The Science of the Total Environment, 39 (1984) 209—235
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

TISSUE AND SUBCELLULAR DISTRIBUTION OF ENZYME ACTIVITIES OF MIXED-FUNCTION OXYGENASE AND BENZO [*a*] PYRENE METABOLISM IN THE COMMON MUSSEL *MYTILUS EDULIS* L.*

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(Received 10th March, 1984; accepted 4th April, 1984)

ABSTRACT

A survey of the tissue and subcellular distribution of some enzyme activities of mixed-function oxygenase (MFO) and benzo[*a*]pyrene (BP) metabolism in *Mytilus edulis* has been carried out. Subcellular fractions were characterized by marker enzymes (pyruvate kinase, β-*N*-acetylglucoseaminidase, succinic dehydrogenase and glucose-6-phosphatase) and electron microscopy. The tissues examined in male and female mussels were the digestive gland, gills, mantle, posterior adductor muscle, foot, 'rest' fraction and blood cells. Evidence was obtained that in the female digestive gland in particular a significant fraction of the microsomes co-sedimented with the mitochondria at 12 000 × *g* and some solubilization of the endoplasmic reticulum also occurred during homogenization and extraction. MFO activities and cytochromes were localized in the microsomes. Cytochrome P-450 was present only in the digestive gland and the P-450-associated activities were highest in this tissue. In contrast cytochrome b_5 was also present in the gills and mantle. The activities were higher in the digestive gland microsomes of female mussels than of males and were in females (means ± SEM, $n = 4$): 7.5 ± 0.7 (glucose-6-phosphate dehydrogenase), 930.2 ± 54.6 (NADH-ferricyanide reductase), 156.5 ± 9.5 (NADH-cytochrome *c* reductase) and 18.4 ± 4.6 (NADPH-cytochrome *c* reductase) (all in nmol min^{-1} mg^{-1} protein at 25°C); 31.0 ± 3.9 (BP hydroxylase, pmol min^{-1} mg^{-1} protein at 25°C); 75.7 ± 11.7 (b_5) and 134 ± 64 (P-450) (in pmol mg^{-1} protein and both the pooled material of both sexes, $n = 4$). Digestive gland microsomal BP hydroxylase activity was partially inhibited by carbon monoxide, SKF-525A and α-naphthoflavone. Based on this evidence and an observed relationship between microsomal BP hydroxylase and NADPH-cytochrome *c* reductase activities, it is concluded that a cytochrome P-450 mediated MFO system is present in mussels.

INTRODUCTION

Despite early reports to the contrary, benzo[*a*]pyrene (BP) hydroxylase activities have recently been detected in bivalve molluscs although the levels

*An abstract of this work has been published in the Proceedings of the 2nd International Symposium on the Responses of Marine Organisms to Pollutants, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, 27—29th April, 1983 (Mar. Environ. Res., in press).

0048-9697/84/$03.00

were considerably lower than in vertebrates or in invertebrates such as crustaceans (see Neff, 1979; Lee, 1981; Stegeman, 1981a). In the most recent study, on the bivalve *Mytilus galloprovincialis*, significantly higher BP hydroxylase specific activities have been recorded (Ade et al., 1982). The implied existence of a mixed-function oxygenase system in bivalves is of interest both from the comparative viewpoint, and in particular in the context of the monitoring of biological effects where the possibility exists that changes in the enzyme activities or components of the system, in response to organic pollutants, could be used as a specific indicator of biological impact of this group of chemicals (Bayne et al., 1979; Lee et al., 1980). Such an index of stress would have immediate application in programmes of environmental impact assessment (Cairns and Van der Schalie, 1980). In the absence of any detailed information on the tissue and sub-cellular distribution of these enzyme activities in bivalves a baseline study has been carried out on the common mussel *Mytilus edulis*. Emphasis in the study has been placed on a characterization of the subcellular fractions and on an examination of the reliability and sensitivity of the assays used; low enzyme activities being anticipated.

Subcellular fractions were characterized in terms of electron microscopy and marker enzymes (pyruvate kinase (PK) (E.C.2.7.1.40), β-*N*-acetylglucose-aminidase (NAGASE) (E.C.3.2.1.30), succinic dehydrogenase (SDH) (E.C.1.3.99.1) and glucose-6-phosphatase (G6PASE) (E.C.3.1.3.9)); a ratio of enzyme activity at pH 5.5 to 6.5 was used to distinguish between microsomal G6PASE and other non-specific phosphatases (see Results). Preliminary studies were carried out on the digestive gland (hepatopancreas) and the methodology then applied to the other tissues in the tissue survey. In the survey, the following enzyme activity or concentration measurements were made in the mitochondrial, microsomal and cytosolic fractions of the major tissues (digestive gland, mantle, gills, posterior adductor muscle, foot and 'rest') and also in whole blood cells of male and female mussels, viz. glucose-6-phosphate dehydrogenase (G6PDH) (E.C.1.1.1.49), NADH and NADPH-dependent cytochrome *c* reductase (NAD(P)H-CYTCRED), NADH and NADPH-dependent ferricyanide reductase (NAD(P)H-FERRIRED), cytochromes b_5 and P-450 and BP hydroxylase. In the case of the digestive gland microsomes, a more detailed study was carried out including inhibition (by SKF-525A, α-naphthoflavone and carbon monoxide) studies of the BP hydroxylase activity in order to investigate whether the reaction was cytochrome P-450-mediated or not. The results are discussed in relation to the other information available for bivalves and to fundamental aspects of mixed-function oxygenase systems in vertebrates and invertebrates.

MATERIALS AND METHODS

Chemicals

Dithiothreitol (DTT), phenylmethylsulphonyl fluoride (PMSF), α-1-antitrypsin, phosphoenolpyruvate (trisodium salt), adenosine 5′-diphosphate

(sodium salt), β-nicotinamide adenine dinucleotide, reduced form, rabbit muscle L-lactic dehydrogenase, *p*-iodonitrotetraxolium violet (INT), grade I glutaraldehyde, D-glucose-6-phosphate (disodium salt), *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, *p*-nitrophenol, β-nicotinamide adenine dinucleotide phosphate, oxidised form and reduced form (NADPH), horse heart cytochrome *c* and α-naphthoflavone (7,8-benzoflavone) were obtained from the Sigma Chemical Co. Ltd. (London); benzo[*a*]pyrene from the Aldrich Chemical Co. Ltd. (U.K.) and [G-^{3}H]-benzo[*a*]pyrene (24 Ci mmol^{-1}) from Amersham International PLC (Amersham, U.K.). Osmium tetroxide, Taab resin and uranyl acetate were obtained from Taab Laboratories (Reading, England) and SKF-525A (2-diethylaminoethyl-2,2-diphenylvalerate) was a kind gift from S. O'Hara, Marine Biological Association, Plymouth, England. All other chemicals were obtained from BDH Ltd. (Poole, U.K.).

