**CYTOCHROME P450 GENE EXPRESSION IN
THE COMMON MUSSEL *MYTILUS EDULIS***

Being a thesis presented in accordance with the regulations
governing the award of the degree of Doctor of Philosophy in
the University of Surrey

by

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to my family,
without whose encouragement and sacrifices
I could never have achieved so much

.....So I turned my mind to understand,
to investigate and search out wisdom
and the scheme of things...

(Ecclesiastes 7:25)

ABSTRACT

The cytochromes P450 are a ubiquitous family of enzymes involved in metabolism of a wide variety of compounds, both naturally occurring and man-made. A number of isozymes of cytochrome P450 are induced by characteristic compounds. Some of these groups of compounds commonly occur in the marine environment as pollutants. It was proposed, by studying the mechanisms of gene regulation, to develop methods of monitoring marine pollution by using levels of certain P450 isozymes in marine animals (in particular the widely used marine mussel Mytilus edulis) as indices of seawater quality.

In this project I have:

- 1) used radiolabelled vertebrate cDNA probes from cytochrome P450 isoenzymes and other proteins to detect homologous sequences in marine invertebrates, particularly in M. edulis.
- 2) successfully detected mRNA and DNA sequences related to the cDNA probes used in nucleic acid samples purified from mussel and other marine species.
- 3) performed assays to determine whether the isozymes identified were inducible, comparing mussel with fish for a number of different P450 cDNA probes.
- 4) performed PCR amplification of mussel DNA using primer pairs derived from published vertebrate P450 sequences.
- 5) synthesized a mussel genomic DNA library in the vector lambda EMBL3a to facilitate further examination of which P450 genes are present in the molluscan genome.

From this work I have determined that sequences homologous to probes from the vertebrate P450I family and P450IV family isozymes are expressed in the mussel, whereas sequences homologous to probes derived from mammalian P450II family isozymes appear to be absent. In comparison with the vertebrate equivalents however, the molluscan P450s appear to be regulated differently: a variety of experimental and field conditions failed to produce clear induction of invertebrate P450, as detected using these probes.

Sequences sufficiently similar to fish P450IA1 were present in mussel DNA to allow in vitro amplification using primers derived from the fish P450 sequence. However primers to the rat P450IVA1 did not amplify a fragment in mussel.

The gene library synthesized will allow clones encoding mussel P450s to be isolated, following identification by either probes derived from mammalian P450s or probes derived from in vitro amplification of mussel P450 sequences. In the latter case, sequencing of the amplified fragment will first be required.

This work complements existing data from studies using other techniques and facilitates additional study to isolate and further analyse the functioning of mussel P450s.

Addenda

The following information has come to light since the completion of this thesis;

First, the cockroach P450IVC1 sequence has been published, describing a protein with 32-36% similarity to mammalian P450IVA & B subfamilies (Bradfield et al., 1991). Interestingly, there is a 13 amino acid conserved sequence described which is uniquely present in IV family proteins. This may prove a useful target for an upstream primer site in PCR studies (though the problems of introns described in section 3.5 still persist).

Second, the problems in sequencing (3.5.2) were most likely due to destruction of the DNA following uv irradiation prior to removal of the band from the agarose gel. A possible route around this problem is to run duplicate lanes on a gel. The bands could be located by exposing only one to uv light and predicting the position of the other. After excision of the non-irradiated band and subsequent purification, an aliquot could be run on a gel to check it was successfully isolated before performing sequencing reactions on the remainder.

References

Promega Notes 30 5 (Apr 1991)

Bradfield, J.Y.; Lee, Y.-H.; Keeley, L.L. (1991):
Cytochrome P450 family 4 in a cockroach: molecular cloning and regulation by hypertrehalosemic hormone
PNAS 88 4558-4562

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ABBREVIATIONS

AHH	aryl hydrocarbon hydroxylase
BNF	β -naphthoflavone
B[a]P	benzo[a]pyrene
BPH	benzo[a]pyrene hydroxylase
cdNA	complementary deoxyribonucleic acid
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DDE	1,1-di(p-chlorophenyl)-2,2-dichloroethylene
DEHP	diethyl hexylphthalate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dsDNA	double stranded deoxyribonucleic acid
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
ECOD	ethoxycoumarin-O-deethylase
EDTA	ethylene diamine tetraacetic acid
EROD	ethoxyresorufin-O-deethylase
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
GPX	glutathione peroxidase
HCBP	hexachlorobiphenyl
HFI	human fructose intolerance
ip	intra-peritoneal
kb	kilobase
kD	kiloDalton
LMP	low melting point
mAP	messenger affinity paper

3MC	3-methylcholanthrene
MFO	mixed-function oxidase
MOPS	morpholinopropanesulphonic acid
Mr	molecular weight
mRNA	messenger ribonucleic acid
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
nm	nanometre
PAGE	polyacrylamide gel electrophoresis
PAH	polycyclic aromatic hydrocarbons
PB	phenobarbital
PCB	polychlorinated biphenyl
PCN	pregnenolone-16 α -carbonitrile
PCR	polymerase chain reaction
pfu	plaque-forming units
rfDNA	replicative form deoxyribonucleic acid
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
TCBP	tetrachlorobiphenyl
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TE	10mM Tris/ 1mM EDTA pH8
TEMED	N,N,N',N'-tetramethyleneethylene diamine
TMP	trimethylpentane
TMV	tobacco mosaic virus
tRNA	transfer ribonucleic acid
UV	ultra violet

CHAPTER 1 : INTRODUCTION

1.1 Cytochrome P450 and Monitoring of Marine Pollution

As the impact of the activities of mankind on the environment increases, a greater understanding of the ability of ecological systems to accommodate various types of pollution is necessary. Therefore, a fundamental knowledge is required of the mechanisms by which organisms deal with the chemicals to which they are exposed.

The effect of chemicals (natural and man-made) on marine systems has been studied in some detail (reviewed in Stegeman, 1989 and Livingstone, 1991), and schemes such as the "Earthwatch" and "Mussel Watch" programmes have been adopted to monitor marine environmental quality (Jensen et al., 1975; Goldberg et al., 1978). In spite of this, newer more sensitive and/or more specific methods of pollution detection are constantly being sought.

The existence of various intracellular biotransformation pathways can result in a wide range of metabolic fates for the compounds to which an organism may be exposed. The primary response of an organism tends to be to metabolise a compound to a biologically inert, easily excretable form. This response may involve a number of enzymes and is normally divided into two phases;

- a) Phase 1: functionalisation reactions (which include those catalysed by cytochromes P450) introduce a functional group (-OH, -NH₂ etc.) into the compound, thus preparing it for metabolism by;
- b) Phase 2: conjugation reactions which facilitate excretion (and subsequent elimination from the body).

Paradoxically, some reactions (especially phase 1 reactions) produce metabolites or intermediates which are more toxic than the parent compound. These may be mutagenic, carcinogenic, or cause free radical damage and/or redox cycling.

The study of cytochromes P450 in higher animals has produced a degree of understanding of how many animals deal with xenobiotics (both those intentionally introduced e.g. drugs and those arising from accidental/environmental exposure e.g. benzo[a]pyrene, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [T.C.D.D.]) and the potential deleterious effects which may arise. This distinct group of haemoproteins has been observed to catalyse the oxidation of numerous structurally diverse substrates, endogenous (e.g. steroids, sterols, fatty acids, bile acids, vitamins) as well as those occurring in the environment. In addition, different substrates induce different characteristic members of the P450s and therefore provide a route by which effects of chemicals on both individual animal fitness and populations may be measured. Indeed some P450's have no known endogenous substrate and so have been purported to exist solely as a mechanism of introducing polar groups into non-polar compounds, thereby facilitating excretion (Ioannides & Parke, 1987,1990). However, a recent paper by Nebert (1990) proposes that the effect of such chemicals is due to similarity with as yet unidentified endogenous ligands, mimicking their effect in intracellular signalling pathways and thereby interfering with normal regulation to produce their effect.

Cytochromes P450 and P450 dependent activities are widely distributed in living systems (table 1.1). To date they have

been found in all eukaryotes in which adequate work has been carried out. Apparent lack of P450 in an organism may simply reflect insufficient study, as was the case with early studies in molluscs (Lee et al., 1972; Payne 1977; Vandermeulen & Penrose 1978). However, much of the current knowledge of the activities and control of cytochromes P450 is based on studies in mammals.

Table 1.1 : Cytochrome P450 Phylogenetic Distribution

P450 Phenomenon detected					
	Metabolism	P450	Isoenzymes	Inducibility	Sequence(s)
		(*)			
Bacteria (1)	+	+	min. 2	+	+
Fungi (2) (exc. Yeast)	+	+	ND	ND	ND
Yeast (3)	+	+	min. 2	+	+
Plant (4)	+	+	+	+	+
Amoebae (5)	+	+	ND	ND	ND
Annelida (6)	+	+	ND	ND	ND
Mollusca (7)	+	+	min. 2	ND	ND
Crustacea (8)	+	+	min. 3	+	ND
Insecta (9)	+	+	min. 6	+	+
Chordata (10)	+	+	min. 5 to min. 33(**)	+	+

39 + 1 = 40 genes now isolated (Nebert 1991)

ND = not done (though an unpublished sequence in a mollusc, the pond snail has been cited by Nebert et al., 1991)

+ = present or detected by one or more groups

References listed overleaf

(*) Spectral determination

(**) Higher figure is for rat, though this figure is subject to change in response to either isolation of new genes or establishment that some genes already isolated are allelic variants rather than separate gene products.

References to Fig 1.1:

- (1) Wiseman, 1980; Hanui et al., 1982; Poulos et al., 1986.
- (2) Wiseman, 1980.
- (3) Kalb et al., 1986; Sanglard et al., 1987.
- (4) Rueffer & Zenk, 1987; Fonne-Pfister et al., 1988; Cottrell et al., 1990; Bozak et al., 1990.
- (5) Gupta et al., 1987.
- (6) Lee, 1981.
- (7) Livingstone & Farrar, 1984; Kirchin, 1988; Livingstone et al., 1989b.
- (8) Elamlouk et al., 1974; Pohl et al., 1974; James, 1984, 1989, 1990.
- (9) Stanton et al., 1978; Clarke et al., 1987; Ronis et al., 1988; Feyereisen et al., 1989.
- (10) See references to Tab. 1.3/1.4 and also reviews as given in section 1.4.1

1.2 Cytochrome P450 Enzymology

Cytochrome P450 is the name given to an individual member of a distinct group of haemoproteins showing absorption maxima at or around 450nm in dithionite-reduced carbon monoxide-difference spectra. These enzymes were first reported independently in 1958 by two research groups as "carbon monoxide-binding pigments" (Klingenberg, 1958; Garfinkel, 1958). It was not until 1962 that Omura and Sato elucidated the haemoprotein nature of the enzyme, however, and evidence of involvement in hydroxylation reactions was then presented by Estabrook et al. (1963). These P450 isozymes have monomeric molecular weights of approximately 45-55kD (kiloDaltons), and form the terminal component of an electron transport chain, often termed the mixed-function oxidase (MFO) system.

In mammalian and plant systems, P450s are membrane bound and have been detected in the endoplasmic reticulum, mitochondria and the nuclear envelope. In bacterial systems, the enzymes are soluble, a fact that facilitated the crystallisation of a P450 from Pseudomonas putida (Poulos et al., 1986). This is the only P450 crystallised to date but has provided much information about the likely 3d structure and function of mammalian P450s (reviewed in Gonzalez, 1989). Although in mammalian species cytochromes P450 are most abundant in the liver (both in terms of relative amount and number of identified isozymes), they also occur in extrahepatic tissues, including kidney, lung, intestine, steroidogenic tissues, skin, and white blood cells (Burke & Orrenius, 1982), and nasal epidermis (Kimura et al., 1989).

However, they are apparently not present in striated muscle or erythrocytes. (P450IVA in rbc?)

In the classical mammalian MFO reaction, the electron donor, reduced NADPH/H⁺, transfers reducing equivalents to the substrate via a 78kD NADPH-linked flavoprotein, NADPH-cytochrome P450 reductase and cytochrome P450. The reductase, a flavin adenine dinucleotide (FAD)- and flavin mononucleotide (FMN)- containing protein is capable of accepting two electrons from the donor, and these are transferred sequentially to the haemoprotein. The net overall reaction catalysed by cytochromes P450 can therefore be represented as:

(where S denote the substrate)

A more detailed representation of the sequence of events is represented in fig. 1.1, showing the stepwise nature of the reaction cycle. Much of the detail of the individual steps is only poorly understood, due to the complexity and instability of the intermediates, although the subject has been recently reviewed (Ruckpaul et al., 1989).

Figure 1.1 : Cytochrome P450 catalytic cycle

Catalytic cycle of cytochrome P-450. RH represents the drug substrate, and ROH the corresponding hydroxylated metabolite. (Adapted from White, R. and Coon, M. J. (1980) *Ann. Rev. Biochem.*, 49, 315-56.)

(Gibson e Skell 1986)

In non-bacterial systems the presence of a lipid component is also required for catalytic activity, as demonstrated by the lack of activity in reconstituted mammalian or yeast systems without lipid (Lu & Coon, 1968; Gibson et al., 1982). In bacterial and mitochondrial systems, the system is slightly different in that adrenodoxin, rather than the P450 reductase, transfers the reducing equivalents to the P450.

In addition to the classical reaction, a number of alternative reaction routes are possible. For example cytochrome b_5 can act as a donor of the second electron, using NADH/ H^+ as its electron source. Further, it is also known that the degree to which cytochrome b_5 is involved in mammals and fish is both isozyme and substrate specific. Experimental data suggests however that, in mammalian systems, the first electron must always come from the reductase (Sato & Omura, 1978; Noshiro & Omura, 1978; Klotz et al., 1983 & 1986).

Although the classical reaction shows P450 as a sequential two electron donor, it is possible for only one electron to be transferred to the substrate. This route, apparently still utilising the NADPH/ H^+ and reductase system, is observed e.g. in the oxygenation of sulphur and nitrogen in sulphides and amines (Watanabe et al. 1982, Cavalieri et al. 1987). This is presumed to occur via abstraction of an initial electron from the substrate to the haem-oxygen complex at the active site of the enzyme, followed by attack of the oxygen on the resulting electropositive centre.

Additionally, in the absence of molecular oxygen, a single electron transfer mechanism may operate in the transfer of an oxygen atom from a peroxy-compound, rather than molecular oxygen, to an organic substrate viz.;

P450

From the mechanism of cytochrome P450-mediated monooxygenation, a stoichiometry of one mole of reduced cofactor and one mole of oxygen consumed per mole of product formed would be expected. However cofactor and oxygen consumption is in excess of the expected levels, and purified P450 in solution is observed to slowly form hydrogen peroxide in the absence of any substrate. This is presumed to arise from autooxidation of oxidised P450, with the resulting hydrogen peroxide having the capacity to react non-enzymically with compounds within the cell to yield oxygenated products (Cavalieri & Rogan, 1982; Ortiz de Montellano, 1986).

The relative importance of each of these different routes of metabolism depends primarily on the specific isozymes present and inducible in the tissue being considered, and the chemical nature of the substrate. Certain compounds are metabolised by both single-electron and two-electron transfer routes, others by only one. Benzo[a]pyrene, a common environmental pollutant and model compound for carcinogen studies, is metabolised by both routes, yielding different metabolites for each mechanism; two-electron transfer yields mainly diols, epoxides and other phenols, whereas in single-electron transfer, quinones are the main product. Indeed it has been suggested that the metabolite profile can be used as an indicator of single- versus two-electron transfer mechanism in a tissue, giving information about the isozymes present in the tissue.

One of the surprising characteristics of the cytochromes P450 is the wide range of different types of reaction that are

carried out on widely differing substrates. For example oxidation (including C-, N- and S- oxidation), reduction, hydrolysis, dehalogenation, epoxidation, desulphation, dealkylation and deamination are all known to occur.

The unique properties of the cytochromes P450 are due, in part, to the nature of its haem ligation and the ability of the haem-iron to exist in hexa- and penta-coordinated forms (fig. 1.2).

In the absence of substrate, cytochromes P450 exist as an equilibrium of the two forms, referred to as low- and high-spin states, respectively, from the electron distribution in the d-orbitals of the haem iron. Changes of spin state can be measured spectrophotometrically, and are characteristic of the compound binding to the P450 and also of the individual isozyme (fig. 1.3). It is considered that the changes in haem ligation arise as a change in apoprotein conformation arising as a result of substrate binding.

Figure 1.2 : Representation of the Cytochrome P450 active site, depicting in plane and out of plane haem.

Haem iron co-ordination in cytochrome P-450. (a) Hexa-co-ordinated, low-spin P-450 with in-plane iron. (b) Penta-co-ordinated, high-spin P-450 with out-of-plane iron. Note that the fifth ligand is a cysteine residue from the apoprotein and that cytochrome P-450 exists as an equilibrium mixture of low-spin (6-co-ordinated) and high-spin (5-co-ordinated) forms.

Gibson & Skell (1986)

Figure 1.3 : Cytochrome P450 binding spectra and example compounds

(From Schenkman et al., 1981 Pharmacol. Ther., 12)

1.3 Cytochrome P450 isozymes

1.3.1 Separation and Identification

Although the existence of more than one form of drug metabolising enzyme was predicted as long ago as 1959, it is only recently that the complexity of the cytochrome P450 superfamily of enzymes has been fully appreciated. After the realisation that more than one P450 form existed, groups of workers developed various criteria to differentiate and classify P450s (Åstrom & dePierre, 1986).

As mentioned above, spectroscopy had an important role in the discovery of P450s, and P450s have been characterised in terms of absorption maxima, spin state and spectral changes with ligand binding. For example, the rat liver P450IA1 (this system of nomenclature is described in section 1.4 below) reduced carbon monoxide difference spectrum maximum is at 448nm, whereas that of rat liver cytochrome P450IVA1 is at 452nm.

Different isozymes often have different or overlapping substrate specificities and/or show positional and stereoselective metabolism of substrates. For example;

- a) 7-ethoxyresorufin is a specific substrate for P450IA1 and 5-pentoxyresorufin a specific substrate for P450IIB1, but 7-ethoxyresorufin is metabolised by both isozymes and
- b) both P450IIB1 and IIC2 metabolise testosterone, the former at the 16B- position, the latter at the 16a- and 2a- positions (Waxman, 1988).

Electrophoretic mobility has been used to separate isozymes on SDS PAGE gels. Different isozymes have different monomeric molecular weights and thus migrate at different rates. However, many of the less abundant P450s are not

observed by this technique or are masked by more abundant isozymes of similar molecular weight.

A more sensitive and specific way of detecting and identifying isozymes is to use antibodies raised to a specific form of purified P450. When suitably purified, these antibodies often show little or no cross-reactivity with other forms of P450. For example, the antibody of Favreau et al. (1987) to the diabetes-inducible RLM6 (shown to be equivalent to the ethanol inducible form, P450IIE1 [Richardson, 1990]) shows no cross-reactivity to P450s IA1 or IIB1. In contrast, the phenobarbital inducible isozymes IIB1 and IIB2 are sufficiently similar (97% at the amino acid level) to be immunologically identical (Gonzalez, 1989; Ryan et al. 1982).

Following limited proteolysis, peptide fragments of isolated proteins can be separated by SDS PAGE. The resulting peptide map can be used to differentiate between the primary sequences of different cytochrome P450 isozymes (Ryan et al. 1982).

The most definitive method of distinguishing between isozymes is to determine the full primary amino acid structure. However isolation and sequencing of the cDNA corresponding to the specific mRNA is preferred to the more tedious amino acid sequencing technique. Thus nucleic acid sequences (and deduced amino acid sequences) provide the main basis of comparisons between P450 isozymes and the currently accepted system of nomenclature (see below).

1.3.2 Cytochrome P450 Nomenclature

Until the late 1980s, P450 isozymes were named variously, as they were discovered, by different laboratories, according to their spectral properties/molecular weight/inducer/metabolic activity. It was certainly the case that different laboratories purified the same protein but named it differently (See tab. 1.2). This gave rise to much difficulty and confusion, particularly when comparing work from different workers or different species.

By the late 1980s however, the growth in the number of cDNA sequences available led to development of a new system of nomenclature based on primary amino acid sequence (Nebert et al., 1987). Comparison of sequence homology by this method facilitates comparison of the spectrum of P450s, both within and between species (fig. 1.4). It has also allowed speculation as to the gene duplication events from which individual P450s arose (Nebert and Gonzalez, 1987; Nelson and Strobel, 1987). ^(Nebert 1991) However, apparent divergence rates appear different for different P450s when species are compared, emphasising the difficulty in constructing relationships between gene duplication events from molecular biological and fossil data (Nebert, 1990).

This system of nomenclature (detailed below) was based on sequences available at the time, and hence per cent similarity values for deliniation between categories are somewhat arbitrary. However it provided a basis for rationalisation which is now widely accepted and which has proved capable of assimilating newly discovered sequences without altering the

Table 1.2 : Comparison of different nomenclature systems for some rat hepatic Cytochrome P450 proteins (Adapted from Waxman, 1988)

Nebert	Waxman	Levin	Guengerich	Schenkman	Wolf
IA1	BNF-B	c	BNF-B	-	MC1b
IA2	ISF-G	d	ISF-G	-	MC1a
IIA1	3	a	UT-F	-	UT1
IIB1	PB-4	b	PB-B	PBRLM5	PB3a
IIB2	PB-5	e	PB-D	PBRLM6	PB3b
IIC6	PB-1	k	PB-C	RLM5a	PB1b
IIC7	-	f	-	-	-
IIC11	2c	h	UT-A	RLM5	PB2a
IIC12	2d	i	UT-I	fRLM4	-
IIC13	-	g	-	RLM3	-
IIE1	-	j	-	RLM6	-
IIIA1	PB-2a	p	PCN-E	-	-
IIIA2	2a	-	-	-	-

Figure 1.4 : The Cytochrome P450 gene Superfamily

existing framework. This is due at least in part ~~due~~ to the fact that all genes within any subfamilies so far examined in human and mouse genomes occur in clusters (Nebert, 1990).

The recent availability of genomic DNA sequences has also been incorporated into this system (Nebert et al., 1989) and the existing situation most recently reviewed by Gonzalez and Nebert, 1990.

The criteria used by Nebert et al., 1987, in the sequence based system of nomenclature is as follows;

a) A characteristic highly conserved region around the haem binding region must be present for the gene product to be classified as a P450

b) Sequence homology of less than 36% at the amino acid level assigns two P450s to different gene families, denoted by Roman numerals viz;

FAMILY P450I

c) Sequence homology of between 40% and 65% assigns two P450s to different sub-families within the same family viz;

SUBFAMILY P450IA

d) Sequence homology of greater than 68% assigns two P450s to different individual genes within the same sub-family viz;

INDIVIDUAL P450IA1

The updated system to include the chromosomal loci (Nebert et al. 1989) recommends;

e) CYP designation for P450 genes,
followed by family in arabic numerals and
subfamily and individual number as before,
all in italics, viz; CHROMOSOMAL LOCUS CYP1A1

Note 1: The suffix letter P is used to denote defective or non-functional Pseudogenes e.g. CYP2C6P (Kimura, H. et al. 1988).

Note 2: In keeping with existing gene locus terminology, CYP is used for human sequences and cyp for mouse sequences.

The expansion of sequence data available from use of molecular biology techniques has led to problems of its own. Many minor forms of P450 not previously resolved by protein biochemistry and immunological techniques are now being identified faster than catalytic activities, in vivo functions or tissues of expression can be identified. Also, for sequences very close to 100% homology (so called microheterogeneity; Komori et al. 1988) consideration must be given as to whether they are in fact the same gene product (Gonzalez, 1989). Certainly there is the possibility of allelic variation, given the evidence from the study of the debrisoquine-4-hydroxylase polymorphism in humans (Gough et al., 1990). In this instance, affected individuals have been shown to possess a number of sequence alterations within the P450 CYP2D gene. In one group of individuals a single nucleotide deletion causing frameshift and subsequent premature chain termination is the cause of the clinical

manifestation (difference in level of isozyme activity). Whilst in this case the variation is small but profound in its effect, there may be other cases where small sequence alterations have occurred with no clinical manifestation. Currently the criterion adopted is that a 3% or less divergence within a species represents allelic variation; greater than this and the sequences are assigned to two different genes. However there are already exceptions; firstly, two catalytically different forms of mouse P450IIA3 (coumarin-7-hydroxylase and testosterone-15 α -hydroxylase) differ at only 11 amino acid residues (circa 2.2%), though formal evidence that they are distinct gene products has not been presented; secondly, rat P450IIB1 and IIB2 (previously b and e under the Levin nomenclature) differ by only 2.6% even though they are clearly distinct gene products (Nebert, 1990).^a

(or
Comparison between species also remains more difficult than comparison within species under the sequence homology system, which takes little account of catalytic function or mechanisms of control. Obviously it should be expected that closely related species would have very similar proteins performing the same functions (orthologues). These can be assigned the same gene designation only if it is proven they correspond to one another both in terms of activity and sequence similarity. At the current rate of progress, sequences are becoming available faster than such proof can be given. For example, in the case of the IVA sub-family, 3 rat and 4 rabbit sequences (assigned IVA1-3 and IVA4-7 respectively) have been identified, but it is not certain which if any of the rabbit proteins corresponds to which rat protein! With species that are less closely related, the problem persists; For example, the only fish P450 to be

sequenced showed approximately equal similarity to both the human IA1 and IA2 genes (57% and 53% respectively) (Heilmann et al., 1988). Should it be named IA1 as the first fish gene in the IA subfamily, or should it be named according to activity and/or regulation compared to the human proteins? In this case, the result is the same since the induction and role of this P450 seem to show characteristic IA1 type activity. However, as more lower vertebrate and invertebrate sequences become available, the picture may become increasingly complex.

1.4 Non-mammalian cytochromes P450

1.4.1 Vertebrates

As previously commented, much of the current understanding of the P450s is based on mammalian data. The number of publications is extensive, and the reader is referred to recent reviews (Guengerich, 1987; Waxman, 1988; Schenkman et al., 1989; Gonzalez, 1989; Gonzalez & Nebert, 1990; Ioannides & Parke, 1990).

Most of the other data is for birds, frogs and fish, with less known for other amphibia and the reptilia (Fig 1.7; the references given are representative of the current knowledge, and not a comprehensive list). It should be noted also that the specific activities of many of the P450 enzymes reported are generally lower than those for rat, probably reflecting the lower overall metabolic rates in poikilothermic animals, but possibly also due to non-optimisation of assay conditions. In addition, much of the data concerning the regulation of P450 levels shows considerable differences with mammalian counterparts.

Much of the fish work has been done in marine species (though some freshwater data is included here), again with a view to understanding the biological fates of compounds released into the environment. Probably the most profound demonstration of the persistence of some compounds in the environment was the detection of PCB residues in the deep sea fish Coryphaenoides armatus (rattail) by Stegeman et al.

Table 1.3 : Non-mammalian vertebrate cytochrome P450

	Avian ⁽¹⁾	Amphibian ⁽²⁾	Reptilian ⁽³⁾	Fish ⁽⁴⁾
P450-type activity	+	+	+	+
P450 detected	+	+	+	+
Inducible/ Seasonal change	+	+	+	+
Purified/ part purified protein	+	ND	ND	+
Isozymes detected	6+	suggested by data	suggested by data	5+
Orthologues identified	IIH1, XIXA1 XVIIIA1, IA1	ND	IA1	IA1
Sequence available	IIH1, XIXA1 XVIIIA1	ND	ND	IA1

This table summarises the published knowledge of cytochrome P450 in non-mammalian vertebrate animals. Source references are listed overleaf.

(ND = not done + = present or detected by one or more groups)

References to tab 1.3;

(1) Dalvi et al., 1987; Ronis et al., 1987; Ronis & Hodgson, 1989; Sinclair et al., 1989; Hobbs et al., 1986; Ono et al., 1988; McPhaul et al., 1988.

(2) Schwen & Mannering, 1982a, 1982b, 1982c; Harri, 1980; Noshiro & Omura, 1983; Miura, 1985; Miura et al., 1986.

(3) Schwen & Mannering, 1982a, 1982b, 1982c; Jewell et al., 1989.

(4) Schwen & Mannering, 1982a, 1982b, 1982c; Heilmann et al., 1988; Others as listed in tables 4 & 5 and in text.

(1986), and the observation that levels of the P450E (P450IA1 orthologue) present as detected by antibody methods correlated with the PCB levels present. However P450 activities have been detected in fish from a wide range of species and from a number of different habitats. Importantly, once again, these studies have demonstrated both differences and similarities when the fish P450s are compared to the mammalian forms. The teleost P450IA1 is, like mammalian P450IA1, inducible by BNF, the model P450IA1 substrate (Klopper-Sams & Stegeman, 1989; Klopper-Sams, 1989). There is a suggestion that this induction may be mediated by a receptor mechanism, since a TCDD-binding protein similar to that found in rat has been observed (Heilmann et al., 1988). *→ Halobiphenyls - found in all vertebrates - not in invertebrates* However a difference in sensitivity to induction of P450IA1 when compared with rat has been observed for halobiphenyls (Gooch et al., 1989). *spatial orientation of Cl⁻ affects activity*

One of the characteristics of studies of fish P450 has been the apparent lack of any response to phenobarbital-type inducers. Induction of a large number of P450II family isozymes by phenobarbital is observed in the rat, whereas fish P450s are apparently refractory to induction by such compounds. This had been purported to be a result of different selective evolutionary pressures on the two groups of animals, particularly as a result of diet (Stegeman, 1981; Nelson & Strobel, 1987). However, enzyme activity characteristic of mammalian P450II family isozymes has now been detected (Stegeman, 1989) and homologous sequences have recently been shown to be present in rainbow trout genomic DNA (Haash et al., 1990).

From examination of the P450 "Family Tree" (fig. 1.4) and a knowledge of intermediary metabolism, it might be expected that other P450 isozymes and activities will be present in

marine organisms. Indeed, this is the case. Table 4 summarises the activities identified for proteins purified by SDS PAGE, and the following additional activities have been detected:

(i) Aromatase, though this has not been proven to be P450 dependent (Timmers & Lambert, 1987)

(ii) Ethylmorphine-N-demethylase (Goksøyr et al., 1987)

(iii) Nitrosamine activation (a P450IIE1 dependent activity in mammals) (Schultz & Schultz, 1984).

Induction has been observed in a number of species with the P450IA1 model inducers β -naphthoflavone and 3-methyl-cholanthrene, and with compound mixtures or environmental mixtures (Tab. 1.5).

Other factors producing changes in P450 levels include season (James & Bend, 1980; Walton et al., 1983; Stegeman & Kloepper-Sams, 1987) and starvation (Andersson et al., 1985). Levels of individual isozymes and/or P450-dependent catalytic activity also vary, as do mammalian P450s, between species (Goksøyr et al., 1987; Monod et al., 1987; Swain & Melius, 1984).

