultural values of our national parks and historical places; and providing for the enjoyment of life through outdoor recreation. The Department assesses our energy and mineral resources and works to ensure that their development is in the best interests of all our people by encouraging stewardship and citizen participation in their care. The Department also has a major responsibility for American Indian reservation communities and for people who live in island territories under U.S. administration.

The Minerals Management Service Mission

As a bureau of the Department of the Interior, the Minerals Management Service's (MMS) primary responsibilities are to manage the mineral resources located on the Nation's Outer Continental Shelf (OCS), collect revenue from the Federal OCS and onshore Federal and Indian lands, and distribute those revenues.

Moreover, in working to meet its responsibilities, the **Offshore Minerals Management Program** administers the OCS competitive leasing program and oversees the safe and environmentally sound exploration and production of our Nation's offshore natural gas, oil and other mineral resources. The MMS **Minerals Revenue Management** meets its responsibilities by ensuring the efficient, timely and accurate collection and disbursement of revenue from mineral leasing and production due to Indian tribes and allottees, States and the U.S. Treasury.

The MMS strives to fulfill its responsibilities through the general guiding principles of: (1) being responsive to the public's concerns and interests by maintaining a dialogue with all potentially affected parties and (2) carrying out its programs with an emphasis on working to enhance the quality of life for all Americans by lending MMS assistance and expertise to economic development and environmental protection.