Mussels and sample preparation

Periodically mussels (4.2—5 cm in length) were collected at low tide from Whitsand Bay, Cornwall, near Plymouth and kept in a system of ambient recirculating seawater without food for 2—3 days to clear gut contents. Animals for the tissue survey and the blood analysis were brought in on, respectively, the 12th November and 31st December 1982. The sex of the animals was determined by examining a smear of the mantle tissue under a light microscope for the presence of sperm or ooctyes. In the preliminary and other studies, the digestive gland was dissected out, damp-dried, the crystalline style removed and the tissue either weighed and used immediately or frozen in liquid-nitrogen and stored at −70°C before use. In the tissue survey, the gills, mantle, posterior adductor muscle, foot and the rest fraction were also dissected out and all the tissues were frozen in liquid-nitrogen and stored at −70°C. The dissections were all performed on the same day; the tissues from twelve mussels were pooled to give each sample (in the case of the foot extra mussels were used to give sufficient material for analysis) and four samples were taken for each sex (in the case of the foot and adductor muscle, only two samples were taken per sex). Blood was obtained from the posterior adductor muscle sinus by hypodermic syringe, the material from 4—6 mussels was pooled to give each sample and four samples were taken per sex; the material was kept on ice until sex-determination was complete and then used immediately.

The homogenization procedure routinely used for the preparation of digestive gland microsomes was as follows. Fresh or frozen tissue was weighed and all subsequent procedures were carried out at 4°C. The tissue was homogenized in a 1:4 w/v ratio (tissue weight: buffer volume) in 20 m*M* Tris—HCl pH 7.6 containing 0.5 *M* sucrose, 0.15 *M* KCl, 1 m*M* EDTA (ethylenediaminetetraacetic acid), 1 m*M* DTT and 100 μ*M* of the protease inhibitor PMSF (added to the buffer from a stock solution in isopropanol just before homogenization to prevent its aqueous inactivation; James, 1978) using a Potter—Elvehjem teflon homogenizer; the homogenizer was

electrically-driven and sufficient passes were made to remove any visible particulate tissue material. The homogenate was centrifuged in an M.S.E. Hi-spin 21 centrifuge at 500 x *g* for 1 h and the resulting supernatant at 12 000 x *g* for 45 min. The 12 000 x *g* pellet was resuspended in a reduced volume of homogenization buffer and recentrifuged at 12 000 x *g* for 45 min. The combined 12 000 x *g* supernatants were then centrifuged in an M.S.E. Prepspin 50 ultracentrifuge at 100 000 x *g* for 90 min and the resulting microsomal pellet resuspended in 20 m*M* Tris—HCl pH 7.6 containing 20% w/v glycerol, 1 m*M* EDTA and 1 m*M* DTT to give a protein concentration of about 4—8 mg per ml. The resuspended microsomes were either used immediately or stored at − 20 or − 70°C.

In the tissue survey the samples were processed in random batches of six. The homogenization was as described for the preparation of digestive gland microsomes except that α-1-antitrypsin was included in the homogenization buffer (final concentration of 0.1 mg per ml buffer) and the homogenization w:v ratio varied for the different tissues, viz. 1:3 (digestive gland, gills, mantle), 1:2 (rest), 1:6 (foot) and 1:5 (posterior adductor muscle). The tissues were homogenized while frozen and the 500—12 000 x *g* pellet after the second 12 000 x *g* spin (= washed 'mitochondrial' fraction) was retained and resuspended in the same buffer volume as for the microsomal fraction. The 100 000 x *g* supernatant was also retained (= cytosolic fraction) and after the preparation procedure was completed, the mitochondrial, microsomal and cytosolic fractions were subdivided into a number of aliquots which were either used immediately or frozen overnight at − 20°C before analysis. The freshly prepared aliquots were used for the determination of cytochromes b_5 and P-450 and most of the BP hydroxylase assays, and the frozen aliquots for the measurement of the activities of the marker enzymes, G6PDH and the reductases and the concentration of protein. An exception was cytosolic PK and G6PDH which were determined on cytosolic fractions kept overnight at 4°C.

In the blood analysis study all procedures were similarly carried out at about 4°C. The pooled blood samples were centrifuged at 1000 x *g* for 10 min and the resulting pellet of blood cells resuspended in the same volume of filtered sterilised seawater. After a second spin, the blood cells were suspended in a reduced volume of the microsomal buffer (see before), to give a protein concentration of about 1 mg per ml, and dispersed by minimum sonication. The freshly prepared samples were used immediately for all the assays except protein determination which was carried out later on frozen material.

Measurement of enzyme activities and concentrations and protein concentration

All enzyme assays were carried out at 25°C and all preliminary studies referred to were on digestive gland microsomes. Spectrophotometric and colorimetric measurements were carried out on a Pye—Unicam SP8-200 double-beam scanning spectrophotometer. In the tissue survey all enzyme

reaction rates were linear with the exception of NADPH-FERRIRED activity and some of the cytosolic reductase activities which showed curvilinearity. In the digestive gland microsomes, linearity was observed between sample size and activity over a five fold volume range of the former, for all enzyme activities except NADPH-FERRIRED.

PK, NAGASE and G6PASE were assayed respectively, by the methods described in Livingstone and Bayne (1974), Barrett and Heath (1977) and Baginski et al. (1974); the buffer routinely used for the G6PASE assay was 50 m*M* sodium cacodylate—HCl pH 5.5 or 6.5. SDH was assayed by a modification of the method of Kmetec (1966) and the increase in absorbance of INT at 540 nm was followed. Contained in a final volume of 1.0 ml were 50 m*M* Tris—HCl pH 7.6, 1 m*M* KCN, 0.06% w/v INT and 20 m*M* sodium succinate. The reaction was run against a reference cuvette containing everything except succinate and initiated by the simultaneous addition of sample to both cuvettes. Total protein was determined by the method of Lowry et al. (1951).