Incomplete though these data are, sufficient resources are now available to obtain protocols for assessing the effects of pollution (particularly involving P450IA1-type inducers) in freshwater, coastal and deep sea habitats. Further work is required to elucidate the full spectrum of P450s present in fish. Of particular interest in this regard is the isozyme responsible for the aflatoxin activation, as the fish seem to

Table 1.4 : Teleost hepatic cytochrome P450 isozymes; Physical properties and catalytic activities

<u>Isozyme</u>	<u>X max</u> <u>nm</u>	<u>Mr</u> <u>kDaltons</u>	<u>Major characteristics</u>
Scup (1)			
P-450A	447.5	52.7	testosterone-6 β -OH, ECOD
P-450B	449.5	45.9	tentative 15-a-OH
P-450C	450	49-51	
P-450D	451	50	
P-450E	447	54.3	EROD, ECOD & AHH (4)
Rainbow trout (2)			
LM1	449	50	
LM2	449.5	54	lauric acid-OH, aflatoxin oxidn. (5)
LM3	447.5	56.5	
LM4a	447	58	EROD, AHH (6)
LM4b	447	58	EROD, AHH (6)
Cod (3)			
P-450a	447.5	55	no EROD
P-450b	447.5	54	no EROD
P-450c	448	58	EROD, B-4-OH
P-450d	448	56	EROD

References/Notes to Table 1.4;

- (1) Klotz et al. 1983, 1986
- (2) Williams and Buhler 1983, 1984
- (3) Goksøyr et al. 1985, 1987
- (4) P-450E has been shown by a number of criteria to be the orthologue to rat and trout P450IA1 and cod P450c (Kloepper-Sams, 1989).
- (5) P450IIIA3-dependent in mammals
- (6) P450IA1 (probably 1 isoenzyme)

Abbreviations used;

AHH	aryl hydrocarbon hydroxylase
B-4-OH	biphenyl-4-hydroxylase
ECOD	ethoxycoumarin-7-hydroxylase
EROD	ethoxyresorufin-7-hydroxylase
-OH	hydroxylase (activity)
oxidn.	oxidation

Table 1.5 : Induction of P450 activities in different fish species

BNF/3MC

Rainbow Trout	Williams & Buhler, 1983; Goksoyr <u>et al.</u> , 1987
Cod	Goksoyr <u>et al.</u> , 1987
Scup	Kloepper-Sams <u>et al.</u> , 1987
Plaice	George & Young, 1986
Marsh Killifish	Kloepper-Sams & Stegeman, 1988, 1989
Goldfish	Swain & Melius, 1984
Black Bullhead	"
Brown Bullhead	"

Environmental exposure/Compound Mixes

Rainbow Trout	Payne, 1977
Marsh Killifish	Elskus & Stegeman, 1989
Cunner	Walton <u>et al.</u> , 1983
Rattail	Stegeman <u>et al.</u> , 1986
Spot	Van Veld <u>et al.</u> , 1988

This table summarises published data showing changes in P450 (measured by a number of criteria), in response to experimental and environmental exposure to a range of inducers.

be particularly susceptible (compared to rat) to the effects of this compound (Sinnhuber et al., 1977). Also of interest (and possibly involving the same fish isozyme - see fig 1.8) is the lauric acid hydroxylase activity, which in mammals involves P450IVA1 concomitant with peroxisome proliferation. Peroxisome proliferation is also observed in fish (Yang et al., 1990), and DEHP, an inducer of P450IVA1 and a common environmental pollutant, is metabolised by fish (Melancon & Lech, 1976), so an equivalent isozyme may be present. It is also likely that P450 isozymes responsible for steroid metabolism in mammals may also have teleost equivalents, particularly since good conservation is seen between avian and mammalian sequences.

1.4.2 Invertebrate Cytochromes P450

A reasonably large body of data on invertebrate xenobiotic metabolism has accumulated, on both comparative aspects and responses to chemicals. In comparison to vertebrates, however, understanding of even fundamental aspects of cytochrome P450 function is limited. Reasons for this include low specific contents or activities of P450s, apparent difference in regulation (i.e. non-inducibility compared to mammalian P450s) and technical difficulties resulting in transferring mammalian-derived study techniques to non-mammalian species.

In fact, the only published P450 sequence from a multicellular invertebrate, P450VIA1, was isolated from the housefly (Feyereisen et al., 1989), although works describing two more from the cockroach and the pond snail are in preparation (Nebert, 1991). The housefly isozyme shows little sequence similarity with any of the vertebrate enzymes. This

animal has been extensively studied as a model species and this and other insect species have been recently reviewed (Ronis & Hodgson, 1989). In particular, variation in P450 levels has been shown in response to xenobiotic exposure. Induction of housefly and other insect cytochromes also appears catalytically comparable with mammalian P450s, though the isozymes present (Tab. 1.1) are indicated to be quite different (Clarke et al., 1989).

In comparison, the situation in marine invertebrates is much less clear. However, the current knowledge is comprehensively reviewed by Payne et al. (1987) and Livingstone (1991). In the higher phyla (echinoderms and crustaceans), P450-dependent metabolism is well documented. Den Besten & Livingstone (1989) demonstrated P450 and BPH activity in a number of echinoderms, and endogenous metabolism indicates a requirement for P450-type activity (steroid metabolism in e.g. the starfish Asterias rubens; Voogt et al. 1987). Data on induction responses to foreign chemicals (Xenobiotics) and seasonal variation in P450s are limited. Elevation of P450-dependent monooxygenase activity was seen in a number of crustacea from polluted sites compared to clean sites (Lee, 1981; Lee et al., 1981). Experimental putative induction shows variation between species e.g. Bihari et al. (1984) and Batel et al. (1988) reported increases in BPH activity by IA1-type inducers in the crab Maja crispata, whereas James & Little (1984) detected no change in the lobster Panulirus argus. There are no reports of a phenobarbital-type induction in these two groups of invertebrates. P450-dependent steroid metabolising activity has been observed, e.g. in lobsters (James & Shiverick, 1984) and tissue distribution has been studied in a number of

species e.g. in shore crab (O'Hara et al., 1982). Progress in understanding P450 function and regulation in these species has been hampered, mainly by the lack of purified proteins. Despite repeated efforts, all attempts yielded degraded or inactive protein. This was attributed to the labile nature of the P450 system in the species studied and to the presence of endogenous inhibitors of activity released on homogenisation (James, 1989). However, recently James (1990) published details of the isolation of 3 forms of P450 from the lobster, P. argus. Positive identification of the isozymes isolated was not possible from the data presented. However, it was noted that none of the purified protein activities were inhibited by metyrapone or were inducible by classical P450IA or IIB inducers, indicating that they were probably not orthologues of the mammalian P450IA or IIB families. This evidence is suggestive that these P450s may have a role in endogenous metabolism rather than xenobiotic metabolism. However, positive identification of the gene products responsible for these activities must await the sequencing of the respective genomic or complementary DNA.

The molluscan P450 system is considered in detail in the following sections. Much of the remaining information concerning marine invertebrates is somewhat superficial. In annelids, MFO activity is indicated to be present in Nereis sp. (Payne 1977). Lee et al. (1981) demonstrated increases in P450 specific content and BPH activity in the ragworm Nereis virens but the responses are not consistent and no pattern of dosage or time course is apparent (Livingstone 1991). Of the lower invertebrate phyla (Porifera, Coelenterata and other minor ones), no direct evidence for P450 dependent metabolism is available. However existence of a monooxygenase is indicated from metabolic pathways present e.g. sterol synthesis in sponges (Guella et al., 1988) and aldrin

oxidation in a number of species (Kahn et al. 1972).

Studies in prokaryotes have yielded information to augment the general understanding of cytochromes P450. Particularly useful is the work of Poulos et al. (1986) on the crystallisation and subsequent analysis of the inducible aqueous P450cam protein (P450C1) from Pseudomonas putida. Together with sequence data for this isozyme (Hanui et al., 1982 and Unger et al., 1986), information about the tertiary structure of cytochrome P450cam was obtained. Using computer modelling and the derived polypeptide sequences for other cytochromes P450, it appears that many, if not all, cytochromes P450 show sufficient similarity to be "fitted" to the structure obtained by Poulos et al.. Also, this data provided evidence to confirm that the conserved cysteine residue, present in all P450s, is the binding site of the haem moiety of the active enzyme.

Cytochrome P450s studies include data from a range of other species, some of which include DNA sequence data. These studies include other bacterial species and yeasts (Kalb et al., 1987; Chen et al., 1988; Ruettinger et al., 1989; Sanglard & Loper, 1987), fungi (Breskvar & Hudnik-Plevnik, 1977), protista, (Gupta et al., 1987), and higher plants (Cottrell et al. 1990; Bozak et al., 1990).

1.5 The Common Mussel, *Mytilus edulis*

1.5.1 Ecology, Physiology and Biochemistry

The common mussel, *Mytilus edulis* L., a bivalve mollusc, is a sedentary filter-feeder of widespread global occurrence. It attains a relatively large size (up to approximately 10cm in length), particularly in warmer waters, and is often the dominant organism of intertidal and sublittoral ecosystems of coastal and estuarine habitats (Yonge, 1976; Krieger et al., 1981). Mussel species are found on a wide variety of substrata such as rock, stones, shingle or sediment. Those occupying the intertidal zone experience marked diurnal and seasonal changes in, for example, water- and air-temperature, salinity, oxygen availability and humidity.

An annotated diagram and photograph overleaf (Fig. 1.5) show the major tissues and their organisation in *M. edulis*. The major tissue with respect to the MFO system is the digestive gland (Livingstone & Farrar, 1984 and 1.5.2 below), lying centrally and dorsally near the mouth of the digestive system and cerebral ganglion. The digestive gland has also been called, questionably (Viarengo et al., 1986), the hepatopancreas. However, precise biochemical evidence for pancreatic and hepatic functions are not available, though its involvement in digestive and storage functions is known. The tissue is a system of blind-ending tubules containing a number of cell types. These are rich in lysosomes involved in intracellular digestion, and the sequestration of heavy metals or organic xenobiotics (Owen, 1972; Moore, 1985; Punin & Konstantinova, 1988). The digestive gland and mantle in

Figure 1.5: Physiology of the marine mussel, *Mytilus edulis*

a) Photograph of individual animal after severing of posterior adductor muscle.

b) Section through animal showing tissue organisation.

particular are relatively rich in lipids (Gabbot, 1983). As a consequence of this and the mode of feeding of mussels, lipophilic xenobiotics from the water column are readily concentrated in these tissues (Geyer et al., 1982).

It is these aspects of mussel biology: size, occurrence, numbers, sessile habit and tendency to bioaccumulate xenobiotics (Mix, 1984; Bayne et al., 1985), which make the mussel attractive for use as a sentinel organism to monitor environmental pollution. The "Earthwatch" and "Mussel Watch" programmes (Jensen et al., 1975; Goldberg et al., 1978) have thus stimulated a number of investigations worldwide (Livingstone, 1991) using this organism.

The intermediary metabolism of the Mollusca is comparatively well understood, with a large body of data being available on occurrence of various pathways, regulation and comparison with higher animals (Gabbot, 1983; Livingstone, 1983; Livingstone & Clarke, 1983; Livingstone et al., 1983; Churchill, 1987). In particular, potential endogenous roles for P450s have been identified, for example in sterol metabolism (Khan & Goad, 1983), arachidonic acid metabolism (Srivastava & Mustafa, 1985) and vitamin D₃ metabolism (Lehtovaara & Koskinen, 1986). Understanding of xenobiotic metabolism is somewhat more limited, though a large amount of observational data is available (see 1.5.2).

Knowledge of molluscan genetics and molecular biology is also limited. A number of studies on genetic variation and population dynamics in Mytilus have been carried out (Skibinski, 1985; Dixon & Flavell, 1986; Edwards & Skibinski, 1987). Reports of nucleotide sequences in Mytilus are limited to the 5SrRNA and 28SrRNA (part only) ribosomal RNA sequences (Fang et al., 1982; Qu et al., 1988). There are no reports of

cdNA or genomic DNA libraries for Mytilus species to my knowledge, although genes from the terrestrial molluscs Aplysia (Scheller et al., 1983; Maudale & Axel, 1985) and Lymnaea stagnalis (Smit et al., 1988) have been isolated.

→ Pond Snail - Willbink 1991 xenobiotics

1.5.2 Mixed Function Oxidase and Cytochrome P450 in Mytilus edulis and other molluscs

Presence of various organic pollutants in surrounding water has been demonstrated to cause a number of deleterious effects in mussels (Aarset & Zachariassen, 1982; Mix, 1986; McCormick-Ray, 1987; Moore et al., 1987, 1988; Livingstone, 1989). In mammals the primary route of disposal of many of these would be via the cytochrome P450 system (with concomitant risk of bioactivation and its consequences).

However a number of early studies failed to demonstrate any cytochrome P450 activity in mussels (Lee et al., 1972; Payne et al., 1977; Vandermeulen & Penrose, 1978) or other bivalve species (clams: Carlson, 1972; Vandermeulen & Penrose: oysters: Vandermeulen & Penrose, 1978). It was argued that this accounted for the persistence of, for example, hydrocarbons in bivalve tissues, rather than the comparatively rapid metabolism and excretion observed in mammals. In contrast, other studies did detect in vitro metabolism indicative of a MFO system in bivalves. Aldrin epoxidation was reported by Krieger et al., (1979) in the mussel Mytilus californianus and by Bayne et al., (1979) and Moore et al., (1980) in M. edulis. Biphenyl hydroxylation (Willis & Addison, 1974) and BPH activity (Stegeman, 1980, 1981; Mix et al., 1981; Payne et al., 1983) were also detected in M. edulis and p-nitroanisole-O-demethylation (Trautman et al., 1979) and antipyrine hydroxylation (Krieger et al., 1979) were

demonstrated in M. californianus.

Cytochromes P450, cytochrome b_5 , and NADH- and NADPH-dependent P450 reductase activities were first demonstrated in Mytilus by Ade et al. (1982) (in M. galloprovincialis), and in M. edulis by Livingstone & Farrar (1984). Tissue and subcellular characterisation studies in M. edulis indicated that, in common with other phyla, the P450 monooxygenase activity is primarily membrane bound in the endoplasmic reticulum and, in mussels, primarily located in the digestive gland (Livingstone and Farrar, 1984). Cytochrome P450 has also been detected in gills (Stegeman, 1985), and cytochrome b_5 , P450 reductase and BPH activity have a much wider tissue distribution (Livingstone & Farrar, 1984). Cytochrome P450 reductase and BPH activities, but not cytochrome P450 or cytochrome b_5 , were detected in haemocytes (blood cells) by both these groups of workers. Whilst many of these observations suggest similarities between the molluscan and mammalian P450 systems, there were some differences. This included the presence of NADPH-independent monooxygenase activity (Stegeman, 1985; Livingstone et al., 1989b) which has been demonstrated to be inhibited by reduced cofactor and is suggested to perhaps be due to a one-electron transfer from the substrate (Livingstone et al., 1989). This hypothesis is supported by evidence from studies of BPH metabolism which in mussels yielded primarily quinones and phenols (Stegeman, 1985; Livingstone et al., 1988b), known to arise from the one-electron transfer route (Cavalieri & Rogan, 1982; section 1.2). Other differences include the observation that some model compounds show different binding spectra in mussel P450 compared with mammalian P450s (Kirchin, 1988).

Studies to try and purify components of the P450 system

have been attempted but with only partial success (Kirchin et al., 1987; Livingstone et al., 1989a), the problems encountered being similar to those of workers purifying P450 from other lower animals (see 1.4.2). However, the study of Kirchin (1988) suggests the presence of at least two isozymes (2 peaks showing absorbance at circa 450nm in CO-difference spectra) after DEAE-Sephacel chromatography, and SDS-PAGE of the major peak showed a single band at 53kD (Kirchin et al., 1987; Kirchin, 1988; Livingstone, 1989a). The identity of these peaks has not been established.

It has been shown that a variety of factors influence levels of P450 activity in mussels and other molluscs. In Mytilus sp., a slightly higher BPH activity and MFO components is recorded in female than male animals (Livingstone, 1985; Suteau et al., 1985; Livingstone, 1987; Kirchin, 1988) at various times of the year. Indeed, levels of MFO components and activities do show seasonal variation: Livingstone (1985) and Suteau et al. (1985) both found a maximum in the summer/autumn and a minimum in the colder winter months. This has been shown to correlate to a degree with changes in water-temperature; both Suteau et al. (1985), and Kirchin (1988) found correlations between water-temperature and P450 specific content. However, the existing data also suggests that other regulatory factors are involved. Kirchin (1988) found no relationship between water-temperature and BPH and NADPH-independent ECOD activity. Indeed, the ECOD activity was undetectable when P450 content and water-temperature were maximal (June), suggesting of a role for P450 in endogenous metabolism.

It is known that many changes in marine invertebrates are linked to cycles of food storage and gametogenesis (Gabbot,

1983). In the Whitsand population used in this study, gametogenesis occurs in autumn and winter, utilising glycogen stored from the previous summer. The ripening of gametes begins in the spring and levels present remain high until spawning in June-August (Lowe et al., 1982). Kirchin (1988) also noted that in digestive gland, total P450 content was maximal during the spawning period and NADPH-independent ECOD activity correlated with the appearance of a second 51.5 kD microsomal protein through the winter months. This, together with a detected seasonal variation in the carbon monoxide difference spectra for the mussel P450 was considered suggestive of differential regulation of at least two isozymes.

A number of attempts have been made to measure changes in MFO levels in response to exposure to foreign compounds using animals treated experimentally and obtained from field study sites. Table 1.6 below summarises the data published for mussel and other molluscs. There is no clear picture of how molluscan P450s are regulated and levels of "induction" are somewhat lower than for higher species. However the following main results have been obtained:

In M. galloprovincialis, both digestive gland microsomal cytochrome P450 specific content and cytochrome b₅ specific content increased in response to laboratory exposures to paraffin, anthracene, perylene and 3MC and in response to field exposures to hydrocarbons (Gilewicz et al., 1984). In digestive gland microsomes of M. edulis, cytochrome P450 and b₅ specific content and NADPH- and NADH-dependent reductase activity were elevated in both short-term and long-term experimental mesocosm-exposure to PAH (Livingstone et al., 1985; Livingstone, 1987, 1988). However, BPH activity

measured fluorometrically (which is increased by PAH in mammals via IA1 induction), was not observed to be elevated by these workers. The changes in MFO system components of M. edulis were seasonally variable (little or no response was seen when animals were ripe with gametes) and, in the case of P450, accompanied by a 2-3 nm blue shift of the CO-difference spectrum peak, (Livingstone, 1987). Again this is indicative of the presence of more than one isozyme.

Also of interest was the recent data of Galli et al. (1988) demonstrating induction by phenobarbital (PB). It is widely considered that molluscs may not have the range of PB-inducible enzymes observed in mammals (P450II family) (Nebert & Gonzalez, 1987), and other workers have failed to detect a similar response in molluscs (Kirchin, 1988; Livingstone et al., 1988a; Suteau et al., 1988).

These data demonstrate that it is possible to alter MFO content/activity in mussels and indicate that more than one isozyme is present. To date however, no given isozyme has been positively identified within the framework of the known classification system.

Table 1.6: Response of some molluscan MFO components following experimental exposure to xenobiotics

criterion Species (Ref)	Xenobiotic	% increase in		
		Cyt. P450 reductase	Cyt. P450	
BPH (i)	(ii)	(iii)		
<u>Crassostrea virginica</u> (1)	3MC, B(a)P, Aroclors	-	-	130-304
<u>C. virginica</u> (2)	Aroclor 1254	-	-	0-74
<u>Mytilus edulis</u> (3)	3MC (Seasonal)	0-34	0-46	0
<u>M. edulis</u> (3)	PB (Seasonal)	0	0-26	0
<u>M. edulis</u> (4,5)	diesel oil (PAH) (Seasonal)	41-200	0-31	0*
<u>M. edulis</u> (6)	diesel oil (PAH) (Seasonal)	0-100	0-100	0-200*
<u>Mytilus galloprovincialis</u> (8)	various PAH	75-1470	-	-
<u>M. galloprovincialis</u> (8)	B(a)P, TCBP	-	-	up to 93
<u>M. galloprovincialis</u> (8)	HCBP	0	-	0
<u>M. galloprovincialis</u> (9)	PB	367	-	-
<u>Chrytochiton stelleri</u> (10)	BNF	0	0	525 & 0*
<u>Littorina littorea</u> (4,5,6)	diesel oil	200-300	51-191	0*
<u>Thais haemastoma</u> (11)	Crude oil (PAH)	-	61	0*

Abbreviations and references listed overleaf.

(i) Data are for digestive gland microsomes except for 1,2 (digestive gland homogenates) and 8 (whole-body microsomes).

(ii) See refs. for dosages & exposure times, which vary greatly.

(iii) Radiometric assay, except for data marked * (fluorimetric assay).

Table 1.6 (contd.)

References:

- 1 Anderson, 1978
- 2 Anderson, 1985
- 3 Livingstone et al., 1988a
- 4 Livingstone et al., 1985
- 5 Livingstone, 1988
- 6 Livingstone, 1987
- 7 Gilewicz et al., 1984
- 8 Suteau et al., 1988
- 9 Galli et al., 1988
- 10 Schlenk & Buhler, 1988
- 11 Livingstone et al., 1986

Abbreviations:

B(a)P	benzo(a)pyrene
BNF	β -naphthoflavone
BPH	benzo(a)pyrene hydroxylase activity
HCBP	hexachlorobiphenyl
3MC	3-methylcholanthrene
PAH	polycyclic aromatic hydrocarbons
PB	phenobarbital
TCBP	tetrachlorobiphenyl

1.6 Outline of studies undertaken

At the time of commencing these studies no single cytochrome P450 isozyme had been positively identified in Mytilus species. Several attempts to isolate and purify a P450 protein had only limited success due to technical difficulties (sections 1.4.2 & 1.5.2), thus preventing the possibility of isolating the mussel P450 genes(s) by antibody-dependent methods.

In my study, an alternative route was tried in order to identify which P450s are present in Mytilus, namely the technique of nucleic acid hybridisation. Using radiolabelled cDNA probes to P450s isolated from other species, it was hoped to detect similar nucleic acid sequences in mussel RNA or DNA, and in gene libraries containing mussel nucleic acid sequences. It is important to note however that to allow the probe DNA to bind to the molluscan nucleic acid sequences, experimental conditions of "low stringency" are used which allow the probe to tolerate a number of mismatching bases in the sequence.

By this method an understanding of the similarity of mussel P450s to P450s from higher species and, most importantly, of aspects of their regulation could be gained. This would be of significance, not only in examining the usefulness of mussel P450 levels in monitoring pollution, but also in establishing the interspecies relationships between P450s at a genetic/ evolutionary level, and in providing further information about the role of cytochromes P450 in endogenous metabolism.

This technique of cross-species DNA hybridisation has previously been used successfully to study protein orthologues

in a number instances, for example in the β -globin gene family (Barrie et al., 1981), and in the cytochrome P450IV family (Hawkins, 1987).

Initial investigations focused on attempts to detect sequences homologous to rat P450IVA1 and P450IIE1 cDNA. Sequences corresponding to other P450s were examined as and when probes became available to us. P450 probes which gave positive signals were then used to study induction of mussel P450 mRNA using an inducer known to increase the relevant P450 in mammals. Other marine animals were also studied in conjunction with this part of the investigation.

Concomitant with the gene expression studies, attempts were made to prepare a library of M. edulis nucleic acid sequences. Initial attempts to make cDNA were unsuccessful due to problems associated with isolation of intact mussel mRNA. However synthesis of a genomic DNA library was completed successfully shortly before the end of my work. This has yet to be screened for the presence of P450 and other genes.

The advent of the polymerase chain reaction (PCR) technology during this investigation provides another route by which mussel gene sequences may be isolated. PCR amplification of mussel DNA sequences using trout-derived primers was demonstrated in preliminary experiments, thus confirming the feasibility of this type of approach for future studies.

The studies have thus demonstrated that the techniques of genetic engineering may be of more success in the characterisation of M. edulis P450 isozymes than previous attempts to purify the enzyme directly.

2.1 Introduction

In this chapter, the experimental procedures used throughout this investigation are described in detail. Any techniques common to two or more procedures are described in section 2.3. Recipes for the buffers, media and solutions used in the procedures are given in Appendix 1. In all cases high quality (Millipore R.O.) water was used, with sterilisation by autoclaving and DEPC treatment where appropriate. Most procedures used are modifications of previously reported methods, with original references being given where appropriate. Experiments were performed with regard to appropriate safety guidelines and with routine use of gloves to prevent nuclease contamination of samples. Details of procedures are given with a brief discussion of the principles involved in the method.

2.2 Materials

The suppliers of enzymes and kits are given in the appropriate methods sections, together with the suppliers of equipment and any other reagents specific to a particular technique.

2.2.1 Chemicals

All chemicals were obtained from BDH, Analar grade or higher, with the exception of the following:

Sigma Chemical Company; Citric acid (trisodium salt), Sodium chloride, MOPS, EDTA, Ethidium bromide, Polyvinyl pyrrolidone, Bromophenol blue, Xylene cyanol, Spermidine trihydrochloride. Biorad; Acrylamide, N,N'-methylenebisacrylamide, TEMED, Ammonium persulphate.

BRL (Ultra Pure reagent grade); Agarose, LMP Agarose, Guanidine chloride, Guanidine isothiocyanate, Urea.

Difco; Bacto-tryptone, Bacto-agar.

Beta Labs: Yeast extract.

Pharmacia; Ficoll 400.

ICN Biomedicals; NusieveTM agarose.

2.2.2 Radiochemicals

All procedures involving radiochemicals were carried out in accordance with the guidelines described in "Local Rules for Protection Against Ionising Radiation", published by the University of Surrey Safety Office. The radiochemicals were obtained from Amersham International (1,2,3,4) or ICN biomedicals Ltd. (1), and were used within one half life of the release date (14 days for ³²P or 40 days for ³⁵S).

The following radiochemicals were used:

1. Deoxycytidine-5'-[alpha-³²P]-triphosphate, triethylammonium salt at 370MBq/ml. Specific activity approx. 3000TBq/mmol. Abbreviated to ³²P-dCTP.
2. Adenosine-5'-[gamma-³²P]-triphosphate, triethylammonium salt at 370MBq/ml. Specific activity approx. 3000TBq/mmol. Abbreviated to gamma-³²P ATP.
3. Deoxyadenosine-5'-[alpha-³⁵S]-triphosphate, triethylammonium salt (Sp isomer) at 370MBq/ml. Specific activity approx. 37TBq/mmol. Abbreviated to ³⁵S-dATP.
4. L-[³⁵S] methionine at 555MBq/ml. Specific activity approx. 46.7TBq/mmol.

2.2.3 Bacterial strains

The strains of E. coli used, together with genotypes and preferred culture media, are shown in Figure 2.1

2.2.4 Cloning vectors and cDNA Probes

The restriction maps of the plasmid and phage vectors used together with information on antibiotic selection etc. are shown in figures 2.2 and 2.3. Schematic diagrams of the probes used and references for their isolation are included in figure 2.4.

Figure 2.1: Details of E. coli strains used in this study

STRAIN	USAGE	GENOTYPE	REFERENCE
	[MEDIUM]		
TB1	Plasmid host	<u>ara</u> , <u>hsdR</u> , <u>thi</u> , r_k^+ ,	BRL
	[TYN-broth]	<u>strA</u> , δ <u>lac-pro</u> ,	(1989)
		ϕ 80dlacZ δ M15	
LE392	lambda EMBL3 host	F^- , <u>hsdR</u> 514, r_k^- , m_k^+ , Maniatis	
	[LB-Mg broth]	<u>supE</u> 44, <u>supF</u> 58, <u>galk</u> 2, <u>et al.</u> 1982	
		δ (<u>lacIZY</u>)6, <u>galT</u> 22,	
		<u>metB</u> 1, <u>trpR</u> 55, λ^-	
P2392	lambda EMBL3 host	F^- , <u>hsdR</u> 514, r_k^- , m_k^+ , Maniatis	
	(selective)	<u>supE</u> 44, <u>supF</u> 58, <u>galk</u> 2, <u>et al.</u> 1982	
	[LB-Mg broth]	δ (<u>lacIZY</u>)6, <u>galT</u> 22,	
		<u>metB</u> 1, <u>trpR</u> 55, λ^- , (P2)	
Y1090	lambda gt11 host	δ (<u>lacU</u> 169), <u>proA</u> ⁺ ,	Young &
	(selective)	δ (<u>lon</u>), <u>araD</u> 139, <u>strA</u> , Davis, 1983	
	[LB-Mg broth]	(pMC9), [<u>trpC</u> 22::Tn10]	
		<u>supF</u>	

Figure 2.2 Plasmid vectors used in this study

Plasmid	Selection	Reference	cDNA insert(s)
pSP64	Ampicillin	Melton <i>et al.</i> , 1984	(IIB1)

pUC18/pUC19	Ampicillin	Norrande <i>et al.</i> , 1983	(pUC19; IA1, IIE1, Yanisch-Perron <i>et al.</i> , 1985	IVA1, GPX, Actin) (pUC18; XIA1)
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Contd. overleaf

Fig. 2.2 (contd.)

Plasmid	Selection	Reference	cDNA insert
pEMBL	Ampicillin	Dente <u>et al.</u> , 1983	(IIC6)

Originally described by Frischauf et al. (1983)

Originally described by Young & Davis (1983)

Figure 2.4 Schematic representations of the probes used in this study

P450IA1 (trout) 1400bp/EcoRI+PstI (Heilmann, 1988)

P450IIB1 (rat) 1700bp/EcoRI (Fujii-Kuriyama et al., 1982)

P450IIC6 (human) 600bp/PstI (Meehan et al., 1988)

P450IIE1 (rat) 1100bp/EcoRI (Richardson, 1990)

P450IVA1 (rat) 2100bp/EcoRI (Earnshaw et al., 1988)

P450XIA1 (human) 1900bp/EcoRI (Chung et al., 1986)

(contd. overleaf)

Figure 2.4 (contd.)

GPX (human) 1100bp/XhoI (Dunn, 1990)

Actin (rat) 2000bp/EcoRI (Gunning *et al.*, 1983)

Key:

A AluI
 Av AvaI
 B BamHI
 Bg BglI
 C ClaI
 E EcoRI
 H HindIII
 N NcoI
 P PstI
 Pv PvuII
 S SstI
 Sp SphI
 X XhoI

* denotes that restriction site is part of the multiple cloning site.

denotes position of conserved haem binding cysteine residue in P450 probes.

Underlined = coding region

Not underlined = non-coding region

Figures relate to deduced amino acid sequence.

2.3 : General Procedures

The following methods were routinely used in a number of different experiments. They are set out here, and are not described in detail in each individual case.