G6PDH was assayed as described by Livingstone (1981); small sample volumes were used to minimize endogenous inhibition effects. Ferricyanide reductase activities were determined by measuring the decrease in absorbance at 420 nm in a reaction mixture containing in a final volume of 1.0 ml, 50 m*M* Tris—HCl pH 7.6, 1 m*M* KCN, 0.2 m*M* potassium ferricyanide and 0.26 m*M* NAD(P)H. The sample was added to the preincubated reaction mixture lacking NAD(P)H and the rate of reaction between sample and ferricyanide recorded; this rate was relatively low and linear except in some cytosolic samples. The NADH or NADPH was then added and the reaction proper recorded. From this net rate was subtracted the previously determined rate of reduction of ferricyanide by NAD(P)H in the absence of enzyme sample and the activity was calculated using an extinction coefficient of 1.02 mM^{-1} cm^{-1} (Ichikawa et al., 1969). Cytochrome *c* reductase activities were determined by measuring the increase in absorbance at 550 nm in a reaction mixture containing in a final volume of 1.0 ml, 50 m*M* Tris—HCl pH 7.6, 1 m*M* KCN, 30 μM cytochrome *c* and 0.26 m*M* NAD(P)H. Following addition of sample to the preincubated reaction mixture lacking NAD(P)H, a rapid reaction was observed between sample and cytochrome *c* which quickly plateaued-off and which varied greatly between samples. The reaction proper was then started by the addition of NADH or NADPH and, after subtraction of the non-enzymic rate between cytochrome *c* and NAD(P)H, the activity calculated using an extinction coefficient of 19.6 mM^{-1} cm^{-1} (Shimakata et al., 1972). The effect of the reaction between cytochrome *c* and sample was minimised by the use of small sample volumes; such reactions have been observed elsewhere and concluded to be non-enzymatic (Pohl and Wiermann, 1981).

BP hydroxylase was assayed by the sensitive radiometric method of Van Cantfort et al. (1977) using a modification of the technique described by Binder and Stegeman (1980). The sample was incubated in a reaction mixture containing in a final volume of 1.0 ml, 50 m*M* Tris—HCl pH 7.6, 0.4 m*M*

NADPH, 2 mg bovine serum albumen, 0.06 m*M* of [G-^{3}H]benzo[*a*]pyrene (about 300 μCi per μmol) and, depending upon the sample, about 0.5—2 mg of microsomal or other protein. The reaction was initiated by the addition of BP in acetone and aliquots of the assay mixture were removed after 1 and 15 min of incubation and the reactions stopped by transfer to tubes containing 1.0 ml of 0.15 *M* KOH in 85% v/v DMSO (dimethylsulphoxide). The stopped reaction mixture was extracted twice with 7.5 ml hexane and the aqueous phase counted after addition of 700 μl 0.35 *N* HCl; counting efficiencies were determined by use of internal standards. Time 1 minute polar counts were kept to a minimum by routine purification of the [^{3}H]-BP substrate (stored frozen at -20°C) (DePierre et al., 1975) and in the most active assays a doubling of polar counts was observed over the 15 min period. The BP hydroxylase activity of digestive gland microsomes was linear with a sample size assay concentration of 0.28—1.4 mg protein per ml, the rate was linear over the first 15 min of the assay and the minimum rate that could be determined accurately by the assay procedure was less than 1.0 pmol per min. No variation due to carrying out the reaction in the absence or presence of light could be determined and no reaction in the absence of sample could be detected (Van Cantfort et al., 1977). The rates of reaction using heat-denatured samples varied between 0—30% of the native rate but the results were not reproducible; such rates are likely to be due to non-enzymatic hydroxylations (Yip, 1964; Trelstad et al., 1981) and, given that they were not reproducible and the conditions producing them might have resulted from the denaturation process, they have not been subtracted from the native rates in calculating the BP hydroxylase activities.

Cytochrome P-450 was quantified in the tissue survey study from the carbon monoxide difference spectrum of sodium dithionite treated samples determined according to the method of Omura and Sato (1964). The buffer was 50 m*M* Tris—HCl pH 7.6, dithionite was added before bubbling with CO and the P-450 was quantified using an extinction coefficient of 91 mM^{-1} cm^{-1} (450—490 nm) (Estabrook and Werringloer, 1978) or 52 mM^{-1} cm^{-1} (460—490 nm) (Estabrook et al., 1972). In later experiments on digestive gland microsomes, a modification of this procedure was used to eliminate spectral interference resulting from an interaction between CO and the 'oxidised' sample (see results): CO was bubbled through the sample beam cuvette before addition of dithionite and any spectral changes removed by automatic baseline-correction. In both procedures, scans were repeated over 5—10 min until maximum peak sizes were observed. Excess dithionite was avoided as it resulted in the formation of a white 'precipitate', which presumably was colloidal sulphur (Estabrook et al., 1972), and a continually changing baseline. Cytochrome b_5 was similarly determined by difference-spectrum, the sample being placed in 50 m*M* Tris—HCl pH 7.6 in both beams of the spectrophotometer and the sample-beam cuvette being reduced by the addition of NADH in water (final concentration: 600 μM). The cytochrome b_5 was quantified using an extinction coefficient of 185 mM^{-1} cm^{-1} (426—409 nm; a correction was applied for the absorbance of NADH at 409 nm)

(Estabrook and Werringloer, 1978) or 112 mM^{-1} cm^{-1} (425—490 nm) (Raw and Mahler, 1959).

In the tissue survey, and other work, unwashed microsomes were generally used in order to minimize centrifugation time and because washing resulted in a more compacted pellet that was difficult to resuspend without significant agitation. Enzyme specific activities and concentrations of the mitochondrial and microsomal fractions have therefore been corrected for cytosolic contamination using the percentage presence of PK (cytosolic marker) and a predetermined factor which represented the amount of PK that could be washed out of the subcellular fraction (= cytosolic contamination) and the amount that was non-specifically bound to the pellet.

Inhibition studies

Inhibition studies were carried out on digestive gland microsomes. Carbon monoxide treatment was effected in two ways: (1) it was bubbled slowly for varying periods of time through the reaction mixture containing microsomes, on ice, and the reaction initiated at 25°C by the addition of [^{3}H]-BP; controls in which air was bubbled instead of CO were also run. (2) CO was bubbled vigorously through the reaction mixture, microsomes were then added and the atmosphere above the mixture on ice perfused with CO for several minutes with occasional gentle agitation before carrying out the reaction at 25°C by the addition of [^{3}H]-BP. α-Naphthoflavone and SKF-525A studies were carried out by adding them mixed in with the [^{3}H]-BP substrate to give a final inhibitor concentration of 0.1 mM.

Electron microscopy

Mitochondral and microsomal pellets were prepared from the digestive gland as described previously and fixed for 1 h in 3% glutaraldehyde solution in 0.1 M sodium phosphate buffer pH 7.2 containing 2.5% NaCl. They were then rinsed in three changes of the 0.1 M phosphate buffer for 15 min each, cut into 1 mm cubes and post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer pH 7.2 for 1 h. Following three more rinses of 15 min each in the 0.1 M phosphate buffer, the pellets were dehydrated through a graded ethanol series, embedded in Taab resin and ultrathin sections cut on a Reichert OMU2 ultramicrotome. Sections were stained with aqueous saturated solution of uranyl acetate (10 min) and lead citrate (Reynolds, 1963) for 10 min and examined in a Philips 200 electron microscope.

Statistical analysis

Mean values are expressed as ± standard error (SEM). Groups of values were compared by one-way analysis of variance. The correlation between two sets of data was examined by determining the Pearson product moment correlation coefficient (Croxton, 1953). In the statistical analyses a level of significance of $\leqslant 0.05$ was accepted as significant.

TABLE 1

PERCENTAGE DISTRIBUTION OF MARKER AND OTHER ENZYME ACTIVITIES AMONG THE SUBCELLULAR FRACTIONS OF DIFFERENT TISSUES OF *M. edulis*, THE G6PASE pH RATIOS OF THE FRACTIONS AND THE SPECIFIC ACTIVITIES OF THE WHOLE TISSUES IN TERMS OF ACTIVITY PER GRAM FRESH WEIGHT[a]

Tissue	Sex	Fraction[b]	PK	NAGASE	SDH	G6PASE	pH ratio[c]	G6PDH	NADH-FERRIRED	NADPH-FERRIRED	NADH-CYTCRED	NADPH CYTCRED	BP hydroxylase
Digestive gland	Male	mito	0.9 ± 0.2	4.1 ± 1.4	85.2 ± 1.9	6.5 ± 3.6	1.15 ± 0.22	3.4 ± 0.8	22.9 ± 1.8	1.9 ± 0.8	36.3 ± 2.3	11.9 ± 2.6	41.3 ± 12.8
		mic	5.4 ± 1.6	4.6 ± 1.3	14.8 ± 1.9	22.4 ± 6.0	0.56 ± 0.13	3.2 ± 0.6	19.7 ± 1.9	4.8 ± 1.7	36.7 ± 1.8	26.5 ± 2.2	58.7 ± 11.1
		cyt	93.7 ± 1.5	91.4 ± 1.1	0	71.1 ± 2.7	1.67 ± 0.22	93.4 ± 0.6	57.4 ± 3.6	93.3 ± 2.5	27.0 ± 0.9	61.6 ± 2.8	0
		S.A.	4.36 ± 0.39	146.8 ± 20.2	0.65 ± 0.05	0.19 ± 0.02		3.15 ± 0.28	18.7 ± 0.95	3.15 ± 0.41	1.23 ± 0.05	0.19 ± 0.01	122.2 ± 16.0
	Female	mito	3.2 ± 0.7	2.4 ± 0.3	79.0 ± 5.6	26.6 ± 3.9	0.33 ± 0.03	2.3 ± 0.5	20.6 ± 2.8	3.3 ± 1.4	30.6 ± 2.6	10.8 ± 2.9	58.3 ± 4.9
		mic	4.5 ± 0.7	3.8 ± 0.1	21.1 ± 5.6	21.3 ± 2.6	0.42 ± 0.01	3.8 ± 0.8	16.1 ± 1.1	6.4 ± 1.0	33.1 ± 0.6	23.0 ± 5.7	35.2 ± 3.2
		cyt	92.5 ± 1.0	94.1 ± 0.3	0	52.2 ± 2.3	1.16 ± 0.10	94.0 ± 1.2	63.4 ± 2.9	90.4 ± 0.9	36.3 ± 2.2	66.2 ± 1.5	6.5 ± 6.5
		S.A.	5.12 ± 0.33	72.2 ± 15.1	0.52 ± 0.04	0.20 ± 0.02		2.47 ± 0.46	20.2 ± 1.1	3.54 ± 0.13	1.50 ± 0.08	0.27 ± 0.02	273.1 ± 18.8
Gills	Male	mito	6.0 ± 0.9	3.9 ± 1.0	83.2 ± 3.8	7.4 ± 1.5	1.54 ± 0.58	2.6 ± 0.8	25.5 ± 0.7	6.1 ± 1.1	48.8 ± 5.2	15.1 ± 3.4	80.9 ± 11.1
		mic	4.4 ± 0.7	4.6 ± 0.5	16.8 ± 3.8	6.9 ± 1.4	0.54 ± 0.31	2.6 ± 0.5	16.5 ± 0.9	5.8 ± 0.3	28.3 ± 1.2	17.2 ± 2.8	19.1 ± 11.1
		cyt	89.6 ± 1.4	91.5 ± 0.7	0	86 ± 2.9	2.01 ± 0.79	94.9 ± 0.6	58.0 ± 1.2	88.1 ± 1.0	22.9 ± 5.4	67.7 ± 5.6	0
		S.A.	2.17 ± 0.15	8.89 ± 1.45	0.17 ± 0.02	0.05 ± 0.004		2.92 ± 0.34	6.39 ± 0.39	1.36 ± 0.1	0.33 ± 0.01	0.05 ± 004	20.04 ± 11.32
	Female	mito	4.2 ± 0.3	5.8 ± 1.0	74.0 ± 3.5	16.4 ± 4.6	0.73 ± 0.05	2.2 ± 0.2	26.3 ± 2.3	3.0 ± 1.6	42.9 ± 1.0	11.0 ± 2.4	26.4 ± 26.4 (3)
		mic	2.7 ± 0.4	4.5 ± 0.9	26.0 ± 3.5	18.0 ± 3.8	0.86 ± 0.07	1.7 ± 0.2	22.7 ± 2.0	4.9 ± 1.3	33.9 ± 1.7	12.3 ± 1.2	73.6 ± 26.4 (3)
		cyt	93.1 ± 0.1	89.7 ± 1.8	0	65.6 ± 8.2	0.93 ± 0.10	96.1 ± 0.3	51.0 ± 4.1	92.2 ± 2.2	23.2 ± 2.6	76.8 ± 3.6	0
		S.A.	3.78 ± 0.47	7.69 ± 1.1	0.19 ± 0.02	0.03 ± 0.006		2.83 ± 0.33	8.20 ± 0.32	1.21 ± 0.1	0.61 ± 0.04	0.07 ± 0.004	7.29 ± 3.16
Mantle	Male	mito	5.4 ± 2.3	3.6 ± 1.3	24.6 ± 4.4	1.8 ± 1.8	1.65 (1)	1.6 ± 0.3	9.0 ± 1.8	1.7 ± 0.4	16.2 ± 0.8	3.8 ± 0.7	50 (0, 100)
		mic	3.2 ± 1.1	5.0 ± 1.7	75.4 ± 4.4	3.5 ± 3.5	0.89 (1)	4.0 ± 0.6	20.0 ± 1.1	3.6 ± 1.2	38.7 ± 4.2	10.6 ± 1.6	50 (100, 0)
		cyt	91.4 ± 2.3	91.5 ± 2.9	0	94.7 ± 3.1	1.30 (1.41, 1.18)	94.4 ± 0.9	71.0 ± 1.7	94.7 ± 0.8	45.2 ± 4.6	85.6 ± 1.7	0
		S.A.	5.60 ± 0.40	2.90 ± 0.82	0.20 ± 0.03	0.04 ± 0.02		1.52 ± 0.08	7.60 ± 0.67	2.05 ± 0.07	0.32 ± 0.04	0.07 ± 0.01	4.16 ± 2.63
	Female	mito	4.2 ± 0.8	11.3 ± 0.5	73.8 ± 3.5	2.0 ± 0.7	1.63 ± 0.23	1.8 ± 0.4	36.0 ± 2.9	1.0 ± 0.6	34.5 ± 5.9	50.1 ± 6.9	49.8 ± 21.5
		mic	3.5 ± 0.4	8.7 ± 0.4	27.3 ± 3.5	3.5 ± 0.4	0.85 (0.64, 1.06)	8.6 ± 4.5	16.4 ± 4.6	1.5 ± 0.8	39.5 ± 1.9	49.9 ± 6.9	50.2 ± 21.5
		cyt	94.4 ± 0.6	79.9 ± 0.8	0	93.8 ± 0.5	0.77 ± 0.11	89.6 ± 4.9	47.7 ± 4.7	97.6 ± 1.4	26.1 ± 6.5	0	0
		S.A.	7.95 ± 0.85	4.58 ± 0.48	0.26 ± 0.02	0.05 ± 0.004		1.10 ± 0.19	18.28 ± 0.93	4.36 ± 0.25	1.89 ± 0.09	0.03 ± 0.003	19.68 ± 6.65
Rest	Male	mito	2.1 ± 0.5	3.1 ± 0.9	64.8 ± 6.0	8.7 ± 1.2	0.69 ± 0.19	2.8 ± 0.8	14.1 ± 1.0	4.5 ± 0.6	25.8 ± 4.8	7.5 ± 2.7	65.0 ± 15.1
		mic	4.4 ± 0.8	4.0 ± 0.5	35.2 ± 6.0	18.1 ± 5.9	0.65 ± 0.03	3.1 ± 0.7	26.7 ± 0.7	10.0 ± 1.1	41.6 ± 5.1	15.1 ± 3.6	35.0 ± 15.1
		cyt	93.5 ± 1.0	93.0 ± 0.6	0	73.2 ± 6.3	1.96 (2.75, 1.16)	94.3 ± 1.0	59.2 ± 1.1	85.5 ± 1.5	32.5 ± 9.4	77.3 ± 6.1	0
		S.A.	2.17 ± 0.32	3.18 ± 0.45	0.16 ± 0.02	0.04 ± 0.02		1.32 ± 0.20	5.48 ± 0.43	0.78 ± 0.06	0.35 ± 0.04	0.07 ± 0.04	6.48 ± 0.59
	Female	mito	5.0 ± 1.4	4.4 ± 0.3	71.3 ± 2.1	11.3 ± 2.2	0.50 (0.7, 0.3)	2.8 ± 1.0	18.5 ± 3.1	9.5 ± 2.7	26.2 ± 2.0	20.3 ± 12.4	17.3 ± 9.0 (3)
		mic	2.8 ± 0.2	5.2 ± 0.4	28.7 ± 2.1	17.8 ± 2.4	0.61 ± 0.03	2.7 ± 0.6	21.9 ± 1.2	4.7 ± 0.9	35.6 ± 1.8	30.9 ± 11.3	82.7 ± 9.0 (3)
		cyt	92.3 ± 1.5	90.4 ± 0.3	0	70.9 ± 3.9	2.62 ± 1.29	94.5 ± 1.5	59.6 ± 1.9	85.9 ± 2.2	38.2 ± 3.1	48.9 ± 20.1	0 (3)
		S.A.	2.38 ± 0.21	3.53 ± 1.27	0.10 ± 0.01	0.02 ± 0.003		1.35 ± 0.12	6.17 ± 0.28	0.91 ± 0.08	0.38 ± 0.10	0.05 ± 0.02	4.48 ± 2.31