2.3.1 Phenol extraction

The standard procedure was as follows: An equal volume of room temperature TE-saturated redistilled phenol was added to the aqueous solution and mixed. The resulting emulsion was centrifuged at 14k rpm in a microfuge (Eppendorf) (samples less than 1.5ml original volume) or 9k rpm, 10°C for 10 minutes in the JA17 or JA20.1 rotor of the Beckman J2-21 centrifuge (samples greater than 1.5ml original volume).

Protein, SDS and agarose separate into the lower (phenolic) phase, whereas nucleic acids remain in the upper (aqueous) phase. Often a white or off-white interface was also present. The aqueous phase is then re-extracted with phenol until the interface is clear, and then extracted with a 1:1 mixture of TE-saturated phenol:chloroform/isoamyl alcohol (App. 1). A final extraction with an equal volume of chloroform/isoamyl alcohol removes the phenol from the solution.

2.3.2 Ethanol precipitation of Nucleic acids

Where no details are given, precipitation was achieved by addition of 0.1 volume 3M sodium acetate and 2 volumes of 100% ethanol. The mixture was then left at -70°C for at least 20 minutes or at -20°C overnight. The nucleic acid was collected by centrifugation at 14k rpm (Max. speed)/4°C in a microfuge or 10k rpm/4°C in the J2-21 for 20 minutes. The supernatant

was immediately removed and the resulting pellet washed with 70% ethanol and vacuum dried in a Univap for 15-20 minutes.

2.3.3 Univap Operation

The Univap (Uniscience Ltd.) is a centrifugal evaporator which dries samples by heating while centrifuging at low speed under vacuum. There are two rotors which will take either microfuge tubes (1.5ml or 0.5ml) or 14ml polypropylene tubes. The sample is placed in the preheated (45°C) rotor and spun to 2k rpm. The vacuum pump (Aquavac, Uniscience) is activated once the rotor reaches 1k rpm, and samples with a residual volume of 50 μ l ^{70% ethanol} are usually dry after 15-20 minutes.

2.3.4 Agarose gel electrophoresis

Agarose gel electrophoresis of nucleic acids was carried out using standard methods as described in Maniatis et al. 1982. In this procedure, the molecules migrate and are separated according to size.

In the case of DNA samples, appropriate DNA markers are run alongside so that the length of the sample DNA(s) can be estimated. Lambda DNA, Hind III fragments (BRL) were used for sizing DNA molecules larger than 0.5kbases, while ϕ x174 rfdNA, Hae III fragments were used for sizing molecules of between 0.1 and 1.0 kbases. The buffer system used throughout was TAE buffer (App. 1) and DNA samples were prepared in DNA running dye (App. 1). Agarose concentrations of between 0.3% and 4% were used, depending on the size of DNAs to be separated (i.e. low concentrations to separate large fragments and vice versa). For most purposes, concentrations of 0.8-1.0% were used.

In the case of RNA samples, these were run on denaturing

agarose gels after preparation in RNA running dye (App. 1). Gels were routinely made up to a concentration of 1.5% in 1x RNA gel buffer (App. 1)/ 18% formaldehyde (37% solution in water). In house preparations of rat liver RNA were used as markers.

For most studies the Pharmacia GNA-100 submarine apparatus was used, but for larger gels, either the BRL Model H5 Submarine gel apparatus or a "home grown" equivalent was used.

The gels were stained with ethidium bromide (Maniatis et al., 1982; App. 1), taking appropriate precautions against spillage, and visualised under UV light using a Model TM20 transilluminator (Genetic Research Instrumentation Ltd.).

2.3.5 Photography

Gels were photographed through an orange filter using a Polaroid Land Speed MP-4 camera and Polaroid type 55 positive/negative single sheet film (occasionally a type 665 8-sheet cartridge was substituted). Photographs in either case were exposed through maximum aperture for 12 seconds and allowed to develop for 25 seconds. Negatives were then soaked overnight in 0.1% sodium sulphite before rinsing in water and air drying.

2.3.6 Recovery of DNA from agarose gels

After size fractionation in agarose, it is often necessary to recover DNA fragments e.g. for radiolabelling as hybridisation probes. A variety of techniques were employed. After electrophoresis in a low melting point (LMP) gel and subsequent staining, the fragment of interest was excised under UV-illumination in the smallest volume of gel possible.

1. Phenol extraction (Maniatis et al., 1982)

An equal weight of water was added, the agarose melted at 70°C, and the resulting mixture cooled to 37°C. After 20 minutes an equal volume of ice cold TE-saturated phenol was added, mixed, and left on ice for ≈ 1 minute to precipitate the agarose. After centrifugation, the agarose interface was re-extracted with an equal volume of TE, re-centrifuged and the two aqueous layers combined. The 2.3.1 protocol was then followed, and prior to precipitation, the volume was reduced by sequential extractions with an equal volume of butan-2-ol to reduce the volume to 400 μ l or less. After precipitation and drying, the DNA is dissolved in TE to a final volume of 0.1 μ g/ μ l. In my experience, good DNA was obtained by this method, but yields can sometimes be erratic, especially from small samples.

2. GenecleanTM Method

(Performed according to manufacturers protocol; UK Distributors; Stratech Scientific Ltd.)

After melting the gel slice into a concentrated sodium iodide solution, the DNA is adsorbed onto a silica matrix. The matrix is pelleted, washed, and the DNA eluted into a small volume. This method is rapid and gives good yields of molecules up to 10kb. However, some difficulty in removing the last traces of sodium iodide was observed by other workers in this laboratory, which may make the resulting DNA unsuitable for ligation reactions. It is however, sufficiently pure for e.g. labelling reactions with DNA polymerases.

3. Use of Spin-X Columns

The gel slice was placed in the top section of the Spin-X (Costar) column. The column was then spun at max speed (Microfuge) for 40 minutes at 4°C. During spinning of the gel slice above the support membrane, it compresses down. The electrophoresis buffer, together with the DNA sample, is eluted into the lower compartment. Whilst it is impossible to extract all the buffer (or all the DNA) from the gel, a good yield can be obtained, particularly of small molecules. This is the least labour intensive method and has the added technical advantage that ordinary agarose gels, rather than the somewhat fragile LMP gels, can be used. Good yield from larger molecules, for example phage arms of 20kb or more, was not obtained using this method.

4. Agarase Digestion

The enzyme agarase (Calbiochem) digests agarose into its constituent oligosaccharides. The resultant liberated DNA can then be precipitated and used. Potentially, this is a useful method for isolating DNA, especially larger molecules. However, I could not after several attempts, get this system working, using the manufacturers protocol or several variations thereof.

2.3.7 Autoradiography

In all cases, Kodak X-Omat AR high speed X-ray film was used. For autoradiography of ^{32}P and ^{35}S , the filters (or gels dried onto Whatman 3MM paper) were wrapped in cling film or clear plastic, taking care to minimise the amount of air bubbles. They were then placed with the film (under dark room conditions) in a film cassette (X-ray Accessories Ltd.)

containing Cronex "Lightning Plus" enhancer screens. Exposure was at -70°C for ^{32}P and room temperature for ^{35}S , for the required amount of time. Typical exposures were overnight for sequencing gels, 3 days for slot blots and up to 10 days for Northern, Southern and phage screening.

For developing, films were allowed to return to room temperature. A Fuji Medical Film Processor RGII was then used, providing automated developing, fixing washing and drying of autoradiograms. RG fixer and RG developer were from Wardray Products Ltd.

2.3.8 Spectroscopic determination of nucleic acid concentration

Accurate determination of sample concentration is required for comparative expression studies and in a number of reactions where relative amounts of components need to be maintained. In this investigation, spectroscopic determination of A_{260} and A_{280} levels were used to measure concentration and assess purity according to the following relationships (given in Maniatis et al., 1982):

$$1 \text{ A.U. } (A_{260}) \equiv 50\mu\text{g/ml dsDNA}$$

$$40\mu\text{g/ml RNA}$$

$$20\mu\text{g/ml oligonucleotides}$$

$$A_{260}$$

$$\text{-----} = 1.8 \text{ for pure DNA, } 2.0 \text{ for pure RNA}$$

$$A_{280}$$

(deviations are usually due to impurities such as salt, protein or ethanol contamination.)

2.4 : Infection of plasmids into *E. coli*

The cDNAs to be used in the investigation, unless isolated in this laboratory, were supplied as a plasmid and therefore had to be infected into a host bacterium.) (

2.4.1 Preparation of competent cells

Cells from the TB1 strain of *E. coli* were made competent (capable of taking up extraneous DNA) by a modification of the magnesium chloride/calcium chloride method originally described by Lederburg and Cohen (1974). 2ml of a fresh overnight culture of cells were inoculated into a 100ml of TYN broth. The culture was then incubated at 37°C with shaking until the OD₆₀₀ was 0.35AU (approximately 2 hours). The culture was cooled on ice for 30 minutes and the cells harvested by centrifugation (10 minutes/ 5k rpm/ 4°C in the JA17 rotor of the Beckman J2-21). The supernatant was decanted and the cells gently resuspended in 20ml of 100mM MgCl₂ using a pasteur pipette. After leaving on ice for 5 minutes the cells were pelleted as before, the cells being resuspended in 10ml of 75mM CaCl₂ in 10mM Tris.HCl (pH8). After a further 35 minutes on ice, the cells were again harvested and resuspended in a fresh 4ml of CaCl₂/Tris.HCl. These cells were kept on ice (0-4°C) until needed, but in any case not longer than 20 hours.

2.4.2 Transformation of competent cells

The appropriate plasmid DNA was made up to 25ng in 25μl CaCl₂/Tris.HCl and 100μl of competent cells added. After standing on ice for 30 minutes, the cells were heat shocked by incubating at 42°C for 2 minutes with shaking. The cells were

cooled on ice for a further 15 minutes and then 1.2ml of warm TYN broth was added. Incubation at 37°C followed for 1 hour (20 minutes unshaken then 40 minutes with shaking). Selection of recombinants, the usual method of subclone isolation, was not required here since all the transformed bacteria should contain cDNA bearing plasmids. This was verified by restriction enzyme digestion and electrophoresis prior to use as a probe. The bacteria were then either grown up for isolation of plasmid DNA or glycerol stocks prepared by addition of an equal volume of sterile glycerol and storing at -70°C.

2.5 : Isolation of Plasmid DNA

Two different methods of plasmid isolation were employed, the second superseding the first during the course of these investigations. The critical factor in plasmid preparation for e.g. use as a hybridisation probe, is the separation of the low Mr plasmid from the high Mr bacterial genome

2.5.1 Cesium chloride centrifugation method

This method, originally described by Radloff et al. (1967) relies on the principle that plasmid DNA saturated with ethidium bromide has a lower density than chromosomal DNA saturated with ethidium bromide, and can therefore be separated by isopycnic centrifugation. The disadvantages of this procedure are that it is time consuming, expensive and prone to error when removing the plasmid band, thus contaminating the plasmid band with chromosomal DNA. The procedure used is a modification of that reported in Maniatis et al. (1982). 500ml of the appropriate culture medium was inoculated with 1ml of a fresh 10ml overnight culture of E. coli containing the plasmid required. The culture was incubated, with shaking, overnight at 37°C and the cells harvested by centrifugation (10 minutes/ 3k rpm/ 4°C) in a JS 4.2 rotor (Beckman J6 centrifuge). The supernatant was discarded and the cells resuspended in 17ml Tris/Sucrose solution. This suspension was transferred to two 35ml centrifuge tubes and 1.3ml of a freshly prepared lysozyme solution (20mg/ml in 0.25M EDTA pH8) added to each. The solutions were kept on ice and swirled intermittently for 5 minutes, followed by the addition of 8.6ml of 0.25M EDTA to each and a further 5 minutes of swirling on ice. 13ml of

Brij/Doc solution was then added rapidly and mixed thoroughly by aspiration and the mixture left to stand on ice for 30 minutes. The cell debris and most of the chromosomal DNA was then pelleted by centrifugation (45 minutes/ 15k rpm/ 4°C) in the J2-21 centrifuge. The supernatant was collected and 0.2ml of 20mg/ml ethidium bromide added. Caesium chloride was added to 0.95g/ml and the solution transferred to 13.5ml Beckman polyallomer "Quickseal" tubes which were then centrifuged (56 hours/ 40k rpm/ 18°C) in a Beckman L7-65 ultracentrifuge (70ti rotor). The upper plasmid band was visualised under UV-illumination and collected by side-puncture of the tube. Ethidium bromide was removed by extraction with equal volumes of caesium chloride-saturated isopropanol.(1:1 mixture of isopropanol and 0.95g/ml CsCl in TE) until the organic layer was clear. The DNA was precipitated by the addition of 0.25 volume of 3M sodium acetate, 1.25 volumes of water and 2.5 volumes of isopropanol. The DNA precipitates out of this mixture within an hour at room temperature and at the end of this time the DNA was collected by centrifugation at 14k rpm in the microfuge for 15 minutes. The pellet was rinsed with 70% ethanol, dried in the Univap, and resuspended in an appropriate volume of water (typically 100µl).

2.5.2 Qiagen method

This method separates high and low molecular weight DNA by gel filtration, using prepacked gravity or pressure flow columns. For preparations of plasmid used in labelling the pressure flow midi-Qiagen column was used with 150ml cultures of cells (since late 1990 all Qiagen columns have been modified to gravity flow). The yield is sufficiently good to be comparable with the larger 500ml culture used in the CsCl

method.

In this method the cells were grown up and pelleted as before. They were resuspended in 4ml of buffer P1 (RNase A in TE), 4ml of buffer P2 added (200mM NaOH / 1% SDS) and the resulting suspension mixed gently and incubated at room temperature for 5 minutes. 4ml of buffer P3 (2.55M KAc) was then added to the (then lysed) cells and the suspension centrifuged (10k rpm / 4°C / 30 minutes) in the J2-21 with JA02.1 rotor. The supernatant (containing the nucleic acids) was immediately removed without disturbing the pellet (containing bacterial cell debris) and applied to a pre-equilibrated (Buffer QB - 750mM NaCl, 50mM MOPS pH7.0, 15% Ethanol) Qiagen plasmid Midi column. The sample was then pushed gently and slowly through the matrix, using a syringe, and the column washed with 2x 4ml of QC buffer (1.0M NaCl, 50mM MOPS pH 7.0, 15% ethanol). This removed the cellular RNA and remaining protein. The plasmid DNA was then eluted with 2ml of buffer QF (1.25M NaCl, 50mM MOPS pH8.2, 15% Ethanol), leaving the bacterial genomic DNA on the column. Of course if shearing had occurred, contamination with bacterial DNA would have occurred at this stage. The plasmid DNA was aliquoted into eppendorf tubes, precipitated with 0.5 volume of isopropanol, stood at room temperature for 30 minutes, and the precipitate collected by spinning at 14k rpm / 4°C for 30 minutes. After washing the pellet with an equal volume of 70% ethanol and drying in the Univap (2.3.3), the DNA was resuspended in water, pooled and stored at -20°C.

2.6 : Restriction Enzyme Digests

Where no specific details are given, restriction enzyme digests were carried out using $1\mu\text{l}$ of enzyme per $1\mu\text{g}$ of DNA in a volume of not less than 10x the volume of enzyme used (typically in total volume of $20\mu\text{l}$). An appropriate volume of 10x reaction buffer was included to provide optimal salt concentration for the enzyme. Where two enzymes requiring different salt conditions were used, equal volumes of each 10x buffer were combined and this appeared to work satisfactorily (Tab 2.1 overleaf gives a table of enzymes and their reaction buffers). $1\mu\text{l}$ of 40mM spermidine is also included to stabilise the net negative charge carried by the DNA. The reaction mixture was then incubated overnight at 37°C . After incubation, $3\mu\text{l}$ (greater than 0.1x reaction volume) of running dye was added. This performs a number of functions; firstly it stops the enzyme reaction. secondly it increased the density of the mixture to facilitate application to the gel and thirdly it provides a visual aid to the progress of the gel electrophoresis (2.3.4). Prior to application to the gel, the mixture is heated to 65°C for 3 minutes to denature any tertiary structure or aggregations of the DNA.

Table 2.1 Restriction enzymes

Enzyme	10x Buffer (BRL "React" system; BRL, 1989)
BamHI	3
EcoRI	3
HindIII	2
PstI	2
Sau3AI	4
XbaI	2
XhoI	2

2.7 : Isolation of RNA from tissues

Whilst perfectly possible to isolate mussel digestive gland RNA using a number of different methods, it has proved problematic using existing methodology to isolate mRNA suitable for reverse transcription and subsequent cDNA synthesis. This is discussed at greater depth in Chapter 5. However, sufficiently intact RNA has been reproducibly isolated for use in Northern and slot/dot blot studies.

Methods developed from Chirgwin et al. (1979), Palmiter (1974), Cathala et al. (1985), Feramisco et al. (1982), Han et al. (1987) and Chomczynski and Saachi (1987) were compared for isolation of mussel digestive gland RNA. It was observed that the methods of Chirgwin et al. and Cathala et al. gave comparable quality of RNA, but that the latter method is technically easier. A modified form of the Cathala et al. method is therefore the standard method used, and is described below.

Approximately 1g of tissue is pulverised under liquid nitrogen using a pestle and mortar. The powder is then quickly transferred to a tube containing 8ml of guanidinium isothiocyanate homogenising buffer (App. 1) with 0.5ml β -mercaptoethanol: The isothiocyanate and β -mercaptoethanol destroy RNAase activity. This suspension was immediately homogenised using a Polytron homogeniser (Kinematica GmbH, Switzerland) for 30 seconds at maximum setting. After standing at room temperature for 20 minutes to ensure complete dispersion, 50ml of 4M LiCl is added. After mixing, the tube is incubated at 4°C for 20 hours, during which time the RNA is selectively precipitated. The precipitated RNA is collected by spinning at 6.5k rpm/ 4°C for 1.5 hours in a Beckman J2-21

using a JA14 rotor. The supernatant is discarded and the pellet resuspended in 30ml 3M LiCl and respun at 6.5k rpm/ 4°C for 30 minutes. The pellet was then resuspended in 4 ml TE/0.1% SDS, phenol extracted according to section 2.3.1 and precipitated according to 2.3.2. After drying (2.3.3) and resuspending, concentration and purity was assessed spectrophotometrically (2.3.8).

In comparison, the method of Chirgwin et al. (1979) used the same homogenisation method, but the homogenate was layered onto 3.5ml CsCl in a 13.5ml quickseal tube. This was then spun at 36k rpm/ 20°C/ 16 hours in a Beckman L5-65 ultracentrifuge using a Ty65 rotor. The tubes were then drained, taking care not to contaminate the RNA pellet with material from the supernatant, and the pellet resuspended in 2.5ml 4M guanidinium hydrochloride. After transferring to a fresh tube, the suspension was then heated to 68°C briefly prior to adding 125µl EDTA (0.5M), 125µl potassium acetate (2M) and 1.25ml cold (-20°C) ethanol. After precipitating at -70°C for 20 minutes, the RNA was pelleted by spinning at 10k rpm/ 10°C/ 10 mins. The pellet was then resuspended in 1ml RNAase-free water and heated to 68°C prior to reprecipitating according to 2.3.2. Clearly the number of manipulations in this method and the long centrifugation step make processing of large numbers of samples more difficult compared with the Cathala et al. method.

The most rapid method employed was that of Chomczynski & Saachi (1987). in which frozen pulverised tissue (1g) was homogenised in 5ml of denaturing solution (4M guanidinium thiocyanate, 25mM sodium citrate pH7, 0.5% sarcosyl, 0.1M 2-mercaptoethanol). Sequentially, 0.5ml 2M sodium acetate pH4, 5ml phenol (water saturated) and 1ml chloroform/isoamyl

alcohol (49:1) were added with mixing by vigorous shaking. The suspension was spun at 10k rpm for 20 mins at 4°C, the aqueous layer removed and ethanol precipitated (2.3.2). The resulting pellet was resuspended in 0.5ml homogenising buffer then reprecipitated (2.3.2). After spinning down and drying, the pellet was resuspended in water and stored at -70°C. This method did yield RNA in good quantity, but the quality was poor as gauged by electrophoresis.

Another protocol in which the RNA is protected from RNAases by a different method is that of Palmiter (1974), in which the transcriptionally active mRNA is selectively isolated as a polysome complex. This was achieved by homogenising at 0°C, using a Potter-Elvehjem homogeniser, in 9ml/g tissue of PB buffer (App.1) with added heparin and Triton X-100. After centrifugation at 12krpm/0°C/5 mins. in the Beckman J2-21, the supernatant was removed to a new tube and the polysomes precipitated with Magnesium ions, through the addition of 10ml of PBM buffer (PB with 200mM MgCl₂ added; App. 1). After incubation on ice for 1 hour, the polysomes were pelleted by spinning 8ml aliquots through a 4ml sucrose cushion comprising 0.2M sucrose in PB buffer at 12krpm/0°C/10 mins. After draining the tube and wiping the sides to remove all traces of liquid, the pellet was then resuspended in 2ml TE/0.1% SDS. 2ml 0.1M sodium acetate pH5 was then added and phenol extraction (2.3.1) and ethanol precipitation (2.3.2) then performed. This method gave very poor yield from mussel digestive gland tissue.

The method of Han et al. (1987) relies on extremes of temperature to minimise degradation of RNA by RNAase: The tissue (1g) was homogenised in 8ml 6M Guanidinium thiocyanate which had been prechilled to 0-4°C on ice for 5 minutes (longer causes precipitation of the thiocyanate). 6.6ml of prechilled (-20°C)

ethanol was then added and the resultant mixture spun at 10k rpm/-10°C in a prechilled JA17 rotor for 5 minutes in the Beckman J2-21 centrifuge. The protein formed a film on the surface and was thus easily removed. The supernatant was then removed by aspiration and a further 10ml thiocyanate added to the pellet. After re-homogenising the pellet for 10 seconds, the suspension was recentrifuged for 3 minutes at 10k rpm/0°C. The supernatant was then removed and 0.25ml 1M acetic acid plus 7.5ml ethanol (-20°C) added. The suspension was then incubated for 3 hours at -20°C then centrifuged for 10 minutes at 6k rpm/-10°C. The floating material and supernatant were aspirated off and the pellet resuspended in 2 ml prechilled 4M Guanidinium chloride. The vessel was rinsed out with a further 8ml chloride and 7.5µl β-mercaptoethanol added. 0.25ml 1M acetic acid and 5 ml ethanol (-20°C) were then added. This section (this para) was then repeated, and the suspension stored overnight at -20°C. The precipitated RNA was then pelleted by spinning at 10k rpm/0°C for 10 minutes, dried and measured spectrophotometrically (2.3.3, 2.3.8).

In contrast, the method of Feramisco et al. (1982) uses heating of sample to inactivate RNAases: 4M guanidinium thiocyanate and sterile TE saturated phenol were preheated to 60°C. 1g pulverised frozen tissue was then added to 5ml thiocyanate and homogenised immediately. Keeping the suspension at 60°C, the chromosomal DNA was sheared by repeatedly drawing the suspension up and down through a 19 gauge syringe needle until the viscosity of the solution was appreciably lower. 5ml of the preheated TE satd. phenol was then added, together with 2.5ml of 0.1M sodium acetate in TE

and 5ml of chloroform. The mixture was shaken vigorously for 10 minutes whilst maintaining the temperature at 60°C, and then cooled to 4°C on ice. It was then spun down at 2k rpm/4°C for 10 minutes before removing the aqueous phase and extracting with phenol/chloroform as per 2.3.1. After precipitation as per 2.3.2, the pellet was redissolved in 1ml TNE, proteinase K added to 200µg/ml and incubated at 37°C for 1hr (an aliquot was retained prior to the proteinase K digestion for spectrophotometry and analysis by electrophoresis). After digestion, the enzyme was heat denatured at 65°C for 5 minutes and then removed by phenol extraction (2.3.1) and the pure RNA precipitated (2.3.2). This method gave good yield of RNA, but once again, the quality was only comparable with, but no better than the Cathala et al. method.

2.8 : Isolation of DNA from tissues

A number of different variations of standard methods to isolate DNA from tissues were tested. The one which gave the best quality of DNA preparation most consistently was a modification of that described by Hewish & Burgoyne (1973a, 1973b), as used by McMahon et al. (1989) and Kloepper-Sams (1989). This procedure is better suited to extraction of DNA from poikilothermic animals than other methods, being more effective in removing nuclease activity at an early stage in the procedure. Approximately 1g of tissue powder, pulverised under liquid nitrogen, was mixed with 10ml of ice-cold DNA homogenisation buffer (App. 1) by vigorous shaking in a 50ml tube. The mixture was then hand-homogenised (10-20 strokes) using a chilled Potter-Elvehjem homogeniser with a teflon pestle. The homogenate was then layered on to 10ml of ice cold cushion buffer (App. 1) in a fresh 50ml tube. The tube was centrifuged (10 minutes/ 2k rpm/ 0°C) in a Beckman J6B centrifuge to spin the nuclei through the cushion. Generally, a whitish/brown pellet was observed. The supernatant was discarded and the pellet drained and resuspended in 10ml isolation buffer (App. 1). The tube was immediately shaken vigorously and 0.4ml 10% SDS 5ml TE-saturated phenol and 5ml 24:1 chloroform/isoamyl alcohol added. The tube was then shaken gently at room temperature for 1 hour.

The resulting emulsion was spun at 4k rpm/ 10 minutes at room temperature. The aqueous phase was then removed, discarding the interface and phenol layer, and taken through the phenol extraction procedure (2.3.1). To the resulting aqueous phase, solid ammonium acetate was added to 2M, and two volumes of cold (-20°C) 100% ethanol were added. The mixture

was shaken gently, then the tube was tapped on the bench to bring the precipitated DNA to the base of the tube where it was collected using a sterile loop. After rinsing in 70% then 100% ethanol, the DNA was briefly air dried then unwound and dissolved into 5ml TE (pH 8.0), warming to 37°C if required. RN^aase A was then added to 100µg/ml and incubated at 37°C for 1 hour. Proteinase K was then added to 200µg/ml and incubated overnight at 37°C. The DNA was then reextracted with phenol and precipitated as detailed above, then redissolved (with heating if necessary) in 1ml TE (pH 8.0).

2.9 : Radiolabelling of cDNA probes

The use of radiolabelling has been fundamental to the development of molecular biology and is arguably still the most sensitive detection system available for the study of gene structure and expression. Two methods were used in the course of this study; nick translation (originally described by Rigby et al., 1977) and random oligonucleotide priming of DNA synthesis (originally described by Feinberg & Vogelstein, 1983). The former incorporates radiolabelled nucleotide into existing DNA using DNA pol1, after the template is nicked by DNAase 1. The latter synthesises a new strand of DNA from a number of sites where random hexanucleotides bind to denatured, single stranded template. Synthesis in this case is using the Klenow fragment of DNA pol1. Specific activity is higher in the second case and so use of the Multiprime system (Amersham International) largely superseded use of the Amersham Nick Translation kit. In both cases, the labelled DNA fragments produced are of the order 200-300 bases in length, and require purification from unincorporated radiolabelled nucleotides before use to prevent high levels of background signal in subsequent autoradiography.

2.9.1 Nick translation

To the DNA to be labelled (approx. 200ng) was added 10 μ l buffer/nucleotide mix and sufficient water to bring the volume to 35 μ l. 10 μ l of ³²P dCTP and finally 5 μ l of enzyme solution were added, the tube contents mixed gently with a pipette tip and incubated at 14°C for 3 hours. The reaction was stopped by the addition of 2 μ l of 0.5M EDTA. The DNA was then purified from unincorporated nucleotides using a NACS prepacs

column (BRL). NACS is an ion exchange resin which binds nucleic acids at low salt concentrations and releases them at high salt concentrations. The column was hydrated by slowly pushing 10ml of 2M NaCl in TE through using a 10ml syringe, and then equilibrated with 10ml of loading buffer (0.5M NaCl in TE or 0.2M NaCl in TE for dsDNA of greater than or less than 1kb respectively. 1ml of loading buffer (TNE; App. 1) was added to the labelling reaction and the whole sample then pushed through the column. The first pass eluate was reapplied to the column and the buffer pushed through to waste. At this point, the DNA was firmly bound to the column and the unincorporated label was removed by 8x 1ml washes with loading buffer. The DNA was then eluted by 3x 0.2ml washes of elution buffer (2M NaCl in TE). The probe was then boiled for 5minutes to denature the DNA. It was then immediately added to the hybridisation mix (2.11.1) to ensure that the probe remained single stranded.

2.9.2 Multiprime

Multiprime is the commercial name for the Amersham kit system for labelling of DNA by random priming. 50µg of DNA to be labelled was boiled for 3 minutes then cooled rapidly on ice. Water was added to a final volume of 23µl, then 10µl of buffer containing unlabelled nucleotides and 5µl of random hexanucleotide primer were added. 5µl of ³²P dCTP and then 2µl of enzyme were added and the components mixed gently with a pipette tip. Incubation was at room temperature for 3 hours, although the manufacturers advise that it can also be carried out at 37°C for 30 minutes if required.

Unincorporated nucleotides were removed by gel filtration using NICK™ columns (Pharmacia). These are prepacked gravity

flow one piece systems containing Sephadex G-50 and represent a considerable improvement in user safety over the push flow syringe-plus-column for the NACS system. The column was equilibrated using 10ml of NICK column buffer. The reaction mix was then applied directly to the column and the column washed with 400 μ l of NICK column buffer. A second 400 μ l eluted the labelled DNA which was subsequently boiled for 5 minutes before immediate use in the hybridisation mixture. A third 400 μ l wash from the NICK column also contained labelled DNA from the reaction, but was not used. Usually it was contaminated with unincorporated nucleotides, the remainder of which could be eluted in a fourth 400 μ l washing.

2.10 : Preparation of support membranes (filters) for hybridisation

The following methods were used to transfer nucleic acids to solid support membranes (filters) of nitrocellulose [Schleicher & Schuell] or nylon [Hybond-N, Amersham International] for use in hybridisation experiments. Nitrocellulose filters were used originally, but were replaced in favour of the more robust nylon.

2.10.1 Southern Blotting

The method was adapted from Maniatis et al. (1982), after the original technique reported by Southern et al. (1975). Following electrophoresis of DNA, the gel was stained and photographed. At least 10 μ g of genomic DNA is required for detection of single copy genes by hybridisation. In practise, up to 50 μ g of DNA was used to try to detect mussel P450 sequences. The gel was immersed in 5 volumes of 0.25M HCl and shaken slowly at room temperature for 15 minutes. This procedure partially depurinates the DNA, breaking it into smaller pieces to facilitate transfer of larger fragments to the filter. The gel was then transferred to 5 volumes of Southern Blot denaturing solution (1.5M NaCl, 0.5M NaOH) and shaken for 45 minutes, followed by the same procedure with Southern Blot neutralising solution (1.5M NaCl, 1M TrisHCl pH8). The DNA fragments were then transferred from the gel to a filter membrane by capillary action as described in the original procedure using 20xSSC. After transfer overnight the DNA was fixed in situ as detailed in section 2.10.5.