[a]Specific activities (S.A.) are in μmol min^{-1} g^{-1} wet weight except for BP hydroxylase which is in pmol min^{-1} g^{-1}. Values are means ± SEM, $n = 4$ unless indicated otherwise and means and values are given where $n = 2$.

[b]Mito: mitochondrial (500—12 000 × *g*); micros: microsomal (12 000—100 000 × *g*) cyt: cytosolic (post-100 000 × *g* supernatant)

[c]Ratio of G5PASE activity at pH 5.5 to 6.5.

RESULTS

Subcellular characterization

The marker enzymes were chosen to represent the following subcellular components: cytosol (PK), mitochondria (SDH), lysosomes (NAGASE) (Barrett and Heath, 1977) and endoplasmic reticulum (G6PASE) (Nordlie, 1974), although in no case was the localization expected to be exclusive; e.g. SDH is also found in the yellow pigment granules, known as cytosomes, in molluscs (Zs.-Nagy, 1977). The preliminary characterization studies were all carried out on digestive gland with no regard to the sex of the individual and the results obtained (not shown) were intermediate between those for the separate sexes in the tissue survey (see Table 1). PK was almost entirely cytosolic (100 000 × *g* supernatant) and NAGASE had a similar distribution indicating that the lysosomes were being broken up by the homogenization procedure and that contamination of the 500—12 000 × *g* (hereafter termed the 'mitochondrial fraction') and 12 000—100 000 × *g* (hereafter termed the 'microsomal fraction') pellets by whole lysosomes was likely to be small. SDH was observed to sediment as 'early' as 2500*g* and about 80% or more was usually found in the mitochondrial pellet, confirming its identity.

G6PASE was present in all fractions and in highest activity in the cytosol. The cytosolic activity was indicated by the pH-characteristics of the cytosolic and microsomal fractions (Fig. 1A) to be due to 'non-specific' phosphatases released from broken lysosomes, and not to unsedimented microsomes. These pH-profiles appeared to reflect the pH-optima differences of the two types of enzyme (G6PASE is classically pH 6 (Nordlie, 1974) compared with the lower pH 3—5 for most lysosomal hydrolases (Barrett and Heath, 1977)). The microsomal fraction showed little or no increase in activity from pH 6.5 to 5.5 compared with a smoothly increasing activity in the cytosol up to an optimum of pH 4.5; the increase in activity in the microsomes from pH 5.5 to 4.5 presumably represents lysosomal or cytosolic contamination. A ratio of the G6PASE activity at pH 5.5 and pH 6.5 was therefore used in the survey to indicate the source of the phosphatase activity; a value of $\leqslant 1$ being expected for the microsomal G6PASE and considerably greater than this for the lysosomal phosphatase.

The pH ratios obtained for the mitochondrial fractions were generally lower than those for the cytosolic fractions indicating that some microsomes (endoplasmic reticulum) were co-sedimenting with the mitochondria at 12 000 × *g*, e.g. see Table 1. A second important result, indicated from the pH activity ratio data, was that during the homogenization and extraction procedure there was a loss of material from the endoplasmic reticulum into the cytosol. The values for the microsomal and cytosolic ratios varied in different studies and showed a relationship with the percentage of the total G6PASE activity that was present in the microsomal fraction (Fig. 1B). Whereas the declining microsomal ratio is explainable on the basis of a decreased likelihood of cytosolic contamination, the cytosolic ratio should

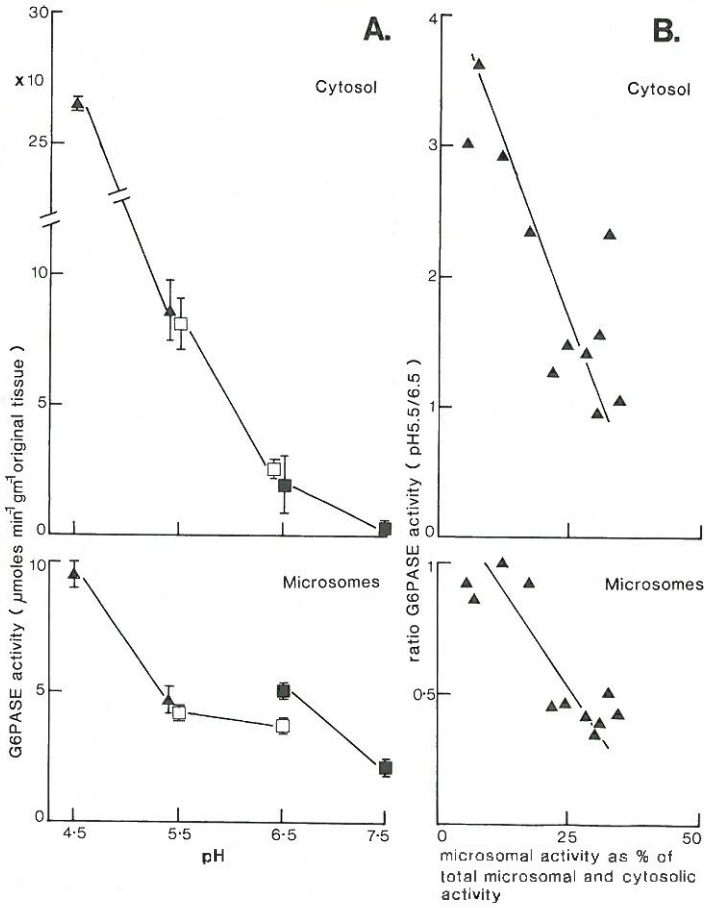

Fig. 1. G6PASE activity of the microsomal and cytosolic fractions of digestive gland. (A) activity vs. pH, means and range shown ($n = 2$); 50 mM buffer, (■) triethanolamine—HCl; (□) sodium cacodylate—HCl; (▲) sodium citrate—citric acid. (B) ratio of activity at pH 5.5 to 6.5 vs. microsomal activity as a percentage of microsomal plus cytosolic activity in whole tissue; correlation coefficient of line (r) = 0.863 (cytosolic pH ratio) and 0.833 (microsomal pH ratio) ($n = 11$ for both data sets).

be unaffected by changes in microsomal composition and the similar decline suggests a release of G6PASE from the endoplasmic reticulum into the cytosol affecting the enzyme activity measurements; this general conclusion was supported by the detection in the tissue survey of cytochromes b_5 and P-450 in the cytosol of certain tissues (see next section).

The results of the electron microscopy were in agreement with the marker enzyme study. The 500—12 000 x g pellet contained mainly mitochondria but with the presence of vesicles of rough endoplasmic reticulum and an occasional large vesicle of smooth endoplasmic reticulum (Fig. 2A and B). In contrast, mitochondria and rough endoplasmic reticulum were absent from the 12 000—100 000 x g pellet which was characterized by many