2.10.2 Northern Blotting

20 μ g RNA samples were electrophoresed on formaldehyde

denaturing gels as described in Maniatis et al. (1982) and 2.3.4. After staining with ethidium bromide and photography, the gel was soaked in 10xSSC for 5 minutes before being taken for capillary transfer to membranes. The blot was set up in the same way as for a Southern Blot with the exception that 10xSSC was used as the transfer buffer. After transfer the membrane was fixed according to section 2.10.5.

2.10.3 DNA dot/slot blots

A nylon or nitrocellulose filter was soaked in 20xSSC and placed in the dot blot (96-well HybriDot™, BRL) or slot blot (Bio Rad) apparatus. After heating to 65°C for 15 minutes, the appropriately diluted DNA sample(s) in 100μl volume of 20xSSC were spotted on. Gentle vacuum was applied, such that a 100μl sample took between 1 and 2 minutes to pass through. Samples wells were washed through with 400μl 20xSSC and the vacuum released. After air drying, the DNA was immobilised on the filter according to section 2.10.5.

2.10.4 RNA dot/slot blots

RNA was denatured for binding to filters according to the formaldehyde method of Lehrach et al. (1977). RNA samples were made up in 0.1ml of DEPC-treated water. They were then denatured by the addition of 0.3 ml 18.5% formaldehyde in 10xSSC (made fresh by the mixing equal volumes of commercially available 37% formaldehyde and 20xSSC) and heating to 65°C for 15 minutes. The filter was pre-wet in 10xSSC and assembled in the apparatus. The 400μl samples were applied and the filter processed in the same manner as the DNA blots.

2.10.5 Treatment of Filters

Where nitrocellulose filters were used, the DNA or RNA was bonded to them by baking at 80°C for 3 hours. Hybond-N filters were UV-irradiated on the TM20 transilluminator for 3 minutes to fix the RNA and DNA. The filters were then stored at 4°C, wrapped in cling film.

Where a filter was saved for reuse after the first probing, it is necessary to remove the hybridised probe from the previous experiment (stripping). This was achieved by either three sequential 30 minute washes in distilled water at 70°C (nitrocellulose) or two washes by adding boiling 0.1% SDS and allowing to cool to room temperature, with shaking in each case. Filters were checked with a geiger counter and where possible by autoradiography. If probe was still bound and could not be removed by further washing, the filters were discarded. Washed filters were washed in 5xSSC and stored wrapped in cling film at 4°C.

2.11 : Hybridisation procedures

2.11.1 Formamide method

All the hybridisation experiments performed using radiolabelled DNA probes utilised the formamide method (adapted from Anderson & Young, 1985). The presence of formamide in the hybridisation mix reduces the melting temperature (T_m) of nucleic acid hybrids and enables hybridisations to be performed at a lower temperature. This is advantageous as at lower temperatures the probe itself is more stable and nitrocellulose filters are less likely to disintegrate. Filters were pre-wetted in 5xSSC and then prehybridised for 7-18 hours at 42°C in 1ml prehybridisation solution per square cm of filter. This ensured blocking of non-specific binding sites on the filter to prevent spurious signals. This operation and the subsequent hybridisation reaction were initially performed in sealed bags in a vessel placed in a shaking water bath. However the acquisition of a Hybaid incubator and rotisserie (with filters layered between mesh spacers being placed in sealed rotating bottles) made this operation less hazardous and more easily reproducible.

The prehybridisation solution was then removed and replaced by hybridisation solution containing the freshly denatured probe (see section 2.9). After thorough mixing, the filter and hybridisation mix were incubated at 42°C, with agitation, overnight. Approximately 0.1ml of hybridisation mix per cm² of filter was used.

2.11.2 Washing of filters

The washing conditions used are described for each separate experiment in the results section as they vary according to the probe used and samples being analysed. Where a weak signal was expected e.g. mussel DNA versus a mammalian P450 probe, low stringency conditions were used (i.e. high salt concentration and low temperature). For experiments where a stronger signal was expected e.g. fish RNA versus a fish P450 probe, more stringent conditions were used (i.e. low salt and high temperature) In all cases however, the format was the same;

1. 1x 5 minutes at room temp. in 5xSSC/0.1% SDS
2. Stringency wash(es) - variable
3. 1x 5 minutes at room temp. in 5xSSC

Where no specific washing conditions are given, the standard stringency washes used were 1x 20 mins. at 48°C, in 1xSSC/0.1% SDS followed by 1x 20 mins. at 55°C in 1xSSC/0.1% SDS.

2.12 : mRNA Isolation and In vitro translation

The isolation of intact mRNA is a necessary prerequisite for synthesis of a complementary DNA (cDNA) library. In the course of these investigations, two methods were used to obtain separation of mRNA from the rRNA and tRNA co-isolated in the total RNA preparation from section 2.7. Both the Hybond-mAP (mRNA Affinity Paper) method and the later examined oligo-dT cellulose method are dependent on the poly-adenylated 3' end of the mRNA hybridising by complementation to either poly-U in the former case or oligo-dT in the latter. A number of criteria exist to verify RNA integrity: whether bands can be detected on gels, whether hybridisation to cDNA can be detected, and specifically in the case of mRNA, whether protein can be produced from the isolated transcript. An in vitro translation reaction was therefore conducted to examine the mussel mRNA isolated.

2.12.1 mRNA purification using Hybond-mAP

Hybond-mAP (messengerRNA Affinity Paper - Amersham International) is a rapid and convenient method for isolation of mRNA. The protocol followed is a modified form of that recommended by the manufacturer. 0.5mg of RNA in 0.623ml water was heated to 65°C for 5 minutes then cooled on ice for 10 minutes. 70µl of 5M NaCl was then added to bring the NaCl concentration to 0.5M. At the same time, a piece of mAP 0.5cm x 1.0cm was wetted in 5ml 0.5M NaCl for approx. 5 minutes with gentle shaking.

The equilibrated paper is then placed into the RNA solution and shaken gently at room temperature for 30 minutes. The paper is then twice washed briefly in 5ml of 0.5M NaCl, and

once in 10ml 70% ethanol. After air drying, the paper is placed into 700 μ l of water and the mRNA eluted at 70°C for 5 minutes. The resulting solution (with the paper removed) is then precipitated (2.3.2) and the mRNA resuspended in a smaller volume (expected yield is circa 10 μ g, hence not more than 50 μ l to keep concentration high enough for use in subsequent experiments) and measured spectrophotometrically (2.3.8).

2.12.2 mRNA purification using Oligo-dT cellulose

This method was used to verify the results of the former method and was essentially an adaptation of the method originally described by Aviv & Leder (1972). 0.5g of oligo-dT cellulose type 7 (Pharmacia) was hydrated in 10ml water. The slurry was allowed to settle and the liquid decanted. The slurry was poured into a 2ml syringe containing a sinter plug to form a 1ml column. The column was then washed with 20ml water, 20ml elution buffer and finally 20ml binding buffer. 500 μ g of tRNA in binding buffer was then applied to the column after heating to 68°C for 15 minutes and cooled on ice (this blocks non-reversible binding sites). The column was then rewashed with 20ml binding buffer. Sample RNA prepared in the same way was then applied. The eluent was collected, reheated to 68°C and reapplied. The column was then washed with a further 20ml of binding buffer. The mRNA was eluted off the column by addition of 2ml of elution buffer at 68°C, and precipitated according to 2.3.2.

2.12.3 In vitro translation

The translation of isolated mRNA in vitro provides a method by which integrity of the RNA can be judged.

Originally described by Pelham & Jackson (1976), the method used was a commercially available kit utilising rabbit reticulocyte lysate to incorporate radiolabelled methionine into a growing polypeptide.

The Amersham kit recommended protocol was followed, which is essentially as follows; A premix is made (dependent on the number of samples to be examined) comprising lysate, radiolabel and sterile water in 10:2:1 proportions. 2.5 μ l of each sample mRNA and the control mRNA (not less than 0.1 μ g each) is then added to 47.5 μ l of premix (later reduced to 17.5 μ g) and incubated at 30°C for 1 hour. The reaction was then cooled on ice and 30 μ l (or 12 μ l) sample buffer (Appendix 1) added. After mixing the samples were then electrophoresed on a denaturing SDS gel as detailed below.

2.12.4 : Polyacrylamide gel electrophoresis

Electrophoresis was performed using a BioRad vertical gel electrophoresis system, using the method of Laemmli (1970). A 12cm separating gel was prepared using Acrylagel and Bisacrylagel stocks (30%w/v acrylamide and 2%w/v N,N-methylene-bis-acrylamide respectively) diluted with separating gel buffer to final concentrations of 7.5% and 0.2% respectively, and polymerised by addition of TEMED (1 μ l/ml gel) and freshly prepared 10% ammonium persulphate (5.6 μ l/ml gel). After casting, polymerisation was allowed to take place overnight under a 10% ethanol solution.

After removal of the alcohol and washing with electrophoresis buffer (E Buffer, App. 1), a stacking gel was then prepared to final concentrations of 5% acrylamide and 0.075% N,N-methylene-bis-acrylamide using stacking gel buffer. After initiating polymerisation as before, it was cast to a

depth of 2-3cm, and the well former inserted. After allowing polymerisation for 2 hours, the well former was removed and the wells washed out with buffer. After boiling for 5 minutes, the samples (30 μ l) were then applied to the gel using a hamilton syringe and electrophoresed. Typically the conditions used were 20mA for 4 hours through the stacking gel, then 40mA overnight (14 hours) for the running gel.

After running, the stacking gel was discarded and the running gel fixed in 7% acetic acid for 15 minutes. It was then washed in water for 15 minutes and soaked in an enhancer solution (Amplify, Amersham International) for 30 minutes. After a further 15 minute wash it was allowed to soak overnight in 10% glycerol. It was then dried under vacuum at 80°C for 2 hours on a BioRad model 483 slab dryer before being autoradiographed for 36 hours (Section 2.3.7).

2.13 : Gene Library Synthesis

Synthesis of gene libraries is the standard method for isolating and purifying a gene of interest from an organism. The library is comprised from short sequences of the whole genome or, in the case of cDNA libraries (Huynh et al., 1985), from the expressed portion of the genome. Two methods were used, firstly to produce the cDNA library and secondly to produce the genomic DNA library. In both cases, bacteriophage vectors from commercially available kits were used. In the former case, cDNA was required to be synthesized from the mRNA purified, and once again a commercially available kit was used. The genomic library was synthesized from restriction digested DNA isolated from mantle tissue.

2.13.1 cDNA Synthesis

1 to 5 μ g of mRNA purified from the mAP method was taken through the Amersham cDNA synthesis system protocol. This method utilises oligo dT primers as templates for first strand synthesis by viral reverse transcriptase. RNase H, which specifically nicks the RNA strand of DNA-RNA hybrids, is then used to digest the mRNA. The short lengths of RNA remaining then act as templates for E. coli DNA polymerase to synthesize the second strand. The reaction was followed as outlined in the protocol booklet, by measuring the incorporation of radiolabelled dCTP into the growing DNA strand. This was achieved by Cherenkov counting of aliquots of the reaction mixture from various stages in an LKB Rackbeta scintillation counter. The DNA was then purified by the geneclean (2.3.6) method prior to resuspending in an appropriate volume of TE

buffer for the cloning reaction.

2.13.2 Cloning of cDNA into bacteriophage lambda gt11

Cloning was carried out according to the method in the Amersham protocol supplied with the kit. The cDNA was prepared for cloning by addition of EcoR1 linkers and methylation of internal EcoR1 sites. The excess linkers were then removed the cDNA by gel filtration. The purified cDNA was then resuspended at approximately 50ng/ μ l and ligated to the phage arms together with the appropriate control reactions for 20 hours at 15°C.

The phage DNAs containing the mussel cDNA were then packaged into infective phage particles using the packaging reactions supplied and incubating at 20°C for 2 hours. 0.5ml SM buffer (App. 1) and 10 μ l of chloroform were then added and the resulting phage stock stored at 4°C.

2.13.3 Cloning of mussel genomic DNA into bacteriophage lambda EMBL3a

In order to reduce the molecular weight of the genomic DNA being cloned to a size capable of being accepted by the vector and to produce the "sticky ends" necessary for ligation, it was subjected to restriction enzyme digestion. Two enzymes were used; a total digest by BamH1 and a partial digest by Sau3A. Although DNA of all sizes is ligated into the arms, only DNA of 9-23kb can be successfully packaged. Therefore in the case of the partial digest, the DNA was under- rather than over-digested. In the case of the BamH1 digest, the DNA was size fractionated by gel electrophoresis (2.3.4) and purified by phenol extraction (2.3.1) after the digest.

To prevent self-ligation and resulting cloning artifacts, the digested DNA was then treated with calf alkaline phosphatase (0.1Units/ μ g DNA) in TE pH9.5 at 37°C for 1 hour. Following this, the enzymes were removed by phenol extraction (2.3.1) and, after ethanol precipitation (2.3.2), the phosphatased DNA was resuspended in an appropriate volume of TE pH8.0.

The bacteriophage vector is supplied (Stratagene) as double digested arms into which the DNA is ligated, using 1 μ g arms per 0.3 μ g of digested DNA. For the control reaction, the reaction components are as follows: 1 μ l (1 μ g) of EMBL3 predigested arms, 0.6 μ l (0.3 μ g) of test (PME) insert, 1 μ l of 5x ligation buffer (inc. ATP) and 1 μ l (2 units) T4 DNA ligase. These were added together in a small eppendorf tube and then water added to bring the final volume to 5 μ l. The reaction was then incubated at 10°C for 48 hours (the longer time and lower temperature of incubation compared with 2.14.2 above is believed to give better yield of the larger fragments). For ligation of sample DNA, the PME test insert is of course replaced by digested mussel DNA.

After ligation into the phage arms, the DNA was packaged directly, using the same protocol as that in 2.14.2 above, and similarly stored.

2.14 : Growth of Bacteriophage

In order to be able to assess the library synthesized, it was necessary to find out how many clones are present and what the average size of the inserted mussel DNA was. Then it is possible to make a statement with regard to whether the library is representative of the whole genome. The protocols followed are from the appropriate commercial manual (Stratagene or Amersham).

2.14.1 Preparation of E. coli cells

A necessary prerequisite for growth of phage is the preparation of suitable host cells. These are various strains of E. coli with appropriate receptors and selection systems.

The lambda gt11 phage was grown with Y1090 cells containing the plasmid pMC9, which confers antibiotic resistance and contains the lacI repressor. Normally the repressor binds to the promoter region of the lacZ gene in the phage genome. Inclusion of 1mM IPTG, which binds to the repressor, resulted in derepression of the lacZ gene in the phage. The inclusion of 0.02% of the chromogenic substrate, X-gal, was then used to indicate presence of β -galactosidase, the product of the gene; plaques produced were blue. If, however, foreign DNA (mussel cDNA in this case) was inserted in the lacZ gene, active protein was not produced and the plaques remained clear. Thus the ratio of blue to clear gave an indication of the numbers of recombinant phage and non recombinants present in the library.

The EMBL3a phage was grown on LE392 and P2392. The LE392 allows growth of all phage containing the correct size DNA for

packaging. In contrast, the P2392 does not allow growth of phage containing active red and gam genes. These are contained in the stuffer fragment of wild type EMBL3, and so were removed in phage where the stuffer was replaced by an insert. The P2392 are therefore selective for recombinant phage whereas the LE392, in which phage grow more rapidly, are used to grow up plaque purified phage.

In all cases the 100 μ l of host cells were taken from a culture in log growth phase and grown at 30°C overnight in 10ml of media containing 10mM MgSO₄ and 0.1% maltose. The low temperature prevents overgrowing of the cells and subsequent entry into stationary phase. The inclusion of magnesium ions and maltose maintains the presence of cell surface receptors to which the phage bind when infecting the host. The cells were then spun down at 1krpm/10 mins in a bench centrifuge and resuspended in 5ml 10mM MgSO₄ and stored at 4°C if not for immediate use. Plating cells used were never more than 24 hours old when used.

2.14.2 Titration of phage

The number of bacteriophage present in the library was assessed by infecting a fixed amount of freshly prepared plating cells (100 μ l of the appropriate host bacteria) with a set of serial dilutions (in SM buffer) of phage. These are incubated at 37°C for 20 minutes before diluting into 4ml of molten top agarose and pouring out the resulting suspension to evenly cover a 9cm diameter agar plate. After allowing to set at room temperature and growing up at 37°C (43°C for Y1090) overnight, the number of plaques present can be used to calculate the number of phage in the library.

2.14.3 Analysis of representative recombinant clones; radiochemical method

This method was used for the analysis of cDNA inserts in the lambdaGT11 vector. The size of 12 clones was determined by growing up individual plaques by adsorbing to 0.5ml plating cells for 20mins at 37°C and subsequent addition of 4ml media. After growing until lysed (circa 4hrs), phage DNA was purified according to the method in 2.15.5 below. The cloned DNAs were then excised by digestion with EcoR1 (section 2.6) and the precipitated fragments labelled with 1 unit Klenow fragment of DNA polymerase and 0.1 μ l gamma-³²P ATP. After incubation for 30mins at 37°C, the samples were run on an agarose gel (section 2.3.4). The gel was fixed in 7% TCA for 30 mins, dried on a BioRad model 483 slab dryer and autoradiographed for 2 hours.

2.14.4 Analysis of representative recombinant clones; phage "miniprep" method

This method was used for analysis of genomic DNA inserts in the lambda EMBL3 vector. Essentially it is the same as that for 2.15.3, but without the radiolabelling step. This was considered unnecessary since the amount of DNA in the insert (due to greater insert size) is greater and should, following electrophoresis, be visible by staining with ethidium bromide.

2.15 : Screening of Recombinant Bacteriophage Libraries

To purify a gene fragment from a library, it is necessary to probe the library with either an antibody or a DNA probe corresponding to the gene of interest. In this case a radiolabelled cDNA probe was used.

Using the information on the titre of the library, obtained from section 2.15 above, bacteriophage were plated out at a density of 100 plaques/square cm, on large (25cm x 25cm) plates without IPTG and X-gal. After growing up of plaques overnight and cooling the plate to 4°C, the plates were overlaid with nitrocellulose filters. Orientation with respect to the plate was recorded using a marker dye, and after 30 seconds the membrane was removed and placed plaque side up for 5 minutes on Whatman 3MM paper soaked in denaturing buffer (Appendix 1, as for Southern blotting). The filter was then placed in 50ml neutralising solution (Appendix 1, as for Southern blotting) for 5 minutes before rinsing in 2xSSC and allowing to dry. Two further replicates were made and taken through the process, but allowing 60 and 90 seconds contact time for the 2nd and 3rd filter respectively. After drying, the filters were baked at 80°C for 2 hours before hybridisation with the appropriate probe (sections 2.9.1 and 2.11).

2.16 : In vitro amplification of DNA (PCR)

2.16.1 Preparation of Oligonucleotides

Oligonucleotides for use in PCR experimentation, were synthesized in the Microbiology Department, University of Surrey, using an Applied Biosystems Model 381A DNA synthesizer, and arrived attached to the solid phase columns used in synthesis.

The sequences of the oligonucleotides used is shown below

	5'	3'
1	P450IVA1 -ve	CTTCT TGACA TACAG GTAGA T
2	P450IVA1 +ve	ATGCA TTGGG AAACA ATTTG CT
3	P450IA1 -ve	GACAC ATTCC TCAAT GGC
4	P450IA1 +ve	CTCAC CGATG CAGCG GCGCT T
5	GPX -ve	GGTGC CTCAG AGGGA CGCCA CATT
6	GPX +ve	GTTTT CATCT ATGAG GGTGT TTCCT
7	HFI -ve	CCATG GATCA GGTAC AAAGG
8	HFI +ve	GGTCC ATTTG TAGTT ATAGT

Oligos 1-5 were purchased by PML for use in this project.

Oligos 6-8 were already available in the laboratory.

Before use, the oligonucleotides were cleaved from the columns and the base protecting groups protecting the 5' end of the oligo were removed. ^{5 places} A disposable 2ml syringe was inserted into one end of the solid phase column and 880 ammonia (ammonium hydroxide, 29.5% NH₃ in water) was drawn up through the column until the syringe contained 1ml. A second syringe was then inserted into the other end of the column and the solution forced backwards and forwards through the column slowly five times.

The column /syringe assembly was then left at room temperature for 45 minutes with the solution in contact with the column support, after which time the solution was again pushed back and forward through the assembly five times. After a further 90 minutes room temperature incubation, the solution was again passed back and forth before eluting into a screw capped vial. By this process, the oligonucleotide is washed into the ammonium hydroxide solution and the cyanoethyl groups protecting the phosphates are removed.

The ammonium hydroxide solution containing the oligonucleotide was sealed tightly in the vial, and incubated at 55°C overnight (this removes the groups protecting the exocyclic amines of A, C [benzoyl] and G [isobutyryl] from undergoing side reactions. The solution was then cooled to room temperature and transferred to a microcentrifuge tube. The tube was placed in the Univap and switched on without the vacuum. After 30 minutes the vacuum was switched on and drying allowed to continue until the sample was completely dry (approx. 2 hours). The sample was suspended in 0.5ml water and incubated at 4°C overnight to resuspend. The concentration present was then determined spectroscopically (2.3.8)

2.16.2 The polymerase chain reaction

The Polymerase Chain Reaction (PCR) is a method of enzymically producing a large number of copies of a specific stretch of DNA, bounded by two oligonucleotide primers. The method, originally described by Saiki et al. (1985), uses repeated cycles of;

1) annealing of primers to single stranded DNA template, 2) elongation of DNA strand, and 3) denaturation of new dsDNA to

form a new template. Although possible with E. coli DNA polymerase, the extreme nature of the heating to denature DNA would require addition of new enzyme at each cycle. The method in widespread use today depends rather on the use of the thermostable DNA polymerase from Thermophilus aquaticus.

The method used in these studies follows the standard protocol of the PCR kit supplied by Perkin Elmer Cetus (Norwalk CT, USA), the 100 μ l reaction mix comprising 53.5 μ l sterile water, 10 μ l 10x buffer, 16 μ l of dNTP mix (final concentration 200 μ M), 5 μ l each of +ve and -ve primer for DNA of interest (see 2.17), 10 μ l of 200 μ g/ml template DNA, and 2.5 units (0.5 μ l) of Taq DNA polymerase. The reaction mix was overlaid with 100 μ l of paraffin oil to prevent evaporation. Thermal cycling conditions varied and are given in the relevant results section. At the end of the reaction (6 hours or overnight) the aqueous layer was removed to a new tube and the volume reduced by sequential extractions with Butan-2-ol. The sample was then electrophoresed (2.3.4) on a 4% (3:1) NuSieve/Agarose gel after addition of 3 μ l of loading dye.

2.17 : DNA sequencing

Sequencing of a product of the PCR reaction was attempted using a modification of the dideoxy method first described by Sanger et al. (1977). In this method, growing strands of newly synthesised DNA are randomly terminated by incorporation of a dideoxy NTP. By electrophoresis of the resulting (radiolabelled) fragments, a "ladder" is generated and the position of each component nucleotide of the DNA can be determined. Reactions were performed as described in the Sequenase™ protocol booklet and using the Sequenase™ kit (U.S. Biochemicals). The P450IA1 primers used in the PCR reaction were also used in the sequencing reaction, and the ³⁵S-dATP was purchased from Amersham.

Direct sequencing of double stranded DNA, first described by Chen & Seeburg (1985), was performed on the band after purification from agarose according to the Spin-X method of section 2.3.6. The advantage of this approach is that sub-cloning into M13 to produce single stranded DNA is not necessary. The method uses a genetically modified form of T7 DNA polymerase rather than the original system where the Klenow fragment of E. coli DNA polymerase I was used. The T7 polymerase is better able to copy template DNA as it polymerises more rapidly and is less impeded by secondary structure, and therefore more useful in sequencing double stranded templates.

The sequencing reaction and gel preparation are described below.

2.17.1 Sequencing gel preparation

The sequencing gel tank was made in the University

workshops and, in this laboratory, gave better results than various commercial apparatus tested. Glass plates 58 x 20 x 0.5cm were obtained from a local glass cutter. the front plate having a U-shaped recess to accomodate the well former. The plates were scrubbed with Decon and hot water, rinsed and dried. 10ml of the siliconising agent was spread over the inside surface of the plates and left to dry in a fume cupboard (approx. 5 minutes). The plates were then wiped, first with ethanol and then with acetone. The gel spacers and comb/well formers (0.4mm thick) were cleaned using ethanol and the plates assembled. Bulldog clips were used to clamp the plates together and the plates were sealed with glass sealing tape (The 3M Company). Resiliconising is necessary after every 5 or 6 uses of the plates.

6% polyacrylamide gels were prepared according to the method of Sanger & Coulson (1975). 11.25ml of 40% acrylamide stock solution and 7.5ml of 10x Tris-borate buffer (TBE; App.1) were added to 31.5g of urea. Distilled water was added to 75ml and the urea dissolved by heating to 37°C. The mixture was degassed and cooled on ice for 20 minutes. The assembled plates were inclined diagonally on a 30° incline so that the gel would fill the plates upwards from one corner. This prevented the formation of large air bubbles when pouring the gel. 420μl of freshly prepared 10% ammonium persulphate and 75μl TEMED were added to the acrylamide solution, mixed quickly and drawn into a 50ml syringe. The gel was expelled gently and quickly from the syringe so that it flowed evenly into the gap between the plates. When the space between the plates was full, the well former was inserted into the gel and the top of the plates clamped firmly. The gel was left to polymerise and then stored in a 7°C cold room until required

(more than 2 and not more than 72 hours). Prior to use the gel was positioned in the apparatus and allowed to warm to room temperature, the sealing tape removed from the bottom (but not the sides), and the reservoirs filled with buffer.

2.17.2 Preparation of single stranded DNA and annealing of primers

4 μ g of double stranded DNA purified from the products of three PCR reactions was denatured to provide single stranded template for the T7 DNA polymerase to copy. This was achieved by the addition of 2 μ l of 2M NaOH to the sample (in 18 μ l of water) and incubation at room temperature for 5 minutes. The denatured DNA was then precipitated by addition of 8 μ l of ammonium acetate pH7.5 and 100 μ l of absolute ethanol. After standing at -70°C for 20 minutes, the precipitated DNA was collected by spinning at 14k rpm (15 minutes) and the supernatant decanted. The pellet was then resuspended in 100 μ l of 80% cold (-20°C) ethanol and recentrifuged. The pellet was then dried in the Univap and stored dessicated at -20°C until use (if not immediately used).

The denatured DNA was reconstituted in 14 μ l water and 4 μ l of sequencing buffer added. 9 μ l was then added to 1 μ l of each of the primers (IA1 oligonucleotides from section 2.17 diluted to 40ng/ μ l) to sequence the fragment in both directions. A control reaction with M13 universal and reverse primer was also run according to the manufacturers protocol. The mixtures were then heated to $T_m - 10^\circ\text{C}^*$ for 15 minutes to anneal the primers then cooled by placing in a water bath at 37°C for 10 minutes then cooling to room temperature. This slow cooling ensured homologous base pairing and annealing took place.

* T_m for primer calculated according to the theoretical equation given in Maniatis et al., 1982:

$$T_m (^{\circ}\text{C}) = 4(nG + nC) + 2(nA + nT)$$

For the nucleotides used: IA1 +ve, $t_M = 54^{\circ}\text{C}$

IA1 -ve, $t_M = 70^{\circ}\text{C}$

2.17.3 Labelling reactions

In these reactions, the primer is extended in the presence of limiting concentrations of deoxyribonucleotide triphosphates and ^{35}S dATP, so that initiation of polymerisation by the T7 DNA polymerase predominates over chain extension in the reaction mix. This ensures uniform incorporation of radiolabel into the individual strands regardless of the final length of the extension, thus enabling even the shortest fragments to be detected.

The labelling reaction comprised the DNA/primer mixture plus $1\mu\text{l}$ of DTT, $2\mu\text{l}$ of labelling mix (dTTP, dCTP & dGTP), $0.5\mu\text{l}$ of radiolabel and $2\mu\text{l}$ of SequenaseTM (added last). The reaction was allowed to proceed for 5 minutes at room temperature.

Four termination mixes (A,T,C,G,) containing $80\mu\text{M}$ concentrations of all four dNTPs plus an $8\mu\text{M}$ concentration of one of the dideoxyribonucleotide triphosphates were then prewarmed to 37°C . $3.5\mu\text{l}$ of the labelling reaction was added to each tube and the reaction allowed to proceed at 37°C for 5 minutes and was then stopped by addition of $4\mu\text{l}$ of loading buffer, which also contains the electrophoresis marker dyes bromophenol blue and xylene cyanol.

2.17.4 Electrophoresis of samples and Autoradiography

The samples in loading buffer were heated to 75°C for 2

minutes before loading on to the gel. The well former was removed, inverted, and the teeth positioned so that they just entered the flat top surface of the gel. The wells formed were then rinsed thoroughly to remove all traces of urea and 3 μ l of each sample loaded on using a Drummond sequencing pipette (Drummond Scientific Co.). The apparatus was run at 50W constant power to ensure constant temperature conditions. The gel was allowed to run until the bromophenol blue had migrated 45cm (A "short" gel - longer sequences can be determined by running the gel until the slower migrating xylene cyanole has travelled the same distance).

The clamps and the remainder of the sealing tape were removed and the plates gently prised apart so that the gel remained attached to one plate. The gel and plate were then immersed in 10% methanol/10% acetic acid for 20 minutes to fix the gel. This was then rinsed with water and then soaked in water for a further 20 minutes to leach out the urea which would otherwise have interfered with the autoradiography. A sheet of Whatman 3MM paper was then wetted and placed on top of the gel. Two further dry sheets were then placed on top and the gel was then peeled off the plate, sticking to the paper. The gel was then cut into two pieces to fit onto a Model 413 slab drier (Biorad) and dried at 80°C with vacuum for 60 minutes. Autoradiography was then performed essentially as in section 2.3.7.

CHAPTER 3 : DETECTION OF CYTOCHROME P450-RELATED SEQUENCES IN MYTILUS EDULIS AND OTHER MARINE ANIMALS

3.1 : Introduction

The following experiments were performed to obtain more evidence about the occurrence and complexity of the cytochrome P450 system in the mussel. As discussed in chapter 1, the difficulty in obtaining pure protein was to be bypassed by the use of cDNA probes to study the spectrum of P450 mRNAs and genes present.

Initially, only the P450IVA1 (lauric acid hydroxylase) and P450IIE1 (diabetes & ethanol inducible) probes were available to me, hence my initial data concentrates on these isozymes (figs. 3.6-7, 3.11-13, 4.1). As access to more probes became possible, other mRNA and genes for different P450 isozymes were studied.

In some experiments, RNA normalisation using an actin cDNA was not conducted. In later experiments, when I became aware of the utility of the approach, this technique was employed as a matter of course.

The details of all the probes used in this study are given in Section 2.2.4.

3.2 : Isolation and Preparation of cDNA probes coding for vertebrate cytochrome P450 isozymes

Plasmid containing the relevant cDNA was isolated from the host bacterium (Section 2.5). An aliquot of the plasmid DNA was then taken for gel electrophoresis, to assess its purity with respect to contamination by host genomic DNA (fig. 3.1). An aliquot of the plasmid was then digested using the appropriate restriction enzyme(s) and the success of this reaction measured by gel electrophoresis, as shown for the P450IIE1 and P450IVA1 cDNA inserts (Fig. 3.2). It was confirmed that the cDNA fragment migrated the correct distance according to its size by comparison with bacteriophage lambda DNA fragments (HindIII digest - BRL or NBL). A preparative digest was then set up and the resulting DNA fragments separated by agarose gel electrophoresis (fig 3.3). The resulting cDNA band was then excised and purified (Section 2.5), and an aliquot of the purified cDNA insert was run on a gel. This was done to verify that the cDNA preparation was absent of contaminating plasmid and bacterial DNA, and also to obtain some estimate of the insert DNA concentration.

Figure 3.1 : Agarose gel showing uncut cytochrome P450IIE1 & P450IVA1 plasmid DNAs

Plasmid DNAs were electrophoresed on a 1% Agarose gel
Lane 1; Marker (Lambda HindIII fragments)
Lanes 3 & 4; pUC19 containing rat P450IIE1 cDNA
Lanes 5-7; pUC19 containing rat P450IVA1 cDNA

Figure 3.2 : Agarose gel showing fragments following restriction enzyme digestion of plasmid DNA to release cDNA inserts

Digested DNA fragments were electrophoresed on a 1% Agarose gel.

Lanes 1-3; P450IVA1 cDNA (2.1kb) released by digestion with EcoR1.

Lane 4; Marker (Lambda HindIII fragments)

Lanes 5-7; P450IIE1 cDNA (1.1kb) released by digestion with EcoR1.

Figure 3.3 : Agarose gel showing cytochrome P450IIE1 cDNA following a large scale preparative digest of plasmid DNA

DNAs were electrophoresed on a 1% Agarose gel.
Lane 1; Marker (Lambda HindIII fragments)
Lane 2; Plasmid DNA following digestion with EcoR1
(Lane 3; Mussel genomic DNA- not directly related to this experiment)

3.3 : Detection of Cytochrome P450-Related Sequences in RNA isolated from marine animals

DNA-RNA hybridisation is a sensitive method of detecting mRNA sequences transcribed from a particular P450 gene or genes with closely related sequences. The high specific activity of the probes used, and the ability to moderate the specificity of the reaction by alteration of hybridisation and washing conditions gives this approach an advantage over other approaches, for example methods dependent on antibodies. This is especially true when detection is "cross-species" and when enzyme activity is low, as is the case in this study.

These experiments describe the initial attempts to detect P450 mRNA in marine animals.

3.3.1 Purification of RNA from mussel tissues

Total RNA was prepared according to the method of Chirgwin et al., 1979, from approximately 0.5g of mussel digestive gland tissue (frozen under liquid Nitrogen and stored at -70°C prior to extraction). Measurement of RNA purified by this method (fig. 3.4) gave a good yield, comparable with that obtained for rat liver (circa

Figure 3.4 : Example spectrophotometric trace of mussel RNA following extraction and purification

Scanning spectrophotometry was performed using a Uvikon 860 split-beam spectrophotometer and 0.5ml quartz cells (Starna, UK).

Using the data obtained and the equations given in 2.3.8;

$$A_{260} = \frac{1.337}{1.97} = 1.97$$

$$1.337\text{AU} \approx 53.5\mu\text{g/ml}$$

$$A_{280} = 0.677$$

$$\approx 1.78\text{mg/ml}$$

$$\approx 1.78\text{mg/g tissue}$$

1-6mg RNA/g tissue depending on tissue type and quality). O.D. 260/280 ratios also suggested that the purity of the product was good.

20 μ g of the mussel digestive gland RNA was then electrophoresed, together with 20 μ g of control rat liver RNA, on a 1.5% gel under denaturing conditions (Section 2.10.2). It was observed however (Fig 3.5), that the banding pattern was different from that expected. The anticipated 28S rRNA band was completely absent from the mussel sample, though the smaller 18S rRNA band could be observed. In comparison both of these larger rRNA bands were visible in the rat liver RNA sample.

It is not the case that the 28S rRNA band is absent in mussels, since this fundamental component of the cellular machinery is conserved in all known eukaryotes, and is qualitatively similar to the corresponding prokaryote ribosomal RNA. Indeed, other workers using methods specific for rRNA have purified 28S rRNA from mussels (Viarengo et al., 1986), other molluscs (Cammarano et al., 1980) and lower invertebrates (Loening, 1968). Loening in particular noted that the large rRNA band from lower orders was somewhat smaller than the mammalian equivalent and indeed was less stable in the extraction conditions used. Cammarano et al., (1975) observed that the size of the small rRNA fragment in molluscs was comparable with the mammals at a m.w. of 650kD, whereas the large rRNA component was smaller than the mammalian equivalent (1350kD, equivalent to 26S, compared with 1650kD). Recently, a partial sequence for the large ribosomal subunit RNA from a Mytilus species has become available (Qu et

Figure 3.5 : Gel of mussel digestive gland RNA following purification by the CsCl method of Chirgwin et al.

RNA samples were electrophoresed on a 1.5% Agarose gel under denaturing conditions.

Lane 1; Rat Liver RNA (20 μ g)

Lanes 3 & 5; Mussel digestive gland RNA (20 μ g) following extraction using the Chirgwin method (3) and Cathala method (5).

al., 1989). Thus it is unlikely that the 26S rRNA component was absent, but rather that partial degradation of the RNA had taken place (although usually this is accompanied by a general smearing of RNA down the gel below the expected band size).

However despite repeated attempts, using different conditions and methods (examined in more detail in chapter 5), I was unable to produce results similar to those obtained by Viarengo et al., or indeed observe the presence of the 26S subunit RNA in anything approaching the expected intensity levels of 2:1 relative to the 18S rRNA. Some indication of intact 26S rRNA was observed at very low levels in some of the samples examined.

In view of the presence of apparently undegraded 18S rRNA, it was considered that the mRNA itself may not be degraded, or only partially so. In support of this is the fact that there is no strong RNA smear close to the end of the gel, which is usually observed in a badly degraded sample. I therefore decided to proceed in attempting to detect P450-related sequences in the RNA samples prepared.

3.3.2 Detection of sequences homologous to different Cytochromes P450 in mussel digestive gland tissue.

Note: This data has, in part, been published (Spry et al., 1989).

Samples were applied to nitrocellulose membranes using a dot blot apparatus, and the filter membranes probed sequentially with radiolabelled P450IVA1 and P450IIE1 cDNAs (Fig. 3.6). The stringency of washing conditions used (Section 2.11.2) were 48°C/1xSSC (low stringency) and

Figure 3.6 : Dot blot to compare hybridisation of the rat Cytochrome P450IVA1 and P450IIE1 cDNA probes to rat liver and mussel digestive gland RNAs

i) P450IVA1 vs. filter #1

ii) P450IVA1 vs. filter #2

iii) P450IIE1 vs. filter #2

Probe synthesis was by nick translation.

Hybridisation was for 14hrs at 42°C.

Washing conditions were 55°C/1xSSC (high stringency) and 48°C/1xSSC (low stringency).

55°C/1xSSC (high stringency).

As can be seen from the data, a positive signal is visible after hybridisation to the P450IVA1 probe under both stringency conditions, and signal strength is dependent on mRNA concentration (though saturation does occur). This implies a relatively close sequence similarity between the cDNA probe and the mussel mRNA. This may be indicative of a P450IVA1-type mRNA in this tissue, but this remains to be determined.

Hybridisation to the P450IIE1 cDNA was not detected in these experiments, which may be due to one of two possibilities. Either, in mussels such a gene (and hence mRNA and protein) does not occur (this would be in agreement with the current understanding of P450 taxonomic distribution, where more primitive organisms do not possess the variety of P450s expressed in the rat, particularly those of the highly diversified P450II family [fig 1.6]), or the gene for this or a similar/orthologous protein may be present in mussel, but was not expressed in control digestive gland tissue.

At stringency conditions higher than those shown above (i.e. at 60°C/ 0.5xSSC), the Mytilus mRNA signal with the P450IVA1 probe was not visible even after extended autoradiogram exposure, whereas the rat signal was not visibly decreased. This presumably reflects the higher T_m of the rat RNA - rat cDNA hybrid compared with the mussel RNA - rat cDNA hybrid, confirming the expected greater sequence similarity.

A further set of blots was used to examine hybridisation of other P450 cDNA probes to mRNA expressed in mussel digestive gland. The washing conditions used were 55°C/1xSSC.

The results verified the data previously obtained (i.e. the presence of signal after probing with P450IVA1 cDNA, but not following probing with P450IIE1 cDNA). Additionally, the presence of mRNA hybridising to the fish P450IA1 (Polynuclear aromatic hydrocarbon inducible form) cDNA probe was detected (see later). No hybridisation to rat P450IIB1 (major phenobarbital inducible form) and P450IIC6 (constitutively expressed phenobarbital inducible) cDNA probes or a human P450XIA1 (cholesterol side chain cleavage) cDNA probe was detected (data not shown).

In many studies of mammalian P450 gene expression, a probe for a non-inducible mRNA such as actin would routinely be used as a control in order to ensure that the hybridisation detected is in fact representative of the level of expression and not due to spurious binding. Thus the filters were routinely probed when possible (see p.103) with an actin cDNA probe to check dilutions. However, this approach is difficult with the mussel for two reasons; Firstly, in the mussel, actin is not necessarily expressed at a fixed levels in a given tissue, whereas in the mammalian liver (the tissue most used in studies of mammalian xenobiotic metabolism) the level of expression is relatively constant. Secondly, the degree of hybridisation is dependent on the sequence homology as well as amount of specific mRNA present. Hence while the use of actin cDNA can confirm the correct application of RNA to the filters, the level of hybridisation may vary between tissues, samples taken at different times of the year (see p120), and between diverse species.

It is important to consider whether or not the

hybridisation observed is specific. One indication that the binding is specific to mRNA species related to rat P450IVA1 or fish P450IA1 has already been noted i.e. the comparison between P450IVA1 and P450IIE1 hybridisation. In dot and slot blot methods, however, under conditions of low stringency, it is possible that the autoradiography signal observed may in fact be due to weak binding of the probe to other mRNAs. Northern blots were were therefore prepared as detailed in Section 2.10.2. It was hoped that these would demonstrate discrete mRNA bands corresponding to the expected size of cytochrome P450 mRNA (i.e. in the same size range as for other species).

In the initial Northern blot studies with the P450IVA1 and P450IIE1 cDNA probes, P450IIE1 mRNA was again undetectable, whereas the P450IVA1 probe gave a hybridisation band in the region expected, slightly below the equivalent 2.1kb rat band (fig. 3.7). This is good supportive evidence for expression of a P450IVA1-like mRNA and protein within the mussel digestive gland. This particular Northern blot was nitrocellulose and unfortunately disintegrated. Hence further probing with other cDNAs e.g. Actin and GPX was not possible. Instead, the expression of other P450s in digestive gland was investigated in parallel with other mussel tissues using a new set of blots.

3.3.3 Detection of Cytochrome P450-related sequences in RNA isolated from different mussel tissues

As mussels contain a number of comparatively large and easily distinguishable tissue types/groups, it is possible

Figure 3.7 : Northern blot of mussel and rat RNA probed with Cytochrome P450IVA1 cDNA

Probe synthesis was by nick translation.

Hybridisation was for 14hrs at 42°C.

Washing conditions used were 55°C/1xSSC

The position of the rat ribosomal RNA bands are as indicated.

Also, the approximate size of the bands hybridising to the probe are shown.

to prepare RNA from different tissues to study the differential expression of genes. The different tissue groups used in this study were the same as those of Livingstone & Farrar (1984) for the study of Cytochrome P450 activity in the mussel. These are;

i) Digestive gland - soft tissue containing numerous blind tubules. Responsible for digestive functions and correspondingly rich in digestive enzymes and lysosomes. Typically 10-15% of total wet weight. (co-dissected with kidney)

ii) Mantle - soft tissue containing reproductive and storage cells. Particularly rich in lipid. Variable, according to nutritional state and season circa 5-40% of total wet weight.

iii) Gills (2 pairs) - fibrous, with respiratory function. Typically <5% of total wet weight.

iv) Foot - sensory organ and used in locomotion. Fibrous, comprising circa 5% of total wet weight.

v) Adductor muscle - Approximately 5% of total wet weight, and the primary regulator of valve opening.

vi) Rest - remaining tissue, including connective, mantle edge and remaining soft tissue. Typically 20-30% of total wet weight.

The yield of RNA varied according to tissue type, the digestive gland yielding the greatest amount per gram (fig 3.8), despite probably containing the largest amount of degradative enzymes. The relative amount from the mantle is

Figure 3.8 : Comparison of mussel total RNA yield by tissue

Bars represent the yields of total RNA (mg) obtained per gram of tissue (wet weight). The tissues used were isolated and pooled from a group of 4 (unsexed) animals.

Key: 1. Digestive gland
2. Mantle
3. Gills
4. Foot
5. Adductor muscle
6. Remaining tissue

probably affected by the high lipid content, and the others, particularly the gill and adductor muscle, by the fibrous nature of the tissue.

The quality of the RNA was, as monitored by electrophoresis and staining, similar from all tissues. Namely, the lower 18S band could clearly be seen, but the 28S upper band was at best very faint or (more usually) not visible at all (Fig. 3.9). RNA slot blots were then prepared and hybridised to the cytochrome P450 cDNA probes as detailed previously (Section 3.3.2). The resulting autoradiographs are shown overleaf (Fig 3.10).

From the data obtained, the most prominent result was the strong hybridisation of the P450IA1 probe to mRNA species from mussel digestive gland, mantle and gill tissues. The level of mRNA expression was apparently higher than for control uninduced rat liver.

Hybridisation to the P450IVA1 probe was also strong in these same 3 mussel tissues, but in this case hybridisation to the rat liver RNA was stronger. However, there is some correlation between the P450IVA1 signal and the actin signal, the one obvious difference is the slight change in relative intensity of the signal from the mantle tissue.

This result from the actin cDNA probe illustrates the problem of using this cDNA as a hybridisation control. Whereas in a mammalian tissue, actin is expressed at a constant level, in mussel (particularly mantle tissue) there is considerable variation. Seasonal actin aggregation and bundle formation associated with cycles of food storage and reproduction are known to occur (Lowe, 1982). Also, seasonal

Figure 3.9 : Gel to compare total RNA isolated from different mussel tissues

RNA samples were electrophoresed on a 1.5% Agarose gel under denaturing conditions.

Quality was gauged by relative intensity of staining in the 28S rRNA and 18S rRNA bands.

Lane 2; Rat Liver RNA (20 μ g)

Lane 3; Mussel remaining tissue fraction RNA (20 μ g)

Lane 4; " adductor muscle RNA (20 μ g)

Lane 5; " foot RNA (20 μ g)

Lane 6; " gill RNA (20 μ g)

Lane 7; " mantle RNA (20 μ g)

Lane 8; " digestive gland RNA (20 μ g)

Figure 3.10 : Detection of P450-related sequences in different mussel tissues

Slot blots of RNA (10ug) isolated from different mussel tissues probed with vertebrate cDNA probes.

Probe synthesis was by Multiprime (Amersham).
Hybridisation was for 14hrs at 42°C.
Washing conditions used were 55°C/1xSSC.

changes in actin gene expression in the mussel have recently been demonstrated at a RNA level in this laboratory (Pilling, 1990). In the present experiment, the higher levels of actin gene expression observed in mantle compared to other tissue types (fig. 3.10) makes acceptance of the P450IVAⁱ signal at face value suspect.

Probing using other available P450 probes (IIB1, IIC6, IIE1, XIA1) did not yield signals which could be distinguished above the high background levels which unfortunately were present during all probings with these filters (data not shown).

3.3.4 Detection of Cytochrome P450-related sequences in RNA isolated from different marine species

In addition to examining whether variation in P450 mRNA expression could be demonstrated between different mussel tissues, the presence of P450 mRNAs in different marine animals was also examined. A number of different species were collected from sites around Plymouth, Devon, and RNA prepared from either whole animal, (in the case of small animal or where dissection was not possible), or from the tissue in which highest P450 protein content was known or anticipated to be present. Usually this was the digestive gland or equivalent organ. In the case of the crab species studied, RNA was purified from the three tissues in which P450 dependent activity had already been identified, namely heart, green or antennary gland, and digestive gland (O'Hara et al., 1982). Yields were very variable, with only small amounts of being obtained from some animals (Table 3.1).

Table 3.1 ; Comparison by species of RNA yield

Animal	Tissue used	Yield (mg/g wet wt.)
Spongiforms		
<u>Halichondria panacea</u>	whole	0.16
<u>Halichondria sanguinea</u>	whole	0.21
Coelenterates (anemones)		
<u>Anemonia sulcata</u>	whole	0.49
<u>Actinia equina</u>	whole	0.59
<u>Bunadactis verrucosa</u>	whole	1.80
Annelids		
<u>Nereis diversicolour</u>	whole	0.93
<u>Arenicola</u> sp.	whole	0.91
<u>Eulalia viridis</u>	whole	1.20
Molluscs		
<u>Patella vulgata</u>	Dig. gland	1.05
<u>Littorina littorea</u>	Dig. gland	1.01
<u>Monodonta lineata</u>	Dig. gland	1.04
<u>Gibbula</u> sp.	Soft tissue	0.99
<u>Calliostoma zizyphinium</u>	Dig. gland	2.12
<u>Nucella lapillus</u>	Dig. gland	1.02
<u>Scrobicula planus</u>	Dig. gland	1.17
<u>Mytilus edulis</u>	Dig. gland	0.97
Crustaceans		
<u>Chthamalus stallata</u>	Whole	0.19
<u>Neomysis integer</u>	Whole	0.18
<u>Ligia oceanica</u>	Whole	0.26
<u>Micropippus puber</u>	Dig. gland	0.94
"	Heart	1.57
"	Green gland	0.55
<u>Carcinus meanas</u>	Dig. gland	0.74
"	Heart	0.88
"	Green gland	0.44
Echinoderms		
<u>Asterias rubens</u>	Pyloric ceaca	1.15
<u>Psammechinus miliaris</u>	Soft tissue	0.70
Tunicates		
<u>Botryllus schlosseri</u>	Whole animal	0.05
<u>Ciona intestinalis</u>	Whole animal	0.12
<u>Aplidium proliferum</u>	Whole animal	0.19
Vertebrates		
Flounder	Liver	0.72

Figure 3.11 ; Dot blot of total RNA samples isolated from a range of species

i) Probed with Cytochrome P450IVA1

(Key overleaf)

Samples are duplicate 10mg dots
Probe synthesis was by Nick translation (BRL).
Hybridisation was for 14hrs at 42°C.
Washing conditions used were 50°C/1xSSC.

contd.

ii) Probed with glutathione peroxidase

(reaction & washing conditions as per i) above)

Key:

a <u>H. panacea</u>	m <u>Gibbula</u> sp.	v(ii) <u>C. meanas</u> (heart)
b <u>H. sanguinea</u>	n <u>C. zizyphinium</u>	v(iii) " (g gland)
c <u>A. sulcata</u>	o <u>N. lapillus</u>	w <u>A. rubens</u>
d <u>A. equina</u>	p <u>S. planus</u>	x <u>P. miliaris</u>
e <u>B. verrucosa</u>	q <u>M. edulis</u>	y <u>B. schlosseri</u>
f <u>N. diversicolour</u>	r <u>N. integer</u>	z <u>C. intestinalis</u>
g <u>Arenicola</u> sp.	s flounder	* <u>A. proliferum</u>
h <u>E. viridis</u>	t <u>L. oceanica</u>	
i <u>P. vulgata</u>	u(i) <u>M. puber</u> (dig. gland)	
j <u>C. stallata</u>	u(ii) " (heart)	
k <u>L. littorea</u>	u(iii) " (green gland)	- Yeast tRNA
l <u>M. lineata</u>	v(i) <u>C. meanas</u> (dig. gland)	+ Rat liver RNA

Hybridisation to RNA from these animals was performed using cDNA probes for P450IVA1, P450IIE1 and genomic probe for GPX, the prepared filter being stripped between each probing. The GPX genomic DNA probe was included both to examine difference in phylogenetic distribution and to act as a control for the cross-species hybridisations (with limitations as previously noted). GPX is thought to be very strongly conserved protein (>80% amino acid conservation in mammals) of ancient origin (similar activity observed in microorganisms) (Chambers et al., 1986; Sukenaga et al., 1987) and, in mammals at least, is not known to be inducible.

Once again, hybridisation to the P450IVA1 cDNA probe was observed (Fig. 3.11), but not to the P450IIE1 cDNA probe (excepting the rat positive control, data not shown). Hybridisation to the GPX genomic DNA probe was also observed (Fig. 3.11 (ii)), however the pattern of relative signal intensity is very similar to that observed for the P450IVA1 cDNA.

A number of problems arise in interpreting "Zooblots" of this nature. Firstly, preparations of RNA are known to be affected by the idiosyncracies of the tissue from which they are prepared (Puissant & Houdebain, 1990). For example polysaccharides may cause interference with quantitation and hence affect the amount of RNA blotted onto the filter. In addition, various colour pigments from invertebrate tissues often copurify when using methods derived from mammalian studies (James, 1984; Kirchin, 1989) and this was my experience when preparing these RNAs. These pigments may or may not affect quantitation and/or the hybridisation

efficiency.

Another potential problem arises from the differences between the organisms themselves. This may be reflected as a difference in the amount of P450 mRNA(s) present compared with the overall amount of RNAs expressed. This may particularly be the case with respect to any differences between RNA from whole body and from digestive gland. There is also the consideration that these organisms were from different sites. The estuarine sediment habitat of the S. planus and N. diversicolor is, in all probability, markedly different in terms of basal pollution levels from the coastal intertidal sampling sites of the other species, which may affect expression levels. Other factors such as reproductive status of the individual animal may also have affected the expressed level of P450 at the time of sampling.

In addition, the difference in position in the taxonomic hierarchy implies differences in P450 sequence between the organisms being studied. Whether these differences arose by genetic drift or more extensive alterations, for example gene duplication, they may have affected the relative signal strength resulting from hybridisation to the rat/trout/human probes. Thus it is not possible to make any quantitative comparisons between the species. However it is possible to examine the strength of hybridisation and the likelihood of this being a reflection of P450 mRNA being present in the animal. It is clear that the duplicate dots for each animal are similar in intensity, and therefore reasonable to assume that the signals observed are due to binding of probe to mRNA, rather than to spurious hybridisation.

The next question is whether or not the binding is specific for sequences closely related to P450IVA1 or GPX. This is more difficult to ascertain. However probing of the filter with the GPX probe (Fig 3.11) showed a very similar pattern of relative intensity between the species compared to the P450IVA1 probe. Some small differences were observed. For example the relative intensities of hybridisation of P450IVA1 and GPX to P. miliaris (sea urchin) RNA were the reverse of that observed for B. schlosseri (tunicate). Also, the signals from the two species of sponge were relatively less intensive following hybridisation to the GPX probe when compared to that observed following hybridisation to the P450IVA1 probe. These suggest that the binding does in fact reflect specific mRNA content.

Having already taken into consideration the problems associated with using GPX as a control, this result perhaps reflected a basal expression of both enzyme types at a "housekeeping" level. The differences in signal between species could well due to the difficulty of quantifying RNA accurately, as previously mentioned.

To try and gain more information concerning the specificity of binding and the likely size of P450s expressed, Northern blots were to be run. However, the samples prepared for this study were lost due to freezer breakdown, and the experiment could not be repeated in the time available.

3.4 Detection of Cytochrome P450-related sequences in DNA isolated from marine animals

One of the problems identified with the analysis of RNA to detect P450-like mRNA sequences is that the presence of a specific mRNA is tissue- and environment-specific. Thus the apparent absence of hybridisation may be a reflection of variation of gene expression rather than an absence of the gene.

To overcome this problem I examined the possibility of probing genomic DNA instead. This carries its own problems, particularly a decrease in signal compared with Northern or RNA dot blots. The main reason is that the number of hybridising copies per microgram of nucleic acid is normally lower for the genomic DNA than the mRNA (dependent on the transcriptional activity of the gene of course). Another contributing factor is the lower thermal stability of the DNA:DNA hybrid compared to the DNA:RNA hybrid, with the melting temperature being typically 10-15°C lower (Keller & Manak, 1989). The stringency of the washing conditions were therefore reduced to allow for this factor.

Initially, the P450IVA1 cDNA probe was hybridised to genomic DNA from rat, herring (Boehringer), mussel and E. coli (Fig. 3.12), to ascertain whether binding of the probe was detectable and to determine under what washing conditions it could be removed. The results indicated that the probe was removed at relatively low stringency (37°C/0.5xSSC) from the marine genomic DNAs. Also, the signal strength was weak compared to the RNA blots though, as discussed previously,

Figure 3.12 : Dot blots comparing hybridisation of a cytochrome P450IVA1 cDNA probe to DNA from four species

Probe synthesis was by Multiprime (Amersham).

Hybridisation was for 14hrs at 42°C.

Washing conditions used were 37°C/1xSSC.

this was to be expected. Under the low stringency washing conditions, the signals from rat and fish DNA were of comparable strength. The signal from mussel DNA was slightly weaker, but was higher than the background hybridisation from the E.coli DNA. This indicated that, even under these conditions, the presence of sequence(s) related to the P450IVA1 probe were being detected in the mussel DNA.

To obtain more information about the sequences to which hybridisation was observed, a Southern blot was prepared. Mussel and rat DNA aliquots were digested overnight at 37°C with a range of restriction enzymes. After reducing to a small volume, the DNAs were electrophoresed on a 1.5% agarose gel according to the method in 2.3.4, and blotted according to 2.10.1 and 2.10.5.

Hybridisation to the P450IVA1 probe, followed by washing according to the criteria used in the DNA dot blot procedure, revealed good hybridisation to the rat DNA; clear banding patterns were observed (Fig. 3.13). However, no hybridisation could be detected with the mussel DNA fragments.

It was considered that perhaps the preferential binding of the probe to the rat DNA might have been the problem with this experiment. The binding could have been so strong that the remaining available probe was not of sufficiently high concentration to be able to detect hybridisation to the mussel DNA. However, a repeat experiment using only restriction enzyme digested mussel DNA also failed to detect any signal, even following extended exposure of the autoradiograph (14 days - data not shown).

This result calls into question the original interpretation of the dot blot data obtained using the P450IVA1 probe, suggesting that the binding originally

Figure 3.13 : Southern blot of rat and mussel genomic DNA probed with radiolabelled cytochrome P450IVA1 cDNA

Key:

m = mussel DNA

r = rat DNA

Samples (50 μ g) were run on a 1% agarose gel following overnight digestion with the restriction enzyme indicated. Probe synthesis was by Multiprime (Amersham). Hybridisation was for 14hrs at 42°C. Washing conditions used were 37°C/1xSSC.

observed may not in fact have been due to homologous sequences. This would make it difficult to isolate P450-containing sequences from the genomic library using mammalian probes, if it were in fact the case. However, the partially degraded nature of the DNA used for the Southern blot (the source was digestive gland - see p199-201) could have been responsible: partially degraded DNA would yield a series of small fragments generated following digestion with EcoR1. This would result in a smear of DNA from the gene of interest, resulting in diffuse bands which could not be detected by autoradiography, thus giving a false negative result. Other alternative explanations for this negative result, for example difference in the ratio of repetitive to unique DNA in mussels compared to rat (which would result in a weaker signal from an equivalent amount of DNA) fail to account for the previously obtained result from the dot blot study.

With previous positive results in mind, a genomic DNA "Zooblot" was done to determine which P450 isozyme gene sequences can be detected in DNA from which species. DNA slot blot filters were prepared using genomic DNA from a number of different animals, and these were probed with a number of P450 cDNA probes (IA1, IIB1, IIC6, IIE1, IVA1, XIA1).

Of the P450s examined it would appear that strongest hybridisation is observed with the P450IA1 and P450IVA1 cDNA probes (Figure 3.14). This is in agreement with existing evidence of P450 evolution and the data obtained from the mRNA studies.

Figure 3.14 : Slot blot of DNAs from different species probed with radiolabelled cDNA probes

Key:

- 1 H. sanguinea (sponge)
- 2 Arenicola sp. (worm)
- 3 M. lineata (gastropod)
- 4 S. planus (bivalve)
- 5 M. puber (crab)
- 6 Echinus sp. (urchin)
- 7 A proliferum (tunicate)
- 8 N. diversicolour (worm)
- 9 L. littorea (gastropod)
- 10 N. lapillus (")
- 11 M. edulis (bivalve)
- 12 P. miliaris (urchin)
- 13 B. schlosseri (tunicate)
- 14 R. rattus (rat)

Blots were prepared with 10 μ g DNA and were probed with multiprime-synthesized probes, hybridised 42°C/14hrs and washed 55°C/1xSSC/15 mins.

3.5 : Detection of Cytochrome P450-related sequences in mussel DNA using the Polymerase Chain Reaction (PCR) methods.

The development of PCR provided a rapid method in which a specific DNA sequence bounded by two oligonucleotide primers can be selectively amplified from genomic DNA. This is a more rapid and direct method for detecting the presence of a given sequence in an organism, compared with the generation and probing of gene libraries. However, the success or failure of this type of approach is dependent on the selection of the oligonucleotide primers themselves, since they provide the "handle" on the gene in question.

It was decided to attempt in vitro amplification of P450 related sequences from mussel genomic DNA (isolated as per 2.8). Having already obtained evidence for the expression in mussel of mRNA with sequence similarity to P450IA1 and P450IVA1, these two isozymes were obvious candidates. Oligonucleotides complementary to the coding regions of the corresponding vertebrate genomic DNA sequences, together with sequences from the conserved GPX gene, were chosen according to the following criteria;

(i) the oligonucleotide primers must be complementary to a species-conserved region within the vertebrate gene of interest. This maximises the chance that the oligo would recognise any corresponding mussel sequences (in the case of the P450 oligonucleotides, one oligonucleotide was to the conserved haem binding region; in the case of the GPX oligonucleotides, one oligonucleotide was to the conserved region around the selenocysteine residue).

(ii) the section of DNA to be amplified (i.e. between the oligonucleotide binding sequences) should preferably be less

than 3kb in length (otherwise efficiency and fidelity are reduced due to limiting nucleotide triphosphate concentrations).

(iii) the section of DNA to be amplified should contain no intron sequences (which are much less well conserved and thus of unknown length and sequence homology).