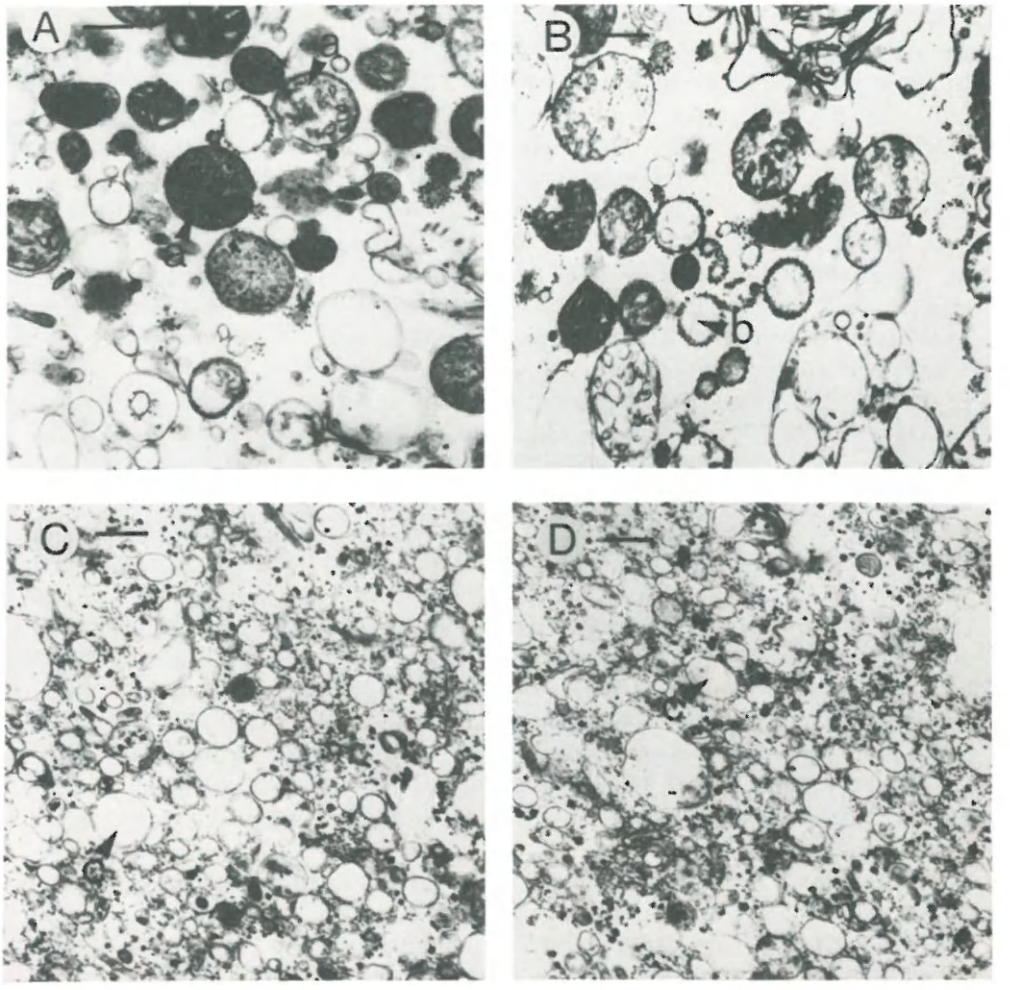

Fig. 2. Electron micrographs of subcellular fractions of digestive gland. (A and B) 500—12 000 × *g* pellet showing mitochondria (a) and vesicles of rough endoplasmic reticulum (b). (C and D) 12 000—100 000 × *g* pellet showing vesicles of smooth endoplasmic reticulum (c) and particulate material. Magnification: × 32 000; scale bar: 0.3 μm.

vesicles of smooth endoplasmic reticulum and various particulate material which presumably is mainly free ribosomes and glycogen particles (Fig. 2C and D). The sedimenting of the rough endoplasmic reticulum before the smooth has been observed for other sources, e.g. rat liver (Dallner, 1963). The diameter of the smooth vesicles varied between about 90 and 500 nm with the mean around 200—250 nm compared with 50—100 nm for rat liver (Dallner, 1963) and 100—250 nm for rat lung (Johannesen et al., 1977); the diameter for the rough vesicles was about 300 nm compared with 100—300 nm for rat liver (Dallner, 1963). Lysosomal vesicles in *Mytilus* are usually considerably larger than those of the endoplasmic reticulum (Moore, 1982, personal communication) and were only occasionally observed in the electron micrographs.

Tissue survey and inhibition studies

The subcellular distributions of the marker and other enzyme activities in some of the tissues are given in Table 1. The distributions of PK, NAGASE and SDH of all the tissues were similar to those already described for the digestive gland with the exception of SDH in the male mantle which was located primarily in the microsomal fraction; SDH differences were also found for the foot and adductor muscle but the total activities were low and the variability high (data not shown). A major sex difference was seen in the distribution of G6PASE in the digestive gland, the activity being greater in the mitochondrial fraction of females and the pH ratio confirming its microsomal nature. The co-sedimenting of microsomal material with the mitochondrial fraction was also clearly indicated for the rest fraction of both sexes but less so or not at all for the other tissues. The specific activity of G6PDH was high in most tissues but the enzyme was primarily cytosolic. NAD(P)H-FERRIRED and NAD(P)H-CYTCRED activities were present in all subcellular fractions as might be expected as, in addition to flavoprotein components of the mixed function oxygenase (MFO) system, the same assay reactions are catalysed by parts of the mitochondrial electron transport chain, by soluble enzymes such as xanthine oxidase (E.C.1.2.3.2.) and by the so-called diaphorases (Lind and Ernster, 1974); non-enzymatic cytosolic reductase activities are also known (Johannesen et al., 1977). BP hydroxylase was generally indicated to be absent from the cytosol, and in the digestive gland was present in both the mitochondrial and microsomal fractions; the same was generally found for the other tissues although the variability was high. The G6PASE activities indicate that a large part of the mitochondrial BP hydroxylase in the female digestive gland is due to microsomal contamination.

The specific activities of the MFO-associated enzyme activities are given in Table 2. After correction for cytosolic contamination a microsomal G6PDH activity was detectable in some tissues but the values were low and showed no consistent variation with tissue-type or sex; a low microsomal NADPH-FERRIRED activity was also detectable in most tissues. The NADH-FERRIRED and NADH-CYTCRED activities were generally highest in the microsomal fractions with no obvious differences between the major tissues. In contrast, the BP hydroxylase and NADPH-CYTCRED activities were highest in the microsomes of the digestive gland and higher in those of female than of male mussels. BP hydroxylase was also present in all the other major tissues but generally not in the foot or adductor muscle. Considering all the microsomal data of the major tissues (digestive gland, gills, mantle, rest) together ($n = 28$), a positive correlation was observed between BP hydroxylase and NADPH-CYTCRED activities (correlation coefficient $r = 0.89$) but not between the former and NADH-CYTCRED ($r = 0.32$) or NADH-FERRIRED ($r = 0.17$) activities.

The results for the cytochrome determinations are given in Fig. 3 and Table 3. Cytochrome b_5 type spectra were found in digestive gland, gills and mantle and in both the microsomal and cytosolic fractions. In contrast,

TABLE 2

SPECIFIC ACTIVITIES OF G6PDH, NADH- AND NADPH-FERRICYANIDE AND CYTOCHROME *c* REDUCTASES AND BP HYDROXYLASE IN THE SUBCELLULAR FRACTIONS OF THE TISSUES OF *M. edulis*[a]