In the case of the gene sequences being studied here, the predicted length of the sequences selected for amplification were much shorter than the upper limit (see on). A diagrammatic representation of the genes and the primers chosen is given in figure 3.15.

3.5.1 Amplification of mussel DNA sequences

A control reaction was set up to discover whether the oligonucleotides primers would recognise and amplify specifically the correct sections of DNA to which they were complementary. 2ng of pUC19 plasmid DNA containing the corresponding cDNA (or genomic DNA in the case of GPX) of interest (see fig. 2.4) were used as a template for each primer pair. The reaction was performed as set out in 2.18, with reaction conditions as follows; denaturation:- 95°C/1 minute, annealing:- 50°C/1 minute, elongation:- 70°C/1 minute.

Each of these control reactions produced a single amplified DNA band of the correct size (Figure 3.16), as predicted from the published genomic sequences, verifying the suitability and specificity of the oligonucleotides selected.

A negative control reaction for each primer pair was not performed here as these are usually only included to show lack of interference from extraneous DNA, which is not a problem when amplifying from high copy number pure DNA preparations.

Figure 3.15 ; Diagrammatic representation of the location of the oligonucleotide probes in the relevant genomic DNA sequences

GPX: 147bp fragment predicted from Ishida et al. 1987

IA1: 207bp fragment predicted from Heilmann et al. 1988

IVA1: 157bp fragment predicted from Kimura et al. 1990

-Solid line denotes exon.

-Dotted line denotes intron.

-Arrows show binding region of oligonucleotides.

-Conserved feature (selenocysteine for GPX, haem binding cysteine for P450s) marked by a hash through the appropriate exon.

Figure 3.16 ; Gel of PCR products from control plasmids amplified using prepared oligonucleotides

DNAs electrophoresed through 4% 3:1 NuSieveGTG agarose:BRL agarose mix.

Lane 1: ϕ x174/HaeIII marker (fragment sizes 1343, 1072, 872, 603, 310, 281/271, 234, 194, 118 & 72)

Lane 2: GPX primers + GPX-pUC plasmid (expected band size = 147bp)

Lane 3: P450IVA1 primers + P450IVA1-pUC plasmid (expected band size = 207bp)

Lane 4: P450IA1 primers + P450IA1-pUC plasmid (expected band size = 157bp)

Debris at the base of lanes 2-4 is unincorporated oligonucleotide primers.

Mussel DNA sequences were then amplified using the P450 and GPX oligonucleotides. At a reduced annealing temperature of 40°C (to allow some mismatching), no PCR product bands could be seen. On reducing the annealing temperature further, to 37°C, a number of bands appear in the IA1/mussel DNA track (fig. 3.17). Of these bands, perhaps the most interesting is the most intense, that labelled no.4, since its size is only slightly larger than that observed for the trout cDNA. This makes it the most likely candidate for a sequence complementary to a mussel P450.

Of the other two enzymes investigated, it is surprising that the GPX reactions did not yield a product, particularly since primers from a less well conserved region of the GPX gene had produced a product from fish DNA (D.D. Howells, personal communication). The lack of amplification of the control GPX plasmid does suggest however that in this case the reaction is at fault rather than their being no template to amplify. If when the control is working there is still no product, it may be that there is a problem with binding of the primers themselves (a comparatively poorly understood process), and maybe moving the primers up- or downstream several base pairs may remedy this situation (Erlich, 1989).

The problem with the lack of amplification using the P450IVA1 primers is, I believe, more likely explained by the structure of the gene itself. As with the P450IA1, the haem binding sequence was chosen as one of the conserved regions for oligonucleotide synthesis. However, a large intron is present immediately upstream in rat genomic DNA (fig 3.15). Since introns are not well conserved across species, the size of this intron in the mussel could not be predicted.

Figure 3.17 PCR products following annealing at 37°C

Lane 1 2 3 4 5 6 7 8

DNAs electrophoresed through 4% 3:1 NuSieveGTG agarose:BRL agarose mix.

Lane 1: ϕ x174/HaeIII marker (fragment sizes 1343, 1072, 873, 603, 310, 281/271, 234, 194 & 118)

Lane 2: GPX primers plus mussel genomic DNA template

Lane 3: GPX primers + GPX-pUC plasmid (expected band size = 147bp)

Lane 4: P450IVA1 primers plus mussel genomic DNA template

Lane 5: P450IVA1 primers + P450IVA1-pUC plasmid (expected band size = 207bp)

Lane 6: P450IA1 primers plus mussel genomic DNA template

Lane 7: P450IA1 primers + P450IA1-pUC plasmid (expected band size = 157bp)

Lane 8: -ve control (mussel DNA template without primers)

Therefore the second oligonucleotide was made corresponding to the relatively less conserved downstream region near the amino terminus of the protein. This oligonucleotide was probably less homologous to the mussel DNA sequences and would not anneal. Hence no amplification product was generated. To investigate this further, a different primer pair might be used. For example a primer corresponding to another sequence conserved in P450 genes together with one specific to the substrate binding region of the P450IVA1 isozyme could be tried. *→ Alternative approach: RNA PCR → oligo dT plus IVA1*

3.5.2 Sequencing of amplified product

The amplified product band No. 4 from lane 6 of figure 3.17 was purified from the gel according to the Spin-X method given in 2.3.6, and reamplified. An aliquot of the resulting product was run on a 2% agarose gel to confirm that it was pure (i.e. it was verified that the single product formed corresponded to the original band isolated from the gel).

The remainder of the reamplified DNA was then precipitated and sequenced according to the method given in 2.17. The conditions used were selected as the most likely to yield data from the single attempt that time allowed. However the resulting autoradiogram did not show a clear ladder but bands across all four lanes. According to the manufacturers handbook, this is often indicative of a contaminated DNA sample. Were sufficient time available for a repeat reaction, it would have been more useful to use two asymmetric reamplification reactions in order to generate large amounts of single stranded DNA for subsequent sequencing.

3.6 : Discussion

The original aim of this section of the study was to positively identify the P450s expressed in mussel digestive gland in order to ascertain which probes to use in subsequent selection of clones from a gene library.

From the data presented, it is evident that firm identification of mussel equivalent to an individual mammalian P450 isozyme has not been achieved. This is due to the absence of sequence-related data, for example similar banding patterns on Southern blots or nucleotide sequence data from PCR generated fragments. In the former case I believe that the probes used were not sufficiently homologous to allow a strong enough signal to be generated. In the latter case, had sufficient time been available, pursuit of this avenue seemed most likely to yield promising data.

However of the data obtained from the hybridisation studies, the following conclusions can be drawn; First, differential detection of mussel mRNA sequences is possible using mammalian P450 cDNA probes. Second, that the spectrum of P450s expressed in mussel digestive gland is different to that in the "equivalent" rat organ, the liver. Third, that the data presented confirms rather than contradicts current understanding of the occurrence and evolution of P450s in the biosphere.

Interestingly, the hybridisation observed was to mRNA from three M. edulis tissue groups (digestive gland, mantle and gills). In comparison, Livingstone & Farrar (1984) detected P450 only in digestive gland, although Stegeman (1985) additionally detected P450 protein in gill tissue.

In addition, candidate P450 probes for use in isolation

of mussel P450 clones and for study of the modulation of P450 gene expression have been identified, namely P450IVA1 and P450IA1.

The presence of sequences related to P450IA1 is an interesting phenomenon in that metabolic studies using EROD activity (the classic indicator of the presence of P450IA1 in mammals) indicate low or undetectable levels of activity in the uninduced mussel (Kirchin, 1988). This, together with the absence of induction (see on) suggests that the regulation of this gene product, if indeed it is a mmolluscan IA1-equivalent, is different from its mammalian counterparts.

Whilst it would seem unlikely that any P450II family proteins are present in mussels (from the predicted P450 "family tree" and from the negative results obtained here in DNA and RNA hybridisation studies), their presence is not yet ruled out entirely. However, it would perhaps be more fruitful to examine whether other more ancient P450 families are represented, should the probes become available.

CHAPTER 4 : INDUCTION OF CYTOCHROME P450 GENE EXPRESSION IN MARINE ANIMALS

4.1 : Introduction

In the ideal animal for use as a biomonitor of marine pollution, the physiological or biochemical changes in response to compound exposure primarily need to meet two criteria. First, changes need to be detectable: ideally, the changes (in this case in levels of gene expression) measured should show a relationship with exposure concentration over a wide range of compound concentrations in the water or tissues. Second, the changes in gene expression would need to be reproducible and specific for the compound or group of compounds being studied. Interference by other factors will obviously make interpretation of data and comparison between samples more difficult. In this chapter the results from a number of experiments are described which examine how well the use of the mussel meets these criteria.

Detection of P450IVA1-related mRNA sequences in the initial stages of this work prompted a study of the response of mussels to known inducers of P450IVA1 mRNA in rodents. The ability of three such compounds to increase P450IVA1 mRNA in preparations from treated animals compared to control animals was measured by hybridisation to the P-450IVA1 cDNA probe.

In further studies, the responses of the mussel and a teleost fish (Scup; Stenotomus chrysops) to a range of P450 inducers were examined, using the relevant P450 cDNA probes to detect changes in level of mRNA coding for the various P450 isozymes.

The measurement of changes in P450 gene expression in

animals from field sites exposed to P450 inducers was also undertaken. Obviously it is necessary for induction under these conditions to be demonstrated successfully before the method can be used on a more routine basis for pollution monitoring. Animals from a site recently contaminated by a crude oil spill were compared with animals from a "clean" (i.e. without apparent chronic or acute contamination from industrial activity or previous oil exposure) reference site.

4.2 : Experimental treatment of *Mytilus edulis* with Cytochrome P450IVA1 inducers

This experiment was performed at the Plymouth Marine Laboratory in Nov./Dec. 1988.

The compounds studied were the plasticiser, diethylhexylphthalate (DEHP) (Lancaster Synthesis); the hypolipidaemic drug, Nafenopin (Ciba Geigy); and the branched chain alkane, 2,2,4-trimethylpentane (TMP) (Aldrich, HPLC grade). For each of these compounds, peroxisome proliferation and/or induction of P450IVA1 has been demonstrated in rodents (Reddy et al., 1974; ~~Gibson et al., 1982~~; Lock et al., 1987; Sharma et al., 1988) and other mammals (Makowska, 1988). The presence of plasticisers and alkanes in the marine environment makes study of these two groups particularly pertinent with respect to the monitoring of pollution responses in marine organisms.

4.2.1 Collection and treatment of animals

no interference with physiological
Mussels of 6-9cm shell length were collected from the Whitsand population in November 1988. After cleaning the shells of barnacles, seaweed and other debris which may have interfered with the experiment, the animals were placed in a holding tank with circulating seawater overnight. This was to allow emptying of gut contents, again to minimise variability in the experiment, and to identify any animals which had not survived *or been damaged by* the collecting procedure.

11 x 40 animals, unsexed and of mixed shell size were then placed in 11 x 10litre static tanks. These tanks were temperature-regulated to 15 ± 2.0 (S.D.) °C by placing in larger tanks containing temperature-controlled recirculating

seawater. The tanks were vigorously aerated in order to provide oxygen and mixing of the study compound. The tanks were covered, to minimise the evaporation resulting from the agitation, and were treated with experimental compounds as follows;

- 1 Seawater control
- 2 Seawater plus 0.001% ethanol (100 μ l)
- 3 DEHP 10ppb
- 4 DEHP 50ppb
- 5 DEHP 250ppb
- 6 Nafenopin 10ppb (in 100 μ l ethanol)
- 7 Nafenopin 50ppb (")
- 8 Nafenopin 250ppb (")
- 9 TMP 20ppb
- 10 TMP 100ppb
- 11 TMP 500ppb

each
In every case the solubility of the test compound in seawater was low. In order to obtain an even distribution within the tank therefore, the compounds were emulsified in 100ml seawater by sonication prior to adding to the tank. The water in the tanks was changed daily over the 12 day exposure period, and more compound added to maintain the dose level. Dosing was staggered over 2.5 hours to allow time for sample preparation on the respective sampling days.

Dosing levels were chosen in the range known to produce an elevation in cytochrome P450 specific content for other organic xenobiotics in mussels, viz. 30 to 125ppb aromatic hydrocarbons (Livingstone, 1988), adjusted upwards for the anticipated lower bioconcentration factors (lower octanol/water partition coefficient) of the putative P450IVA1

inducers.

A number of fatalities were recorded during the course of the experiment, as shown below. However from their random distribution, this most likely reflective of damage caused during the collecting procedures or stress on an individual of poor fitness rather, than effects as a result of exposure to the compound.

Animal deaths during experimental procedure;

Day 4 - TMP 500ppb, tank 11, (1 animal)

Day 7 - Nafenopin 50ppb, tank 7, (1 animal)

Day 7 - Ethanol 0.001%, tank 2, (1 animal)

Day 8 - DEHP 50ppb, tank 4, (1 animal)

Day 12 - DEHP 10ppb, tank 3, (1 animal)

4.2.2 Processing of samples and detection of sequences homologous to P450IVA1

At each sampling day (0, 4, 7 and 12), samples were taken (5 animals) and RNA prepared according to the method of Cathala et al. (section 2.7). After freezing at -180°C , the RNA samples were then transported on dry ice to Guildford. The RNA was then measured spectroscopically and appropriate dilutions made for application to nitrocellulose filters (section 2.10). $50\mu\text{g}$ RNA from each animal was then applied to one of three filters (one for each compound studied), together with the appropriate controls (Rat total RNA [+ve] and yeast tRNA [-ve]), and probed with a nick translated radiolabelled P450IVA1 probe. After washing at low stringency ($48^{\circ}\text{C}/1\times\text{SSC}$), the signal was somewhat weaker than expected, so the final wash was not performed in this case, and the filters put down to autoradiograph directly.

The resulting autoradiographs showed that for all three

compounds examined, the mussels did not exhibit clear induction of P450IVA1-like RNA sequences under this set of experimental conditions. In fact the intensity of the hybridisation signal for different individuals appeared to be extremely variable (Fig. 4.1 for DEHP, other data not shown). The only suggested pattern is for induction to have occurred at day 12 for 50ppb-treated individuals and at day 4 for 250ppb-treated individuals. However, the variability of the control samples, and hence individual variation, must be taken into consideration.

The lack of any clear pattern of induction may have been due to a number of factors. First, the compounds may not have reached the target tissue. The hybridisation signal would thus represent the basal level of expression. This would seem unlikely, however, given the ability of mussels to filter large volumes of seawater and readily bioaccumulate lipophilic organic xenobiotics (Livingstone, 1991), including phthalate esters (Wofford et al., 1981). The bioaccumulation of such compounds usually proceeds as linear uptake from the water column, followed by the eventual reaching of a tissue equilibrium concentration. The latter can be predicted from the octanol/water partition coefficient for the compound (within the range $\log K_{ow} \approx 2$ to 6) but these data are not available for the compounds used. However, a relatively low bioconcentration factor (concentration in tissue/concentration in water) of 11.2 was observed for the uptake of DEHP by the oyster, Crassostrea virginica, after 24 hours (Wofford et al., 1981). Although this factor would more than likely increase with longer time of exposure (i.e. the tissue equilibrium had probably not been reached in this paper), the likely body burden in the mussel exposures may have been less

than those reached by gavage administration in mammalian studies, viz. using the 24 hour oyster figure, about 2.8mg/kg for mussel exposed to 250ppb DEHP compared to 250mg/kg for rodents (factor 100 lower). In contrast more similar dosages are indicated for TMP, viz. using the data of Stegeman & Teal (1973) for the uptake of No.2 fuel oil (mixed aliphatic and aromatic hydrocarbons) by C. virginica, a tissue concentration of 186mg/kg is predicted after 48hrs exposure to 500ppb TMP compared to 1400mg/kg used for mammals (factor 7.5 lower). The dosage levels for nafenopin in mammalian studies was 180mg/kg. This and the other dose levels cited gave detectable increases in P450IVA1 and/or increases in w-hydroxylation of lauric acid. *in mammalian studies*

Second, any response may have been missed in the time course followed, if for example the response was rapid and transient. However, it might be expected that, even in cases where the response is rapid, the elevated levels of RNA would continue in the presence of the inducer. This was clearly not the case in the data obtained, and is a factor which must be taken into account when considering whether the signal from the day4/250ppb samples is a true response.

Third, the response may not be at the transcriptional level, for example protein stabilisation may occur as a means to elevating activity. However, in protein studies by Kirchin (1988), reproduceable increases in lauric acid hydroxylase activity were not observed following exposure to the hypolipidaemic drug clofibrate. It cannot be presumed however that this is a general case for all P450 inducers in mussel; indeed seasonal variation in response to 3-methylcholanthrene is reported for mussels (Livingstone et al., 1988).

The data presented here, together with the results of

Kirchin already described, indicate (if the sequence being detected is indeed the P450IVA1 orthologue in mussel), that the mechanism of gene regulation is different from that observed in rat. Species differences in the regulation of P450 orthologues are known however, as observed between fish and rat for both P450I and P450II as discussed in chapter 1. Indeed, Makowska (1987) notes differences in sensitivity to induction of P450IVA1 in response to peroxisome proliferators even between different species of rodents, so differences between rodents and molluscs are perhaps not surprising.

Another possibility is that the sequence being detected is not the P450IVA1 homologue, but another P450 isozyme, and is therefore not responsive to induction by classical P450IVA1 inducers. This can only be firmly determined by the cloning

Figure 4.1 Dot blot of mussel digestive gland RNA samples from animals exposed to the mammalian cytochrome P450IVA1 inducer, diethylhexylphthalate

Key; 20 μ g total RNA per sample

For box nos. 0-3: Columns of five individual animals corresponding (L-R) to days 0, 4, 7 & 12 respectively.

Box 0; Control mussels

Box 1; Treated mussels (10ppb)

Box 2; " " (50ppb)

Box 3; " " (250ppb)

Box 4: RNA controls; upper = yeast tRNA (-ve), lower = rat liver (+ve)

Probe synthesis by nick translation. Hybridised overnight at 42°C. Washing conditions 48 °C/1xSSC

and characterisation of the relevant gene.

Regardless of the identity of the gene product being analysed, it is apparent that the pollution of water by the classical P450IVA1 inducers used in this study does not lead to an apparent increase in P450IVA1-related mRNA in the mussel. Hence modulation of this gene cannot be used as an indicator for the presence of these compounds. Further work using other P450IVA1 inducers which may be environmental pollutants, for example the phenoxyacetic acid herbicides (Bacher et al., 1989) needs to be undertaken.

4.3 : Comparison of mRNA levels for Cytochromes P450 in Scup and Mussel experimentally treated with inducers of cytochrome P450 families I-IV

These experiments were undertaken during my visit to the Woods Hole Oceanographic Institution (WHOI) during October/November 1989. This set of experiments using P450 cDNA resources pooled from the WHOI and the University of Surrey has yielded comparative data from mussel and fish.

4.3.1 Collection and treatment of animals

Fish (Scup, *S. chrysops*) were caught by angling in Vineyard Sound, Massachusetts in spring 1989 and held in large (approx. 1000 litre) flowing seawater tanks prior to use. They were fed on a diet of chopped, locally caught (Nantucket Sound) squid.

24 hours before dosing, three (controls) or four (treated) animals were placed in each of nine 20 litre aerated static tanks. They were then dosed intraperitoneally as shown below. The cited references were used as a guide to the appropriate dosages (Tab 4.1). A second bolus i.p. dose was given at 36 hours, and the animals were sacrificed after 84 hours. This dosing regimen was used to achieve elevations in mRNA and in protein levels for parallel studies using nucleic acid hybridisation and western blotting techniques.

Two animals died in the course of the experiment (one each in group A and H) due to internal organ damage during the dosing procedure. Further abnormalities were noted in some of the fish. Some reflected the condition of the fish, for

Table 4.1 Dosing regimen for Scup

Tank	Compound	Dose	Vehicle	Reference
A	-	-	Corn oil	(control)
B	-	-	29% saline	(control)
C	-	-	Tween 20/ water (1:1)	(control)
D	Isosafrole	200mg/Kg	Corn oil	Leaver <u>et al.</u> (1988)
E	Mitotane (DDE)	100mg/Kg	Corn oil	Yosahoika <u>et al.</u> (1984)
F	Pregnenelone- 16a-carbonitrile	40mg/Kg	Tween 20/ water (1:1)	Goksoyr <u>et al.</u> (1986)
G	Acetone	400mg/Kg	29% Saline	Clark & Paris (1974)
H	Sodium clofibrate	50mg/Kg	29% Saline	Gibson <u>et al.</u> (1982)
I	3-methyl- cholanthrene	20mg/Kg	Corn oil	Stegeman <u>et al.</u> (1981)

example minor cases of diseased fins and eyes. Other changes were obviously due to the dosing of the animals, and were only observed after killing. In particular, many of the treated animals had full gall bladders. Animals in group F (pregnenolone carbonitrile) had discoloured livers, whereas animals in group H (clofibrate) had increased liver volume compared with control animals. However, when this was compared by weight with control animals, the difference was not significant ($0.5 > p > 0.1$ derived from Students t test).

The livers were frozen in liquid nitrogen immediately after removal from the animal, and stored at -70°C to await RNA extraction.

Mussels (approx. 200) were collected from Scortons Creek, Massachussetts in November, and held in 10 litre tanks with flowing seawater. After clearing the shells of biological material and clearing of the gut contents overnight, the animals (5-8.5cm shell length) were placed in 9 x 5litre static tanks with aeration, 5 animals per tank.

On this occasion (compared with section 4.2), the inducers were injected directly into the animal rather than being dissolved in the seawater. $125\mu\text{l}$ of vehicle containing the appropriate compound was injected into the cavity behind the mantle tissue of each valve of the animal, $250\mu\text{l}$ in total per animal. The total doses per animal were as follows;

Isosafrole	25mg	DDE (mitotane)	12.5mg
PCN	5mg	Acetone	50mg
Clofibrate	6.25mg	3MC	2.5mg

This approach of injecting with the maximal amount of compound possible was used to see if any increase in mRNA or protein for P450 at all in any of the groups could be detected. The animals were taped closed and left out of the water for 1 hour

to ensure compound was absorbed (after Kirchin, 1988). The animals were then returned to the tanks for 48 hours.

Despite the time allowed for the animals to absorb the compound, there was some leakage of the compounds into the surrounding seawater, observed as clouding or as an oil floating on the surface of the water.

Animals were killed in groups of five and digestive glands removed and frozen at -180°C . One group dosed with each compound was transported on dry ice to the University of Surrey and duplicate samples were retained at WHOI.

Of the animals killed, most were apparently healthy with the following abnormalities noted; i) animals in the two groups using Tween as a vehicle were appreciably less healthy (there was in fact one fatality in this group - the smallest individual in terms of shell size). Thus data obtained from this group must be treated with caution. ^(movement no suitable for the work) ii) some tissue ^{available for} damage was observed at the injection site in some animals ^{movement used,} (observed as discolouration of the tissue). iii) in corn oil treated animals, discolouration of the digestive gland was observed, presumably as a result of retention of the corn oil in fatty tissue.

4.3.2 Processing of samples and detection of P450-related sequences

RNA was prepared according to the method of Cathala et al. and, after quantitation, applied to nitrocellulose (scup RNA) or nylon (mussel RNA) membranes using a slot blot apparatus according to the schemes shown overleaf.

Membranes were probed with various cDNA probes by the standard method (2.9-2.11) in order to answer a number of questions. First, is the particular P450 isozyme to which the

probe corresponds expressed in the tissue? Second, is expression modified by the inducer? Third, can induction of a particular isozyme be detected following administration of a compound not associated with this type of induction in mammals? Finally, how do the patterns of induction observed in mussel and fish compare with those in rat?

The washing conditions used (55°C/1xSSC for 15 minutes) were standardised in order to obtain an indication of the relative level of sequence similarity between the two species. It was considered that for the scup, which will in all probability show stronger sequence similarity with the mammalian probes than the mussel (and in any case, stronger similarity to the trout IA1 cDNA), these conditions would be specific enough to differentiate between P450 families whilst still allowing comparison with signal from the mussel mRNA.

Results obtained are shown in fig.4.2 to 4.8. Also the opportunity arose to analyse some filters by scanning densitometry using a machine demonstrated by National Diagnostics. From these data, the following conclusions can be drawn in answer to the questions posed previously;

Probing of scup RNA filters showed strong hybridisation to the P450IA1 (figs. 4.2 & 4.3) and P450IVA1 (fig 4.4) probes, weak hybridisation to the P450IIE1 (fig. 4.5) probe and little, if any, to the P450IIB1 (fig. 4.6) probe.

The verification of induction in response to any of the model compounds was hampered by the absence of control hybridisation data (e.g. actin or albumin) for any of the filters to confirm that the correct amounts of RNA had been applied. However, there was an apparent induction of P450IA1

Figure 4.2 : Detection of hybridisation by trout P450IA1 cDNA to RNA from 3-methylcholanthrene-treated scup

Probe synthesis by nick translation. Hybridised overnight at 42°C. Washing conditions 55°C/1xSSC

Figure 4.3 : Detection of hybridisation by trout P450IA1 cDNA to RNA from isosafrole-treated scup

Probe synthesis by nick translation. Hybridised overnight at 42°C. Washing conditions 55°C/1xSSC

Figure 4.4 : Detection of hybridisation by rat P450IVA1 cDNA to RNA from clofibrate-treated scup

Probe synthesis by nick translation. Hybridised overnight at 42°C. Washing conditions 55°C/1xSSC

Figure 4.5 : Detection of hybridisation by rat P450IIE1 cDNA to RNA from acetone-treated scup

Probe synthesis by nick translation. Hybridised overnight at 42°C. Washing conditions 55°C/1xSSC

Figure 4.6 : Detection of hybridisation by rat P450IIB1 cDNA to RNA from mitotane-treated scup

Probe synthesis by nick translation. Hybridised overnight at 42°C. Washing conditions 55°C/1xSSC

mRNA in response to 3-methylcholanthrene exposure (Fig.4.2), which was verified by analysis using transmission densitometry (Table 4.2 & Fig. 4.9). This data is supportive of previous data from other fish studies using P450IA1 inducers (references given in Tab. 1.5). However insufficient individuals were present (following the death of one of the control animals) for sound statistical analysis. In contrast, no induction was observed in animals treated with isosafrole (a P450IA2 inducer in mammals) when probed with this cDNA (fig. 4.3).

In comparison, induction was not observed when the rat P450IVA1 probe was used to detect P450IVA1 related mRNA sequences (Fig 4.4), even though the signal was stronger in comparison to the P450IA1 samples. This may simply be due to the use of a probe of higher specific activity, or it may be a reflection of stronger sequence conservation of P450IVA1. Additionally, there may be a comparatively high level of basal expression of P450IVA1 in the non-induced animal.

Filters with mussel RNA showed strongest signal with the trout P450IA1 cDNA probe (fig. 4.7). However, the degree of non-specific binding on this filter, both to the negative control tRNA and to the right hand side of the filter generally make interpreting data from this filter difficult. The weaker binding to the rat P450IVA1 probe (fig. 4.8) compared with fig 4.7 (taking the data at face value) is an interesting reversal of the fish data. However it should be noted that the two sets of filters (fish and mussel) were probed separately using different probe preparations. A signal above background was not detectable with the other

Figure 4.7 : Detection of hybridisation by trout P450IA1 cDNA to RNA from 3-methylcholanthrene-treated mussel

Probe synthesis by multiprime. Hybridised overnight at 42°C.
 Washing conditions 55°C/1xSSC

Figure 4.8 : Detection of hybridisation by rat P450IVA1 cDNA to RNA from clofibrate-treated mussel

Probe synthesis by multiprime. Hybridised overnight at 42°C.
Washing conditions 55°C/1xSSC

Figure 4.9 : Densitometer scan of autoradiogram from figure 4.2

(see figure 4.2 for key)

6 white boxes delineated for the $3\mu\text{g}$ sample were used to obtain data used in table 4.2. Smaller box immediately below was used for control measurement. Change in colour denotes change in intensity of signal.

Table 4.2 : Densitometric comparison of mussel and scup RNA following probing with vertebrate P450IVA1 and P450IA1

Readings taken from the 3 μ g RNA band in each case:

Scup (3MC) vs. P450IA1 (Average of values)

Control (n=3) = 31.6

Treated (n=3) = 68.2

Scup (Clofibrate) vs. P450IVA1 (Average of values)

Control (n=2) = 74.8

Treated (n=4) = 77.2

Mussel (3MC) vs. P450IA1 (Average of values)

Control (n=5) = 69.1

Treated (n=5) = 130.16* probably more due to high background rather than a true signal.

Mussel (Clofibrate) vs. P450IVA1 (Average of values)

Control (n=5) = 105.7

Treated (n=5) = 91.72

probes used (IIB1, IIC6, IIE1,XIA1). In the case of probes from the the P450II family, this may be because equivalent genes are not present in the mussel, since this family are believed to be of later evolution. In the case of the P450XIA1 (which is involved in steroid biosynthesis), the negative result may be because the isozyme is not be expressed in the in the digestive gland. P450XIA1 is considered to be an ancient protein (Fig 1.6), and in mammals the gene is expressed in the adrenal gland. Occurrence of sterols, which in mammals require P45XIA1 for synthesis, is also found in mussels (Khan & Goad, 1983). However the site of synthesis has not to my knowledge been identified.

In both the scup and the mussel, no case of induction of a P450 mRNA from one family by an inducer of another (mammalian) family was observed (e.g. 3MC did not show induction of IIB1 in fish). This indicates that the mechanism of gene regulation is also conserved to some degree between species.

4.4 : Detection of mRNA with sequence similarity to Cytochromes P450 in samples taken after acute exposure to crude oil pollution

(This work was carried out with the help and technical assistance of Ed Pilling, an MSc. Toxicology student who performed the work under my supervision).

The grounding of the tanker "Sivand" off the coast of Cornwall in May 1990 resulted in the spillage of a substantial amount of light crude oil, some of which was washed up on local beaches. The control reference site at Whitsand was fortunately unaffected, however the shore at Mothecombe approximately 12 miles further east was particularly badly affected. Oil was beached over a period of several days, and surface oil remained in less accessible sites for several weeks. Samples of several species were collected from both sites 14 days after the oil first came ashore. RNA was prepared and samples from the two sites were compared for levels of cytochrome P450 mRNA expression.

RNA was purified according to the method of Cathala et al. (1983) from the following organisms: Patella vulgata (limpet), Actinia equina (anemone), Mytilus edulis (mussel), Monodonta lineata (topshell), Littorina littorea (periwinkle) and Nucella lapilus (dogwhelk). The yield obtained was somewhat variable, both between animals and between sites (Tab. 4.3). In the case of the A. equina, this is most likely due to the fact that whole animal rather than a single tissue was used to prepare the RNA. In all the other cases, the digestive gland alone was used.

Table 4.3 : Yield of total RNA from tissues of control and oil affected animals

Key: White; Whitsand Bay

Hatched; Mothercombe

1. Patella vulgata
2. Actinia equina
3. Mytilus edulis
4. Monodonta lineata
5. Littorina littorea
6. Nucella lapillus

RNA samples resuspended after extraction in 50µl water per gram wet weight of tissue.

In cases of animals exposed to a large amount of a toxic compound, it is common for tissue levels of total RNA to be decreased, whilst levels of specific mRNAs, for example "stress proteins" may be maintained at elevated levels. This may have been the cause of the low levels of total RNA from limpet and topshell from the Mothecombe site. In the case of the dogwhelk and the periwinkle, the position was reversed; the Mothecombe samples showed the greater tissue RNA concentrations. A biological explanation of this is more difficult to conceptualise. In the case of the dogwhelk, it was most likely due to a simple error in the spectrophotometric quantitation of the sample, since the subsequent RNA gels and blots show overloading of the Whitsand sample, suggesting underestimation of the amount of RNA in the stock RNA sample. No similar explanation for the periwinkle can be offered however.

The results from the Northern blots of the two groups of samples showed no apparent induction of P450IA1- or P450IVA1-related sequences in response to the oil exposure (fig. 4.10). The blots were not of particularly good quality though, reflecting partial degradation of sample RNA during preparation. The autoradiograph of the blot probed with the actin probe did show apparent alterations in expression between sites.

The slot blots yielded more information following probing with P450IA1 and P450IVA1 cDNAs (Figs 4.11 & 4.12). However it showed that there was no detectable response to the oil exposure. The only evident difference between samples from oiled and clean sites was for N. lapilus, where the signal was

Figure 4.10 : Northern blots of RNA from oil-affected and control animals probed with radiolabelled cDNA probes

Probe synthesis by multiprime. Hybridised overnight at 42°C.
Washing conditions 55°C/1xSSC

- Key:
1 Rat liver (+ve control)	8 <u>Monodonta lineata</u> (Whitsand)
2 <u>Patella vulgata</u> (Whitsand)	9 " " (Mothecombe)
3 " " (Mothecombe)	10 <u>Littorina littorea</u> (Whitsand)
4 <u>Actinia equina</u> (Whitsand)	11 " " (Mothecombe)
5 " " (Mothecombe)	12 <u>Nucella lapillus</u> (Whitsand)
6 <u>Mytilus edulis</u> (Whitsand)	13 " " (Mothecombe)
7 " " (Mothecombe)	

reduced in animals from the Mothecombe (oiled) site. This result can be rationalised in terms of less mRNA being expressed by a severely stressed animal. However, it could also be an artifact due to a quantitation error as discussed previously, since this signal was decreased for all three probes used, including the actin probe (fig. 4.13).

It is difficult therefore to draw any definitive conclusions from this "one-off" survey of a heavily oiled site. Indeed it is possible that where the level of fouling is sufficiently high to be toxic, the induction of specific P450 isoenzymes cannot be used as a monitor of pollution level.

Further, the levels of actin expression detected were particularly variable, making interpretation difficult in the absence of an obvious trend. An attempt to normalise the actin signal could be made, for example using a scanning densitometer. Even by this method, assumptions would have to be made to compare data directly between species; First, levels of expression must be presumed to be the same for all tissues. Second, degree of homology with the actin probe must be presumed to be the same for all animals. Third, the level of actin expression at each site must be presumed to be the same for each species.

Figure 4.11 : Slot blot of RNA from oil-affected and control animals probed with radiolabelled P450IA1 cDNA

Key:

1 <u>P. vulgata</u> (Whitsand)	8 <u>M. lineata</u> (Mothecombe)
2 " (Mothecombe)	9 <u>L. littorea</u> (Whitsand)
3 <u>A. equina</u> (Whitsand)	10 " (Mothecombe)
4 " (Mothecombe)	11 <u>N. lapillus</u> (Whitsand)
5 <u>M. edulis</u> (Whitsand)	12 " (Mothecombe)
6 " (Mothecombe)	+ Rat liver RNA
7 <u>M. lineata</u> (Whitsand)	- yeast tRNA

Probe synthesis by multiprime. Hybridised overnight at 42°C.
 Washing conditions 55°C/1xSSC

Figure 4.12 : Slot blot of RNA from oil-affected and control animals probed with radiolabelled P450IVA1 cDNA

- Key:
1 <u>P. vulgata</u> (Whitsand)	8 <u>M. lineata</u> (Mothecombe)
2 " (Mothecombe)	9 <u>L. littorea</u> (Whitsand)
3 <u>A. equina</u> (Whitsand)	10 " (Mothecombe)
4 " (Mothecombe)	11 <u>N. lapillus</u> (Whitsand)
5 <u>M. edulis</u> (Whitsand)	12 " (Mothecombe)
6 " (Mothecombe)	+ Rat liver RNA
7 <u>M. lineata</u> (Whitsand)	- yeast tRNA

Probe synthesis by multiprime. Hybridised overnight at 42°C.
Washing conditions 55°C/1xSSC

Figure 4.13 : Slot blot of RNA from oil-affected and control animals probed with radiolabelled actin cDNA

Key:

1 <u>P. vulgata</u> (Whitsand)	8 <u>M. lineata</u> (Mothecombe)
2 " (Mothecombe)	9 <u>L. littorea</u> (Whitsand)
3 <u>A. equina</u> (Whitsand)	10 " (Mothecombe)
4 " (Mothecombe)	11 <u>N. lapillus</u> (Whitsand)
5 <u>M. edulis</u> (Whitsand)	12 " (Mothecombe)
6 " (Mothecombe)	+ Rat liver RNA
7 <u>M. lineata</u> (Whitsand)	- yeast tRNA

Probe synthesis by multiprime. Hybridised overnight at 42°C.

Washing conditions 55°C/1xSSC

4.5 : Discussion

From the remarks in the previous chapter and the experimental data presented here, it is apparent that the mammalian cDNA probes available cannot as yet be used successfully to detect alterations in the expression of cytochromes P450 in the mussel or other marine invertebrates in response to added inducers.

This may primarily be a reflection of non-responsiveness of the P450s in the animals following exposure to xenobiotics. Indeed, the existing evidence from other studies (Tab. 1.6) suggests that marine invertebrates in general, and mussels in particular, do not show the type of induction observed in rodents, which commonly produces increases of an order of magnitude or greater in protein or enzyme activity. In contrast, the data presented in this chapter suggests that P450 expression in marine invertebrates appears to decrease following acute exposure to crude oil, though as noted, this experiment used severely affected individuals.

Alternatively, it may be that the seawater quality off the coast of Southwest England is such that the xenobiotic-metabolising systems of mussels and other invertebrates are already maximally induced in the environment. Hence, no further increase resulting from laboratory or other exposure to pollutants or inducers will be detected. The existing data does however indicate that this suggestion is unlikely, since similar P450/MFO levels are observed in mussels from a wide variety of habitats worldwide.

Another possibility which must be considered is that the cDNA probes used did not detect the right P450 isozyme, i.e. the P450 isozyme(s) responding to exposure to xenobiotics may

not have been detected with the available mammalian cDNA probes. The most definitive method of studying alterations in expression of P450 in Mytilus and other marine species would be, as was noted at the beginning of this study, to first isolate and identify the specific P450 genes present. To date no conclusive evidence for the expression of any single isozyme has yet been published for the mussel. The efforts I have made to isolate and identify the P450 genes involved are presented in the following chapter.

CHAPTER 5 : cDNA AND GENOMIC LIBRARIES FROM THE MUSSEL

5.1 : Introduction

This chapter details attempts to generate a gene library of cDNA sequences from Mytilus edulis. In research on rodent and human cytochrome P450 the investigator can choose from a number of commercially available libraries to study the gene or cDNA of interest. In contrast there is no such option in mussel P450 research and I therefore decided to produce my own mussel cDNA and genomic libraries.

5.2 : Synthesis of a cDNA library from mussel mRNA

The most important factor in the synthesis of a good cDNA library is the quality of the starting material, the mRNA. A number of commonly used methods were compared to try to obtain intact mRNA from mussel digestive gland and the results are presented. With the failure of any of these methods to yield RNA of a quality comparable to that obtained from rat liver by the same methods, synthesis of the mussel cDNA library was attempted with the best available RNA preparation.

5.2.1 Isolation of RNA from mussel digestive gland using a number of different techniques

As described in chapter 3, total RNA has been isolated from mussel digestive gland using standard mammalian-derived methodology with moderate success. In RNA purified by the initial technique tried, the 18S ribosomal RNA band was present but the 28S band was absent (Fig. 3.5). The methods outlined below were tried in order to obtain a better product. Variable results were obtained as shown.

The methods of Chirgwin et al. and Cathala et al. have already been described in section 2.7. At this stage it was considered that the problem of mRNA degradation encountered may have been due to endogenous ribonucleases. The ability of the mussel to remain physiologically viable under extremes of temperature, in particular at lower temperatures (at which mammalian ribonucleases are inactive), suggested that a likely problem in the extraction procedure could be the activity of the nucleases in the chilled homogenate immediately prior to inactivation by the homogenising buffer. A series of experiments were therefore conducted where 10mM Vanadyl

ribonucleoside (a potent inhibitor of nuclease activity) or 15mg/ml tRNA (as a competitive inhibitor) were included in the extraction procedure at the initial homogenisation stage. These made no apparent difference in the qualitative appearance of the RNA isolated (Fig. 5.1). Indeed, they introduced a potential problem in that the vanadyl ribonucleoside complex, is difficult to remove. This would have been required before cDNA synthesis could take place, as it is also an inhibitor of the enzyme reverse transcriptase.

In addition experiments were conducted to ascertain whether RNA quality from the tissue was adversely affected by the freezing and short term storage at low temperature prior to processing. No apparent difference was observed between RNA from fresh and frozen tissue, as determined by agarose gel electrophoresis under denaturing conditions (2.7).

Another method available in the laboratory was adapted from Chomczynski & Sacchi (1987). When tried, this method did yield RNA from mussel tissue but it was of poorer quality compared to the previous methods (as gauged by the integrity of the lower 18S RNA band).

From the methods examined so far it was clear that the standard method of deactivating nuclease activity (using a chaotropic agent) was not sufficient. Other methods were tried which used an alternative means of protecting the higher molecular weight RNA from degradation. The first of these is the method routinely used for isolation of RNA as a polysome complex, namely that of Palmiter (1974). Although it was

Figure 5.1 : Comparison of mussel RNA samples isolated in the presence of different RNase inhibitors

Key:

1 Standard extraction procedure

2 15mg/ml tRNA added to the homogenisation medium

3 10mM vanadyl ribonucleotide added to the homogenisation medium

The samples were electrophoresed on a 1.5% agarose denaturing gel.

The approximate size of the bands are shown.

possible to isolate RNA by this method, it proved to be particularly badly degraded compared with the rat samples.

Other methods were tried, which relied on temperature extremes to reduce the RNA degradation observed. The method of Han et al. (1987) relies on reduction of temperature, whereas the method of Feramisco et al. (1982) relies on high temperature inactivation of ribonuclease activity.

Of the two methods, the second gave the more promising data. The RNA from the first method was more degraded than the best samples from previous methods. In comparison, the hot phenol procedure resulted in isolation of high molecular weight RNA prior to the proteinase K digestion, however the expected 28S and 18S banding pattern was not visible. The interpretation of this is that the RNA isolated included an amount of riboprotein which gave a higher apparent molecular weight and was less pure (as determined by spectrophotometry) than the corresponding proteinase K digested and purified sample (Fig. 5.2). The purified sample did however give RNA with the now characteristic absence (or at the very least severe diminution) of the 26S ribosomal RNA band.

At this stage a commercially available extraction kit was used to verify that the problem was not simply of a local technical nature. The Amersham RNA extraction kit was used, according to the protocol supplied. This was an adaptation of the methods developed by Han et al. and Cathala et al., but with the benefit of quality controlled reagents. The results once again demonstrated that RNA extraction was possible, and produced the expected banding pattern in RNA isolated from rat liver (Fig. 5.3). Again however, the mussel RNA lacked the

Figure 5.2 : Gel showing RNA isolated by the method of Feramisco et al.

Key:

- 1 Rat total RNA undigested
- 2 " " " digested with proteinase K
- 3 " " " digested with DNase
- 4 Mussel total RNA digested with DNase
- 5 " " " digested with proteinase K
- 6 " " " undigested

The samples were electrophoresed on a 1.5% agarose denaturing gel.

? denotes that some indication of a 26S band may be present

Figure 5.3 : Gel showing RNA isolated by the Amersham method

Key:

1-6 Mussel total RNA purified using the Amersham protocol
7 Rat total RNA

The samples (20 μ g) were electrophoresed on a 1.5% agarose denaturing gel.

upper 26S ribosomal RNA band.

Combining the evidence from all these experiments, it might be concluded that molluscan 26S ribosomal RNA is not as stable under these isolation conditions as its mammalian equivalent. Thus it is possible that whilst the absence of a 26S band in mammalian RNA preparations is indicative of degradation of the sample (Chirgwin et al., 1979), the molluscan equivalent may not be a good indicator of the degradation in the RNA sample as a whole. It does therefore remain a possibility that the extraction procedures used caused selective degradation of the molluscan 26S RNA as the ribosomes dissociated (the other RNA species, including the mRNA, remaining intact). There is some support for this argument in that the 18S ribosomal band does remain as a discrete band on the gel rather than the diffuse smear of RNA observed after digestion by non-specific ribonuclease activity. Indeed Loening (1968) noted that ribosomal RNA purified from some lower animals was less stable than that prepared from mammalian tissue.

If this were the general case for molluscan RNA preparations, then one might expect to observe a similar effect with all tissues, not just those in which a high RNase level is observed. RNA preparations from all tissues were subsequently analysed by electrophoresis for the presence of the 26S ribosomal band. The results in figure 3.8 demonstrate that the loss of the upper ribosomal band was indeed common to all mussel tissues and that it may indeed be less stable than mammalian 28S ribosomal RNAs.

This analysis was extended to RNA from other species, and indeed the loss of the upper ribosomal band was observed in other marine molluscs when extraction procedures developed for

mammalian RNA were used (Fig. 5.4). Interestingly though, in total RNA isolated from spongiforms and coelenterates both ribosomal bands were present.

In summary, the RNA isolated from mussel soft tissue was qualitatively different from mammalian RNA isolated by the same technique. However insufficient time was available to develop a method which could reproducibly produce RNA preparations in which the 26S rRNA band was preserved intact. Instead it was decided to attempt mRNA isolation and cDNA synthesis from the best of the available total RNA preparations. This decision was made on the premise that the larger mRNAs might be more stable than the 26S rRNA and therefore be represented in the total RNA.

Figure 5.4 : Comparison of total RNA samples isolated from marine lower invertebrates

Key:

20 μ g total RNA per track, sources as follows;

1 Rat liver (+ve control)	7 <u>Arenicola</u> sp.
2 -	8 <u>Eulalia viridis</u>
3 <u>Halichondria panacea</u>	9 <u>Patella vulgata</u>
4 <u>Halichondria sanguinea</u>	10 <u>Littorina littorea</u>
5 <u>Actinia equina</u>	11 <u>Monodonta lineata</u>
6 <u>Nereis diversicolour</u>	12 <u>Gibbula</u> sp.

The samples were electrophoresed on a 1.5% agarose denaturing gel.

Note: due to the poor quality of the photograph, some of the signal is obscured from the two end tracks.

5.2.2 In vitro translation of mussel total RNA and mRNA samples

One method of determining whether the RNA isolated contains mRNA useful for reverse transcription is translation of the RNA in vitro. The sizes of the resulting polypeptide products provides an indication of the integrity of the mRNA isolated. The Amersham kit (2.12) was used and the RNA preparations compared with the TMV mRNA control using SDS-PAGE (2.13) to separate the translation products.

However from the results, it would appear that the reaction had not worked with my RNA samples (including the rat RNA control samples) (Fig. 5.5). This may simply have been a technical problem, associated with optimisation of the reaction conditions, or possibly the use of total RNA rather than purified poly A+ mRNA.

Subsequent experiments with poly A+ RNA as isolated below also failed to give large translation products, suggesting translation conditions may not have been optimal.

5.2.3 Purification of poly A+ RNA (mRNA) from mussel total RNA samples

Initial attempts to obtain mRNA from mussel RNA using the Amersham mAP method as outlined in the protocol booklet were unsuccessful in so far as only very low yields were recorded. A modified protocol was then supplied by Amersham and was used as described in 2.12.1.

Results obtained using the revised method were promising, and mRNA of reasonable yield and purity were obtained (6.8 μ g

Figure 5.5 : SDS-PAGE of peptide products produced from in vitro translation of RNA samples

30 μ l samples run on 7.5% gel
Key:
1 -ve control (no template)
2 +ve control (Tobacco Mosaic Virus RNA, 0.1 μ g)
3 Rat total RNA (1 μ g)
4 Mussel total RNA (1 μ g)

poly A⁺ RNA from 4mg total RNA [0.17%]). This was lower than that achieved for rat liver, where a 2% yield was observed (8μg from 0.4mg total RNA), and may reflect a lower mRNA specific content in mussel. This in turn may be a reflection of the lower overall metabolic activity of the mussel, as is observed with many mussel enzyme levels, including cytochrome P450 [Livingstone & Farrar, 1984]). An alternative explanation may be a lower amount of poly A⁺ RNA due to partial degradation.

However, disappointing results obtained with the in vitro translation (i.e. no evidence of product) prompted a closer investigation of the mRNA extraction method. It was considered that part of the product might not be translatable poly A⁺ RNA, but in fact be ribosomal RNA with short lengths of adenosine ribonucleotide-rich regions. Product from one mAP extraction (10.0μg from 6mg) was redissolved and reapplied to a second piece of mAP and the extraction procedure repeated. On this occasion the recovery and purity after the second pass was good (5μg in total- Fig 5.6) when allowance for possible irreversible binding to the mAP and loss in various handling procedures is made. This augured well for subsequent cDNA synthesis.

A comparison of this somewhat new technique was made with the more traditional route to mRNA purification (oligo-dT column chromatography) to obtain the best material for cDNA synthesis. For the rat the best yield obtained was 2.9% (14.52μg from 0.5mg total RNA). Both this yield and the best mussel yield (3%) compare favourably with yields from the mAP procedure, though recovery from "second pass" experiments was

Figure 5.6 : Spectrophotometric trace of mussel poly-A+ RNA following extraction and purification

Scanning spectrophotometry was performed using a Uvikon 860 split-beam spectrophotometer and 0.5ml quartz cells (Starna, UK).

Using the data obtained and the equations given in 2.3.8;

$$\underline{A_{260}} = \underline{0.025} = 2.08$$

$$0.025\text{AU} \approx 1\mu\text{g/ml}$$

$$A_{280} \quad 0.012$$

$$\approx 5\mu\text{g from 6mg tissue}$$

effectively zero (this may have been due to the difficulty in keeping the equipment nuclease free coupled with problems due to extraction of small amounts of mRNA from the larger volumes). The mRNA from the single pass oligo-dT method may therefore have been less pure than that obtained from the mAP method. Hence mAP purified poly A+ RNA was used in the subsequent cDNA synthesis and cloning experiments.

5.2.4 cDNA synthesis from purified mussel poly A+ RNA

5 μ g of mussel mRNA thus obtained was then reverse transcribed in parallel with 1 μ g of control rat globin mRNA as supplied, using the oligo dT primer as supplied.

The mussel reaction was set up to contain 32 P dCTP in order to follow the reaction through its various stages. The data for the incorporation of 32 P dCTP into mussel cDNA was;

First strand reaction; 9.08% incorporation, equivalent to 0.45 μ g ss cDNA

Second strand reaction; 2.24% incorporation, equivalent to 0.23 μ g ds cDNA

The first strand synthesis efficiency is comparable to that anticipated by the manufacturer (15-30%), though perhaps a little low. The second strand reaction efficiency is more disappointing, 40.5% instead of >90% expected.

The resulting cDNA was then purified by the Geneclean procedure (2.3.8) prior to cloning into phage vector.

5.2.5 Cloning of mussel cDNA into lambda gt11

The purified double stranded mussel cDNA was then cloned into the vector, lambda gt11, using the protocol supplied. It was noted however that after the digestion of the EcoR1 linkers, a significant proportion of the resulting "sticky ended" radiolabelled fragments were eluted from the Sephadex G-50 column much later than expected, indicating that the cDNA was of smaller size than would be anticipated from full length mRNA. Indeed when ethanol precipitating the remaining linkered cDNA, much of the remaining radiolabel was not precipitated. This is an indication that the cDNA may have contained a proportion of smaller fragments of nucleic acid (50bp or less).

The estimated amount of linkered cDNA left for the packaging stage was 100ng. Although this was not sufficient to produce a good library, it was decided to follow through the procedure to check the efficiency of the following stages. Four packaging reactions were set up, using only half the reaction volumes recommended in the protocol as the amounts of ligated cDNA were less than expected.

After packaging and plating out of Y1090 cells infected with the recombinant phage (2.13 & 2.14), the results in Table 5.1 were obtained.

From the data, the numbers of recombinants in the library (from tubes 3 and 4 combined) is 3.45×10^4 in total. This is however, unlikely to constitute a complete library of mussel digestive gland cDNAs, since the number of different genes expressed in a cell is typically of the order 10000-15000 and many of these are present as multiple copies (Lewin, 1987).

Table 5.1 Titres obtained for packaged ligation mix after plating out infected Y1090 cells

Tube	Contents	Titre (pfu/ml)	% recombinants
1	lambda gt11 control DNA	4.0×10^7	0
2	gt11 arms + control cDNA (100ng)	5.5×10^6	96
3	gt11 arms + mussel cDNA (66ng)	4.0×10^4	50
4	gt11 arms + mussel cDNA (33ng)	2.9×10^4	50

Infected host cells were plated out with medium containing ampicillin, IPTG and the chromogen X-gal. Host cells infected with parental phage DNA produce the enzyme β -galactosidase which reacts with the X-gal to yield blue plaques. In cells infected with recombinant phage, the lacZ gene (which codes for β -galactosidase) is disrupted, and plaques produced are therefore white.

The % recombinants is therefore obtained from $100 \times (\text{no. white plaques} / \text{total no. plaques})$.

The control cDNA (rabbit globin cDNA) is that supplied in the Amersham cloning system.

This may be particularly so if, as was suggested from the cDNA synthesis, many small cDNAs of less than full length are present.

The library was plated out and screened for the presence of recombinants hybridising to the rat P450IVA1 cDNA probe. However, no positive clones were detected, even after repeated screening.

It would seem therefore that, despite earlier evidence to the contrary (chapter 3), the mRNA used in this procedure was not sufficiently intact for cDNA synthesis and cloning. The fact that a substantial signal was observed in Northern blots does suggest however that, in the total RNA preparation, the mRNA was substantially intact. An alternative explanation may have been a low yield of cDNA from the reverse transcription and/or a preponderance of short cDNAs.

5.3 : Synthesis of a gene library from mussel genomic DNA

As cDNA library synthesis was proving difficult, it was decided to pursue an alternative course to the isolation of mussel P450 genes, namely the generation of a genomic DNA library. Recombinant clones identified as containing putative P450 sequences could then be further analysed to obtain the coding region DNA sequence for confirmation of identity.

5.3.1 Isolation of genomic DNA from mussel tissue and preparation for cloning

A number of techniques are available for the isolation of DNA from animal tissue. However, the continued activity of poikilotherm DNAases at low temperature requires the use of methods which eliminate such activity at an early stage and thus ensure the extraction of intact DNA. The method of Hewish & Burgoyne (1973a&b) achieves this, and good quality DNA can be obtained from mammalian and fish tissues. However, repeated attempts to isolate high molecular weight undegraded DNA from mussel digestive gland tissue were unsuccessful (Fig. 5.7). The decision was therefore made to isolate the DNA from a tissue expected to contain less DNAase activity. The other major homogeneous soft tissue in the mussel is the mantle (which contains the gametes in "ripe" animals). Using this tissue, good quality DNA was obtained with ease and in abundance (Fig. 5.8).

Full length genomic DNA is too large for direct insertion into phage cloning vectors and so is digested with a

Figure 5.7 : Gel to show mussel DNA purified from digestive gland tissue

Key:

1 Lambda HindIII marker

2 -

3 20µg mussel DNA

4 -

5 4µg mussel DNA

Samples were electrophoresed on a 0.5% low melting point agarose gel.

Figure 5.8 : Gel to show mussel DNA purified from mantle tissue

Key:

1 -

2 Lambda HindIII marker (0.2μg)

3 " " " (2.0μg)

4 -

5 10μg mussel DNA (from mantle tissue)

Samples electrophoresed on a 0.5% low melting point agarose gel.

restriction endonuclease into fragments compatible with the vector of choice (9 to 23kb for lambda EMBL3a). Digestion of the genomic DNA also performs the useful function of providing the mussel DNA fragments with "sticky ends" compatible with those of the prepared vector arms. Digestion was achieved by one of two methods, either; i) complete overnight digestion with an enzyme which recognises a six nucleotide cutting site, or ii) a timed partial digestion with an enzyme recognising a more frequent four nucleotide motif. The former has the advantage of being easier technically, but has the disadvantage that some genes may be cleaved by the digestion and that overlapping DNA fragments may not be isolated. Partial digestion overcomes these problems by not cutting the DNA at all available sites, thus providing overlapping fragments. Sufficient digestion time must be allowed however, so that the average size of the DNA fragments produced is within the range accepted by the vector.

A BamHI digest of mussel DNA was set up and digested to completion overnight at 37°C. An agarose gel (0.3%) was run to check that the digestion was successful (Fig. 5.9).

The partial digestion procedure was more complex, and several attempts were required to obtain the correct conditions. It was necessary to establish i) how much enzyme and DNA to add and ii) how long to allow the reaction to proceed. Although laboratory manuals to give "ball-park" figures, the precise conditions are dependent on both the DNA preparation and the restriction enzyme. The method followed was based on that of Herrmann & Frischauf (1987). An analytical partial digest was performed and, as can clearly be seen from Fig. 5.10, average fragment size decreases with time.

From this data it would appear that under these conditions, an incubation time of 40 minutes is required to obtain fragments

of average size 9-23kb. In order to favour the cloning of larger fragments, it was decided to allow the preparative digest to run for 30 minutes only, as the presence of over-size fragments is less disadvantageous than the presence of many under-size fragments.

The reaction was then scaled up 10-fold to allow for losses in subsequent processing. An aliquot of the preparative digest was removed immediately prior to the addition of enzyme for use in control reactions. Uncut and cut were then compared. As can be seen from Fig.5.11, the reaction had not proceeded as far as in the analytical digest, but is sufficiently well digested to obtain fragments of appropriate size for insertion into the cloning vector.

The mussel DNA fragments needed to be further processed in order to prevent artifacts such as multiple insertion and recombination of mussel DNA during ligation. This was achieved by alkaline phosphatase treatment of the mussel DNA to ensure that it was dephosphorylated and thus only one mussel DNA fragment could be inserted per recombinant phage. The efficacy of this reaction was tested by self-ligation of phosphatased and non-phosphatased DNA. Control experiments were also performed using HindIII digested bacteriophage lambda DNA, as shown in Figure 5.12.

The results verify that for the lambda HindIII digested DNA, both the ligase and phosphatase enzyme reactions had

Figure 5.9 : Gel to show BamH1 digested mussel DNA

Key:

- 1 -
- 2 Lambda HindIII marker
- 3 -
- 4 BamH1 digested DNA (1 μ g/lane)
- 5 -
- 6 Undigested DNA (1 μ g/lane)
- 7 -

Samples were electrophoresed on a 0.3% low melting point agarose gel.

Figure 5.10 : Gel to show Sau3A partial digests of mussel DNA purified from digestive gland tissue

Lane	1	2	3	4	5	6	7	8
Time of digest (mins)	-	0	5	10	20	30	40	60

Origin

23.

9.4

6.6

4.4

2.2

2.0

Key:

1 Lambda HindIII marker

2-8 partial digests of DNA ($2\mu\text{g}/\text{lane}$)

Samples were electrophoresed on a 0.3% low melting point agarose gel.

Figure 5.11 : Preparative partial digest of mussel DNA with Sau3A

Key:

1 Lambda HindIII marker

2 Undigested mussel DNA ($1\mu\text{g}$)

3 Sau3A partial digest of mussel DNA ($1\mu\text{g}$)

Samples electrophoresed on a 0.3% low melting point agarose gel.

Figure 5.12 : Gel to assess efficacy of phosphatase and ligation reactions on mussel and bacteriophage control DNA

Key:

- 1 Lambda HindIII marker, unligated
- 2 " " " , phosphatase treated & ligated
- 3 " " " , ligated
- 4 BamH1 digested mussel DNA, unligated
- 5 " " " " , phosphatase treated & ligated
- 6 " " " " , ligated
- 7 Sau3A digested mussel DNA, unligated
- 8 " " " " , phosphatase treated & ligated
- 9 " " " " , ligated

Samples were electrophoresed on a 0.5% agarose gel.

worked. The BamH1 digested mussel DNA also showed the expected results for the respective enzyme treatments. The Sau3A digested mussel DNA however was too faint to be able to ascertain whether the enzymes had worked on these DNA samples. In a repeat experiment using more DNA, no difference could be seen between ligated and unligated DNA samples. The results indicated that this Sau3A digested DNA preparation could not be ligated for some reason, and hence a new partial digest would be required for cloning purposes. It was decided to concentrate on finishing the technically easier cloning of the BamH1 digested mussel DNA.

5.3.2 Cloning of mussel genomic DNA fragments into Bacteriophage lambda EMBL3a

The bacteriophage lambda EMBL3a was supplied with control insert (for checking ligation) and wild type bacteriophage lambda (for checking the packaging reaction). In order to test the packaging reagents (Amersham-which had been stored at -70°C for some time prior to use), the control insert (denoted PME) of 13kb as provided by Stratagene was taken through a dummy ligation run prior to using the mussel DNA fragments. This was performed according to the protocol given in the Stratagene data sheet. Two packaging mixes were then used; one for the PME-ligated lambda EMBL3 DNA and one for the control wild type lambda DNA. The reaction was performed as per the Amersham packaging protocol.

Titration of the recombinant phage was performed using the P2392 recombinant selective host cells. Results obtained are given in Table 5.2. The number of plaques present on the 10^{-2} dilutions was too great to allow the growth of the bacteria, hence no plaques were visible, only bacterial

debris. The results from the lower dilution give packaging efficiencies of 3×10^6 pfu/ μ g and 1.7×10^7 pfu/ μ g for the PME and wild type DNA respectively, assuming the ligation reaction for the PME was 100% complete.

Whilst these are quite respectable figures, they fall some way short of the quoted manufacturers batch analysis of 1.6×10^9 ! No plaques were visible at the lowest dilution as would be expected from a hundred-fold dilution of the samples giving^{ca} the preceding figures.

The success of the cloning was further demonstrated using phage minipreps as detailed in sections 2.15.4 and 2.15.5. The digestion of minipreps by BamH1 demonstrated that the inserted DNA was in fact the control PME DNA which had been ligated into the phage arms.

Cloning of the BamH1 digested mussel DNA was then undertaken using two concentrations of DNA with the remaining EMBL3 phage arms. After packaging by the standard method, titration of the recombinant phage was performed, with results as shown in Table 5.3.

From the data, the titres obtained for the two ligation reactions were 6.8×10^4 pfu/ μ g and 1.0×10^4 pfu/ μ g respectively. This titre is relatively low for a complete genomic library. However, these titres represents a combined total number of 1×10^5 viable clones. Using the relationship:

$$N = \frac{\ln(1-P)}{\ln(1-f)}$$

(where f=fraction of genome/clone and P=probability that a given sequence is represented) a theoretical probability of a given sequence occurring in the library can be obtained.

Table 5.2

Dilution	No. of plaques	
	PME	wild type
10^{-2}	-	-
10^{-4}	15	85
10^{-6}	0	0

Table 5.3

Dilution	No. of Plaques	
	Tube 1	Tube 2
	(0.6 μ g)	(0.4 μ g)
10 ⁻²	41	4
10 ⁻⁴	0	0

The deduced size of the mussel genome is of the order 3×10^9 base pairs (3.2pg DNA/cell; Hinegardner, 1974). If an average insert is taken as 16kb (representing $1/5.3 \times 10^6$ of the genome) and the value of N is 1×10^5 (i.e. the combined titre), then the probability, P, that a given sequence is represented is 40%. Thus it can be seen that at best the recombinant phage produced represent only about half of the mussel genome.

It was not possible in the time available to screen the genomic library for P-450 sequences, and this will be pursued by other workers.

Before screening is done, it would obviously be advantageous to a) amplify the library, so that more than one screen can be done of the available clones and b) await the positive identification (sequencing) of the PCR fragment. If the PCR fragment is a P450, then it would be the best DNA probe to use for detection of mussel P450 sequences present in the library.

5.4 : Discussion

Successful reverse transcription of mRNA is a necessary prerequisite to the sequence analysis of tissue specific genes. However, the data presented in this section shows that adaptation of the existing methodology for mammalian RNA to isolate mussel RNA was only partially successful. Hence it was uncertain whether subsequent synthesis and analysis of a cDNA library from the mRNA obtained would be fruitful. In actuality, a cDNA library was synthesized successfully, but the fragments isolated proved to be too small to be of use and not representative of the mRNAs expressed in the mussel digestive gland. This may have been due to the quality of the RNA isolated or some other factor, for example contaminating salts in the preparation affecting the reverse transcriptase enzyme, leading to premature termination of the first DNA strand.

The alternative approach, using genomic DNA as the starting material for the library, was more successful. However the fact that the library was generated from a total digest may prove a restriction on the subsequent studies. Although the library synthesized may not include clones encoding the whole mussel genome, it may include clones containing P450 sequences. Further studies will therefore include analysis of this library by the use of hybridisation probing.

Should further work on generation of a new library be considered, a number of newer methods could be tried, rather than continuing to use the adapted mammalian methodology used here. Methods now exist where mRNA can be directly separated from tissue homogenates, rather than following a series of

stages. In addition RNA PCR with random primers and/or oligo-dT can be used to generate libraries from single stranded cDNA following reverse transcription rather than using the standard method, which provides for amplification of the sequences of interest before insertion into vectors.

At the outset of this piece of work in 1987, understanding cytochrome P450s in marine invertebrates was confined largely to data obtained from enzymic and related studies. These data suggested that the levels of P450s were low in comparison to mammals. In addition, much of the experimental data suggested that induction response to xenobiotic insult was absent or greatly reduced in invertebrates compared to mammals. This led to speculation by some workers that cytochromes P450 in molluscs were not involved in xenobiotic metabolism to the same degree as observed in mammals. This was supported by chemico-physical and bioaccumulation data, which suggest that uptake and depuration are largely, though not exclusively, passive processes.

However, understanding of the extent to which environmental contaminants affect the mussel cytochrome P450 system and associated endogenous metabolic functions was far from complete. Indeed, no firm evidence for the existence of any single isoenzyme was available, although the data of Kirchin (1988) suggested that more than one isoenzyme was expressed in the mussel. Neither was there any firm indication of differential regulation of isozymes in response to xenobiotic exposure, though seasonal regulation was apparent and Livingstone (1987) had observed spectroscopic shifts after mussels were exposed to xenobiotics.

The potential for study was therefore fairly wide and, as discussed previously, hampered by the lack of suitable antibody probes for particular P450 proteins. The use of molecular biology techniques was therefore engaged, as

described in this thesis. The approach used concentrated on two areas; first, to screen mussel nucleic acid preparations for the presence of sequences with regions homologous to mammalian and fish cytochromes P450, and second, to obtain clones of sequences of mussel cytochrome P450s to compare sequence and regulatory function with mammalian and fish equivalents.

Initial results described indicate that a mammalian cDNA probe, rat liver P450IVA1, does indeed recognise sequences in RNA isolated from mussel digestive gland. These data are in agreement with the existing P450 protein data, derived from several studies, which indicates that cytochrome P450 is present in this tissue at the level of 3-134 pmol/mg protein (Livingstone, 1991). In addition sequence similarity (and therefore hybridisation), was observed between the P450IVA1 probe used and RNA isolated from a range of other marine animals. This is good evidence that a cytochrome P450, possibly with regions of strong homology to the P450IVA1 isozyme, is present and is expressed in a range of diverse organisms. In some cases, this is supportive of previous work, as detailed in ~~the~~ chapter 1. In others cases, for example sponges and coelenterates, this is the first direct evidence that a cytochrome P450-dependent mixed function oxidase system may be present, although previously reported data has described enzyme activity which in higher animals is known to be cytochrome P450-dependent e.g. aldrin epoxidation (Khan et al., 1972).

In addition, the strength of the signal varies somewhat between different species, which may reflect different levels of expression. However, relative intensity of a signal in these studies is not necessarily a good indicator of the

extent of homology to the cDNA probe in question for a number of reasons: A number of workers have observed difficulties in quantifying RNA isolated from various tissues and species (Houdebain et al., 1990); and tissues from marine invertebrates have been observed to have certain idiosyncrasies (James, 1989) which further increases the uncertainty with regard to the true amount of RNA in spectrophotometrically determined samples. In the case of DNA samples, this problem could be overcome by use of an alternative quantitation method, for example that based on the fluorimetric detection using the Hoescht dye procedure, but this approach is not possible with RNA samples. Also, probable differences in the extent of sequence similarity between species further complicates interpretation of the data. These problems could largely be resolved by further method development and the use of probes specific to the organism and gene of interest.

Further method development is also required in order to obtain a solution to the problem of the degradation of the 26S RNA band in the extraction media required for intact mRNA isolation and to overcome the problems of isolating large molecular weight DNA from digestive gland tissue. These problems themselves, however, do not obstruct the analysis of marine invertebrate gene expression to the same extent as does the quantitation problem.

Despite the methodological problems encountered, it was possible to detect differences in the hybridisation between the RNA/DNA samples and the different cytochrome P450 cDNA probes used. The degree of binding was largely as would be predicted from the cytochrome P450 "family tree" of Gonzalez & Nebert (1990). In particular, probes from the large P450II

family of isozymes, which appear to have diversified rapidly in the time between the divergence of mammals and molluscs, do not recognise sequences in the mussel or any of the other invertebrates examined. The P450IVA1 probe by comparison, which is predicted to be of comparatively ancient origin, produces a signal in RNA from the whole spectrum of animals examined, albeit a weak one in some cases. This is not, however, definitive evidence for the presence of a P450IVA1 ^{ortho}homologue in mussels and other marine invertebrates. Rather, it suggests that one or more of the mRNA sequences for the P450 isozymes present show a greater sequence similarity with one or more regions of the P450IVA1 used. However the presence of a true P450IVA1 ^{ortho}homologue is still a possibility given ^{the}its apparent ^{of this isozyme}involvement in fatty acid metabolism in mammals. Experimental data obtained from metabolism studies (Kirchin, 1988) indicates the presence of lauric acid hydroxylase activity, which may be due to the activity of a mussel P450IVA1 ^{ortho}homologue. Most recently, Nebert et al. (1991) mention a paper currently in preparation (Lee et al.), which describes the isolation of a new P450IV family gene from a terrestrial invertebrate, the cockroach.

In addition, a strong signal was also observed for the P450IA1 probe used. ^{In one respect} This ~~also~~ is not unexpected, given that the root of the P450I family is predicted (from vertebrate data) to be prior to the vertebrate/mollusc divergence. ^{because lack of ERD!} Once again however, comparison of relative signal strengths between species is complicated by the problems described for P450IVA1. A further complication in this case is the fact that the probe is derived from trout. This would suggest that the probe cDNA sequence should be less divergent from an equivalent molluscan gene than the corresponding mammalian P450IA1, and might

therefore be expected to give a stronger signal (though a suitable probe was not available to test this hypothesis). It could be speculated that since the deduced protein sequence similarity between fish P450IA1 and rodent P450IA1/2 is only 51-59%, the fish probe may be more similar to an invertebrate P450I mRNA. It would then show as strong or stronger hybridisation to an invertebrate RNA sample than to the rat +ve control. This was difficult to assess however given the problems already described with quantitation of RNA and, in the case of DNA slot blots, information about genome size (& therefore numbers of gene copies per microgram of DNA). When comparing sequence data however, it is important to consider the parallel enzymology data. It is striking that the hybridisation to IA1 is strong when the IA1 protein studies done in vertebrates (including fish) all show concomitant EROD activity, a phenomenon absent in mussels. This is a case which outlines the pitfalls associated with using P450-dependent activities as indicators of the presence of specific sequences and vice versa.

Of the probes used however, it was these two (IA1 & IVA1) which consistently showed hybridisation above the background signal throughout in mussel nucleic acid samples. One which, surprisingly, did not was the P450XIA1 probe. Of the probes used, the P450XIA1 and P450IVA1 are predicted to be the most ancient in origin. The lack of signal to P450XIA1 in the case of RNA samples may in part be ascribed to the appropriate tissue not being probed or by being overshadowed by RNA from other larger tissues. However, in the case of the DNA slot blots, the only conceivable reason is too low a probe activity.

However, bearing in mind that Nebert et al. (1991) also refer to a P450X family gene being isolated from the pond

snail by Y. Teunissen, it would seem likely that a similar gene would be present in other molluscs. Hence further work could be done using this probe.

In comparing tissue expression levels for these isozymes in mussel, it is notable that low (if any) expression was detected in muscular tissue, which is comparable to the situation in mammals. There is some signal in the other tissues digestive gland, mantle and gill, but due to the quality of these blots, accurate quantitation from the autoradiogram is not possible. What the signals represent is therefore difficult to elucidate. Since the signals are approximately the same in all three tissues, maybe both the isozymes examined are expressed at similar levels in all three tissues. This is possible, but other data which indicates differences in P450-dependent enzyme activity between these tissues (Livingstone & Farrar, 1984) should be borne in mind. Alternatively, the probes may both be detecting the same isozyme in a non-specific manner. This is unlikely since subsequently they show differences in hybridisation to DNA from different animals, demonstrating selective binding albeit under different stringency conditions.

Thus the evidence presented suggests that P450 isozymes related to P450IA1 and P450IVA1 are those most probably expressed in mussels, particularly since primers to the P450IA1 gene produce a product following PCR amplification of mussel DNA. The data suggests that P450II family enzymes are probably not being expressed. This is in apparent conflict with the data of Galli et al. (1988), which describes the induction of P450 by phenobarbital in the Mediterranean mussel, M. galloprovincialis. In mammals, this is characteristic of an increase in isozymes of the P450IIB

subfamily, though some other forms e.g. P450IIIA are also induced. Hence the induction observed in mussel may be of the P450III form, a family which is known to be more strongly conserved. Unfortunately, a probe for this P450 was not available at the time of the study. From current understanding, there would be predicted to be an equivalent to P450XIA1 in the mussel. However there exists the possibility that the endogenous role of this protein in mammals could be fulfilled by another P450 or another protein in mussels, and that the corresponding P450XIA1 gene could have been lost. The presence of a closely related isozyme in another mollusc does seem to indicate against this however. Further work would be necessary to investigate this more fully.

With regard to regulation of P450s in marine invertebrates, the studies described here verify what previous workers have found, namely that the cytochrome P450 system is largely refractive to the classical inducers of mammalian P450s. Certainly the ten and hundred fold magnitude of induction observed in higher animals are not reproduced in any of the experimental conditions investigated. However only limited probes were available for these studies and use of further probes would be useful, e.g. those obtained from closely related species such as the pond snail P450X and also from other invertebrates such as the P450VI of the housefly and P450IV of the cockroach. It would be unlikely however, that the cockroach probe would pick up anything missed by the rat P450IV probe, bearing in mind the likely sequence similarity and the stringency of washing conditions used, unless of course that the sequences were much more closely related and hence the stringency of washing could be elevated. It is interesting to consider that for both the fly and pond

snail isozymes, there are no known vertebrate equivalents. This corresponds with data from protein studies which suggests P450 forms are present in invertebrates which have no exact counterparts in higher organisms (James, 1984; Livingstone, 1990). ^{Clarke 1987}
et al.

These other invertebrate sequences, once made available, could be used to design primer pairs for in vitro amplification of mussel DNA or RNA preparations, thus providing a more direct route to the identification of mussel P450 sequences. This would facilitate the positive identification of the P450s present in mussel and allow further work on the effect of xenobiotics on any or all of them. Thus further understanding of both the endogenous and exogenous regulation of the mussel P450 system would be gained.

However, to definitively identify all the P450 isozymes present in M. edulis, a gene library must be probed exhaustively. Of course, the P450s of most immediate interest are those present in the digestive gland which are responsible for the metabolism of compounds such as benzo(a)pyrene, and to this end a cDNA library would be adequate and more selective than using a genomic library. Of course, to achieve this, the problems associated with mRNA isolation must be addressed.

The genomic library successfully synthesized in the course of this work should of course be probed following amplification. This is most likely to yield positive clones if fragments of mussel P450s isolated following PCR can be used as probes as they will have 100% sequence homology with the clone and will therefore generate a strong signal.

In final summary, this work has demonstrated that P450-related sequences can be detected in mussel and other marine invertebrates. Other P450s appear not to be present. The

studies on induction yielded largely negative results, as might have been expected from previous enzymological data. Some methodological data is given on extraction procedures appropriate for mussel nucleic acid extraction. This knowledge provides a springboard from which more specific research into single isozymes can be undertaken, taking advantage of the newer methodology such as PCR and the RNAase protection assay developed during the last few years.

APPENDIX ONE : Media and Solutions

Brij/Doc (2.5.1):

1% Brij 58 (polyoxyethylene-20-cetyl ether)

0.4% sodium deoxycholate

made up sterile in 1x TE.

Burgoynes Buffer (2.8):

(10x stock)

0.6M KCl

0.15M NaCl

0.15M Tris pH7.5

15mM spermidine

50mM spermidine

Filter sterilise before use.

Chloroform (2.3.1):

24/25 chloroform

1/25 isoamyl alcohol

made up fresh on each occasion.

Cushion buffer (2.8):

1x Burgoynes buffer

0.1mM EDTA

0.1mM EGTA

0.5M sucrose

Filter sterilise before use.

DNA running dye (2.3.4):

0.25% bromophenol blue

40% w/v sucrose

made up in 2mM EDTA.

E buffer (2.12.4):

(8x concentrate)

8g sodium dodecyl sulphate

115.2g glycine

24.2g Trizma 7-9 buffer (Sigma)

make up to 1 litre

Ethidium bromide (2.3.4):

0.5mg/ml stock solution diluted 1:100 for use in a staining bath.

Guanidinium isothiocyanate (2.7):

5M guanidinium isothiocyanate

50mM Tris (pH 8.0)

10mM EDTA

Homogenising buffer (2.8):

1x Burgoynes buffer

2mM EDTA

0.5mM EGTA

0.34M sucrose

Filter sterilise before use.

Isolation buffer (2.8):

0.15M NaCl

10mM EDTA

10mM Tris pH 7.5

LB-Mg (2.2.3):

10g Tryptone

5g yeast extract

10g NaCl

2g MgSO₄·7H₂O

PB buffer (2.7):

(10x stock)

250mM Tris

250mM NaCl

50mM MgCl₂

pH adjusted to 7.5 prior to autoclaving.

Phenol (2.3.1):

Redistilled Analar grade phenol melted at 65°C. An equal volume of 1xTE (pH 8.0) was added, together with 0.1% 8-hydroxyquinoline, and shaken. After separation of the two phases, the aqueous layer was removed and replaced with fresh 1xTE and shaken. Reextraction with TE in this manner was continued until the pH of the removed TE was 7.5 or greater. The TE saturated phenol was then stored at 4°C.

RNA gel buffer (2.3.4):

(5x concentrate)

0.2M MOPS (morpholinopropanesulphonic acid) pH 7.0

50mM sodium acetate

0.5mM EDTA (pH8.0)

RNA running dye (2.3.4):

0.25% bromophenol blue

0.25% xylene cyanol

40%w/v sucrose

made up in 2mM EDTA.

SM buffer (2.13.2):

(per litre)

5.8g NaCl

2g MgSO₄·7H₂O

6.05g Tris base

5ml of 2%w/v gelatin

adjust pH to 7.5 before autoclaving

SSC (2.10.1):

(20x concentrate)

3M NaCl

0.3M sodium citrate

adjust pH to 7.0 before autoclaving

TAE (2.3.4):

(10x concentrate)

0.4M Tris-acetate

10mM EDTA

TBE (2.17.1):

(5x concentrate - per litre)

54g Tris base

27.5g boric acid

20ml 0.5M EDTA

TE (2.3.1):

10mM Tris

1mM EDTA

adjust to appropriate pH before autoclaving.

TNE (2.9.1):

10mM Tris

1mM EDTA

100mM NaCl

adjust to pH 8.0 before autoclaving.

TYN (2.2.3):

(per litre)

10g Tryptone

10g Yeast extract

10g NaCl

Detection of mRNA Sequences Homologous to the Human Glutathione Peroxidase and Rat Cytochrome P-450IVA1 Genes in *Mytilus edulis*

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ABSTRACT

In this study we have used cloned gene probes for human glutathione peroxidase (GPX), rat cytochrome P-450IVA1 and rat cytochrome P-450IIE1 to detect homologous sequences in RNA from the hepato-pancreas of Mytilus edulis. The presence of sequences hybridising to the GPX and P-450IVA1 probes, but not to the P-450IIE1 probe, confirms the ancient origin of the former genes and indicates that conserved-sequence DNA probes from higher organisms can be used to examine the structure and function of genes of environmental interest in marine organisms.

The advent of genetic engineering techniques in the mid-1970s resulted in a quantum leap in our understanding of gene structure and function. It also resulted in the application of molecular biology to a wide range of disciplines including pharmacology, toxicology, genetics, developmental biology and neurobiology. However, its application to marine biology has, with a few notable exceptions, been slow. This has been due in part to the difficulty of using the traditional route of isolating genes, namely the initial purification of the enzyme or structural protein of interest. In this communication our attempts to circumvent this problem by using mammalian gene probes to detect homologous RNA sequences in the common mussel *Mytilus edulis* are described.

Genetic engineering techniques can be used both to isolate genes (or gene probes), and (via DNA sequencing) to elucidate the structure and function of

proteins.¹ The use of gene fragments as DNA probes lies in the ability of pieces of single-stranded DNA to bind (hybridise) to homologous sequences in target DNA or RNA by Watson-Crick base pairing. By use of the appropriate conditions the interaction can be made sequence-specific with no mismatches (high stringency) or can be made to tolerate up to about 40% mismatching (low stringency). Usually the gene probe is radioactively labelled with a high-energy β -emitter such as ^{32}P , and the target DNA or RNA is immobilised on a nitrocellulose or nylon filter. After hybridisation and washing at the required level of stringency the interaction between probe and target is detected by autoradiography.

Our studies on selenium-dependent glutathione peroxidase (GPX) have resulted in the isolation of the genes for the human and mouse² forms of the gene and the cDNA for a related polypeptide GPRP (D. Dunn & P. Goldfarb, unpublished results). DNA sequence analysis has shown that in all cases the seleno-cysteine residue in the active site is encoded by the 'stop' codon UGA.² Comparison of the deduced amino acid sequence of GPX with the known sequence of the bovine and rat enzymes has indicated that the sequence of GPX has been highly conserved during evolution and that GPX may be a polypeptide of ancient origin.³ It was thus of interest to determine whether a GPX DNA probe could detect related sequences in the mRNA of *Mytilus edulis*. As GPX is expressed at high levels in the liver of mammals,² RNA from the hepato-pancreas of *Mytilus* was examined. In Fig. 1a the result of an experiment in which a human GPX probe was hybridised to mussel RNA is shown. As can be seen, homologous sequences were detected confirming that GPX-related sequences are of ancient origin. It is not yet known if the detected mRNA sequences code for mussel GPX or for a related seleno-peptide such as GPRP; Isolation of the gene sequences from a library of mussel genomic DNA or cDNA will give further information on this point.

We have also isolated gene probes corresponding to two members of the cytochrome P-450 superfamily of isoenzymes, namely P-450IVA1 (P-450_{LA}, P-452), and P-450RLM6 (probably the same as the ethanol-inducible form P-450IIE1, P-450_j). P-450IVA1 mRNA synthesis is induced by hypolipidaemic drugs such as clofibrate⁴ and by chlorphenoxyacetic acid herbicides (M. Bacher & G. G. Gibson, unpublished). We have also shown that P-450RLM6 mRNA is increased in diabetic rats and returns to basal levels after treatment with insulin (T. Richardson, P. Goldfarb & G. G. Gibson and J. Schenkman, unpublished results). It has been proposed that certain members of the cytochrome P-450 gene family are of ancient origin whereas others are of more recent evolution.^{5,6} This would suggest that cytochrome P-450 gene probes might be used to detect sequences in more primitive organisms such as the mussel.

Fig. 1. Hybridisation of radioactive gene probes to mussel and rat total cytoplasmic RNA. RNA was spotted on to nitrocellulose filters (a, b, c, e) or electrophoresed in agarose and blotted to nitrocellulose (d). Filters were hybridised, washed and autoradiographed to detect probe binding. (a) Human GPX genomic DNA probe; (b-d) rat cytochrome P-450IVA1 cDNA probe; (e) rat cytochrome P-450RLM6 (P-450IIE1) cDNA probe. Low stringency washes: $2 \times$ SSC, 48°C , 2×45 min; high stringency washes: $0.5 \times$ SSC, 55°C , 2×45 min.

As shown in Fig. 1b sequences homologous to a rat P-450IVA1 cDNA probe can be found in mussel RNA under conditions of low stringency. Hybridisation is still present even at high stringency, as shown in Fig. 1c, suggesting that the mussel mRNA sequences are relatively homologous to the rat sequences. However, Northern blot analysis of the size of the mussel mRNA (Fig. 1d) suggests that it is smaller than rat P-450IVA1 mRNA and thus, conclusions as to the identity of these sequences must await their isolation.

The results shown in Fig. 1e show that sequences homologous to a rat P-450RLM6 (P-450IIE1) DNA probe cannot be detected in mussel RNA even at low stringency. This is in agreement with the proposal that the P-450IIE1 gene is of relatively recent origin,^{5,6} and homologous sequences may not be present in more primitive organisms such as *Mytilus*.

The preliminary results reported here suggest that it may well be possible to isolate and study genes for enzymes of interest from marine organisms by using mammalian gene probes. This may particularly be the case where the enzyme of interest is crucial to life, or performs a major protective/xenobiotic-metabolising function, and may thus be of ancient origin. Alternatively, if the enzyme (or structural protein) is of more recent origin but contains functional domains which have been conserved in evolution, then subcloned mammalian DNA probes corresponding to such regions could be used. It may thus be possible to introduce the technique of molecular biology and genetic engineering to marine biology more rapidly than previously anticipated using mammalian gene probes as the starting point.

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COMMUNICATIONS

Cytochrome *P*-450 gene expression in the common mussel *Mytilus edulis*

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The cytochrome *P*-450-dependent mono-oxygenase system catalyses the oxidation of many structurally diverse compounds. In vertebrate systems, different species are known to express a number of different isoenzymes, each showing different substrate specificity. It has been observed that different isoenzymes may be induced in response to exposure to xenobiotics and drugs. It is hoped that this phenomenon, if exhibited in the molluscan *P*-450 system, will allow the mussel to be used as a biomonitor of environmental pollution in the marine environment.

P-450 enzyme activity in the mussel is primarily located in the digestive gland (hepatopancreas), with trace amounts

found in mantle (storage and reproductive) and gill tissues. Levels of enzyme activity and specific content are, however, approximately 10-fold lower than in mammalian tissue [1].

Purification of *P*-450 from mussel digestive gland tissue has been attempted, and partial purification has been achieved [2]. However, problems attributed to low *P*-450-specific content and to amending techniques used for purification of the mammalian enzyme have made the traditional route to gene isolation (i.e. via protein purification) difficult [3].

In this study, we demonstrate the feasibility of an alternative route to gene isolation, using mammalian gene probes to detect homologous DNA and RNA sequences in the mussel.

Mussels were collected from Whitsand Bay (Cornwall, U.K.) and the digestive gland removed and immediately frozen in liquid nitrogen. After storage at -70°C , RNA was purified according to the method of Cathala *et al.* [4] and DNA was purified according to Maniatis *et al.* [5]. Northern blotting was undertaken using the methods given in Maniatis

Fig. 1. Hybridization of ^{32}P -labelled rat *P*-450 cDNA probes to mussel and rat RNAs
(a) *P*-450 RLM6; (b) *P*-450 IVA1; (c) *P*-450 IVA1.

et al. [5] on to nylon (Hybond-N, Amersham). Dot blots using a BRL apparatus were on to nitrocellulose (BRL) for DNA and nylon (Amersham) for RNA.

Gene probes isolated from rat cDNA libraries corresponding to two members of cytochrome *P*-450 superfamily, namely a 1.2 kb fragment of *P*-450 IVA1 (*P*-450 LAw, *P*-52) and a 1.1 kb fragment of *P*-450 RLM6 (probably the same as the alcohol inducible *P*-450 IIE1/*P*-450j; T. H. Richardson, personal communication) were used to detect homologous sequences.

The results shown in Fig. 1(a) demonstrate that sequences homologous to rat *P*-450 RLM6 cannot be detected in mussel, even under low stringency washing conditions (42°C, 1 × SSC [5]). In contrast, the *P*-450 IVA1 probe does bind to mussel RNA (Fig. 1b), even under more stringent washing conditions (55°C, 0.5 × SSC). These observations are in agreement with the proposal that the *P*-450 II family is of comparatively recent origin and might not be present in lower organisms, whereas the *P*-450 IV family is of more ancient origin [6, 7]. It might be expected therefore that sequences homologous to *P*-450 IVA1 would be expressed in lower organisms and indeed preliminary work in this laboratory has detected homology to RNA from a range of marine invertebrates.

We have also detected sequences homologous to the *P*-450 IVA1 probe in mussel genomic DNA; however, much less stringent washing conditions were required (37°C, 1 × SSC) owing to the presence of intron sequences.

In a Northern blot comparing rat and mussel RNA (Fig. 1c), hybridization of the *P*-450 IVA1 probe is again observed. The size of the mRNA would appear to be smaller than the major 2.1 kb rat *P*-450 IVA1 band. Conclusions about the identity of this mRNA and corresponding protein function must therefore await the isolation of the cDNA.

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Metabolic activation of 7,12-dimethylbenz(a)anthracene: role of cytochrome *P*-450 isoenzymes in the formation of DNA and protein adducts *in vitro*

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The carcinogenic properties of 7,12-dimethylbenz(a)anthracene (DMBA) are dependent on metabolic conversion to the bay region diol-epoxide, DMBA-3,4-diol-1,2-oxide (DMBA-DO) and this ultimate carcinogen is formed by sequential reactions which are catalysed by cytochrome(s) *P*-450 *P*-450 and epoxide hydrolase (EH) [1]:

Formation of DMBA oxides at other ring positions produces less potent procarcinogens.

We have identified some *P*-450s which are important in the formation of the proximate carcinogen (DMBA-3,4-diol) and in the formation of diols which are without adverse effect (DMBA-5,6- and -8,9-diols). A major determinant in the formation of DMBA-3,4-diol is *P*-450-PB1 (*P*-450IIC) [2], while *P*-450-MC1b (*P*-450IA1) produces only small amounts of this metabolite, but is highly active in the formation of diols at the -5,6- and -8,9-positions [2, 3]. Indirect evidence from studies of the formation of DMBA-membrane protein adducts *in vitro* suggest that *P*-450-PB3a (*P*-

450IIB1) may play some role in the second *P*-450-mediated oxidation during ultimate carcinogen formation [2].

The current study was initiated to identify other *P*-450s which may be important in the formation of DMBA metabolites and adducts and has utilized microsomal membranes from animals treated with selective inducers of *P*-450-isoenzymes. A protocol has been developed for the measurement of DMBA-protein and DMBA-DNA adducts in the same reaction mixtures. In parallel with these studies, rates of formation of DMBA diols have also been determined. Adduct formation was determined in reaction mixtures containing membranes (1 mg of protein/ml), NADPH, [³H]DMBA and exogenous calf-thymus DNA. After a 1 h incubation, reaction was stopped by cooling and the membranes were recovered by centrifugation at 100 000 *g*_{max} for 1 h. DNA was recovered from the supernatant by precipitation with ethanol and the resulting pellet was washed with 70% (v/v) ethanol before determination of radioactivity and DNA content. The membrane pellet was extracted with chloroform/methanol to remove unreacted DMBA, DMBA metabolites and DMBA-lipid adducts. The solvent-extracted proteins were hydrolysed with sodium hydroxide before determination of radioactivity and protein content. Rates of adduct formation are expressed as pmol of DMBA min⁻¹ mg⁻¹ protein or DNA.

Microsomal membranes were prepared from the livers of untreated Long-Evans rats and animals pretreated with phenobarbital, Sudan III, dexamethasone, pyrazole or clofibrate. The effect of these inducing agents on the rates of formation of DMBA-diols is shown in Fig. 1(a). Pretreatment of animals with phenobarbital and Sudan III produces distinctive changes in the regio-selective metabolism of DMBA which we have previously reported [2, 3]. The formation of

Abbreviations used: *P*-450, cytochrome *P*-450; DMBA, 7,12-dimethylbenz(a)anthracene; DO, 3,4-diol-1,2-oxide.

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My son, there is something else to watch out for. There is no end to the writing of books, and too much study will wear you out.

(Ecclesiastes 12.12, Good News Bible)

There are more things in heaven and earth, Horatio,
Than are dreamt of in your philosophy.

(Hamlet I.5)

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