Tissue	Sex	Fraction	G6PDH	NADH-FERRIRED	NADPH-FERRIRED	NADH-CYTCRED	NADPH CYTCRED	BP hydroxylase
Digestive gland	Male	mito	13.63 ± 2.90	737.6 ± 27.3	3.57 ± 1.18	85.80 ± 9.20	4.05 ± 0.95	5.96 ± 1.27
		mic	8.37 ± 0.14	818.6 ± 33.1	7.62 ± 2.06	119.2 ± 2.1	12.4 ± 1.32	18.59 ± 1.29
		cyt	47.81 ± 4.50	170.5 ± 25.9	47.84 ± 6.48	5.70 ± 0.33	1.95 ± 0.14	n.d.[b]
	Female	mito	3.21 ± 0.16	742.0 ± 61.2	5.36 ± 3.25	86.64 ± 5.95	4.80 ± 1.15	30.52 ± 2.48
		mic	7.47 ± 0.70	930.2 ± 54.6	34.87 ± 5.70	156.5 ± 9.5	18.35 ± 4.56	30.95 ± 3.94
		cyt	38.50 ± 8.00	209.4 ± 16.1	52.51 ± 1.68	8.90 ± 0.71	2.96 ± 0.20	0.31 ± 0.31
Gills	Male	mito	n.d.	854.2 ± 139.1	17.75 ± 2.18	94.25 ± 20.91	3.47 ± 1.12	7.07 ± 3.39
		mic	15.01 ± 2.74	724.1 ± 52.5	32.15 ± 2.98	67.97 ± 4.26	4.98 ± 0.27	4.37 ± 3.39
		cyt	104.2 ± 8.7	152.5 ± 10.1	49.2 ± 3.8	3.00 ± 0.50	1.26 ± 0.19	—[c]
	Female	mito	n.d.	792.6 ± 72.1	3.99 ± 2.10	102.0 ± 10.0	2.17 ± 0.30	1.14 ± 1.14 (3)
		mic	2.79 ± 0.46	1007.2 ± 21.8	19.76 ± 5.78	114.7 ± 3.8	4.16 ± 0.33	2.52 ± 1.33 (3)
		cyt	116.7 ± 11.8	188.5 ± 35.9	48.50 ± 4.50	6.10 ± 0.90	2.23 ± 0.12	—
Mantle	Male	mito	n.d.	173.6 ± 23.0	n.d.	26.12 ± 2.46	0.31 ± 0.05	0.92 ± 0.92
		mic	10.56 ± 1.19	602.9 ± 76.2	10.43 ± 2.98	43.93 ± 3.65	2.27 ± 0.40	1.23 ± 1.23
		cyt	31.50 ± 1.30	120.5 ± 8.8	43.50 ± 2.60	2.60 ± 0.30	1.32 ± 0.15	—
	Female	mito	n.d.	931.9 ± 174.8	n.d.	95.10 ± 21.94	1.86 ± 0.27	1.03 ± 0.39
		mic	14.28 ± 4.87	928.9 ± 281.4	n.d.	247.7 ± 23.70	4.54 ± 0.74	3.97 ± 2.00
		cyt	19.00 ± 4.60	157.8 ± 12.0	78.3 ± 6.9	9.10 ± 2.40	0	—
Rest	Male	mito	10.01 ± 0.87	1025.1 ± 274.7	36.50 ± 9.54	111.6 ± 19.1	3.33 ± 1.55	7.44 ± 1.46
		mic	9.00 ± 1.42	1361.4 ± 116.7	49.96 ± 8.05	133.9 ± 7.2	6.42 ± 2.59	5.10 ± 2.42
		cyt	54.70 ± 7.50	156.0 ± 24.8	31.40 ± 2.10	6.10 ± 2.80	2.82 ± 1.91	—
	Female	mito	n.d.	1868.9 ± 243.2	85.80 ± 15.31	184.3 ± 34.1	7.37 ± 2.01	3.04 ± 1.91 (3)
		mic	9.69 ± 1.29	1445.1 ± 167.7	32.09 ± 3.61	143.4 ± 11.0	8.70 ± 1.51	7.43 ± 3.65 (3)
		cyt	72.30 ± 10.60	206.1 ± 26.9	43.00 ± 4.00	8.50 ± 1.90	2.24 ± 1.35	—
Foot	Male	mito	n.d. (2)	204 (103, 306)	136 (198, 72)	73.5 (41.1, 106)	n.d. (2)	n.d. (2)
		mic	n.d. (2)	201 (74, 329)	55.5 (43.7, 67.2)	92.4 (56.3, 128.6)	n.d. (2)	n.d. (2)
		cyt	87.80 (72.8, 102.8)	297 (335, 259)	192 (317, 166)	5.4 (1.9, 8.9)	15.8 (21.2, 10.4)	—
	Female	mito	n.d. (2)	5735 (9193, 2276)	529 (834, 224)	107 (147, 66.8)	4.8 (3.4, 6.0)	n.d. (2)[d]
		mic	n.d. (2)	2756 (3121, 2392)	227 (311, 141)	195 (241, 149)	16.4 (6.1, 26.6)	n.d. (2)[d]
		cyt	56.70 (81.7, 31.6)	248 (355, 140)	87.1 (104, 70.2)	5.1 (3.6, 6.6)	0.83 (1.4, 0.2)	—
Adductor[e]	Male and Female	mito	2.28 ± 2.28	4476 ± 1324	91.20 ± 38.90	509.0 ± 106.3	20.40 ± 11.59	n.d.
		mic	6.83 ± 4.51	1908 ± 447	10.93 ± 3.68	66.78 ± 23.51	5.65 ± 1.80	n.d.
		cyt	16.30 ± 4.70	106.5 ± 31.2	12.23 ± 1.73	10.75 ± 0.61	2.38 ± 1.15	—

[a]Specific acitivities are in nmol min^{-1} mg^{-1} protein except for BP hydroxylase which is in pmol min^{-1} mg^{-1}. Values are means ± SEM, $n = 4$ unless indicated otherwise and means and values are given where $n = 2$.

[b]n.d. not detected.

[c]In cytosolic samples from tissues other than the digestive gland, 12 out of 31 analysed showed very slight BP hydroxylase activities but the changes were very near the limit of the assay procedure and it was not possible to subsequently confirm these results.

[d]An indication of BP hydroxylase activity was also observed for these samples.

[e]Pooled data are given for this tissue where no sex difference is obvious.

TABLE 3

CYTOCHROME b_5 AND P-450 LEVELS AND λ_{max} OF THE DIFFERENCE SPECTRA IN THE SUBCELLULAR FRACTIONS OF THE TISSUES OF *M. edulis*[a]

TABLE 3
CYTOCHROME b_5 AND P-450 LEVELS AND λ_{max} OF THE DIFFERENCE SPECTRA IN THE SUBCELLULAR FRACTIONS OF THE TISSUES OF *M. edulis*[a]

Tissue	Sex[b]	Fraction	b_5 λ_{max}	b_5 levels	P-450 λ_{max}	P-450 levels	'415' levels[c]
Digestive gland	Male and Female	mito	n.r.s.c.[d]	—[e]	415.0 ± 0 (3)		0.10 (2)
		mic	425.5 ± 0.5 (4)	75.7 ± 11.7 (4)	415.3 ± 0.3 (4); 450 ± 0.7 (4)	134 ± 64 (4)	0.12 ± 0.04 (3)
		cyt	427.9 ± 0.3 (7)	16.0 ± 0.3 (7)	415.9 ± 0.8 (5); F: 450.3 ± 1.2 (3)	F: 8.7 ± 4.6 (3)	M: 0.081 (0.086, .076) F: 0.30 ± 0.04 (3)
Gills	Male and Female	mito	n.r.s.c.	—	—	—	—
		mic	425.0 ± 2.0 (3)	43.6 ± 7.6 (4)	417 (1)	—	F: 0.004 (1)
		cyt	425.5 (425, 426)	4.9 ± 1.7 (6)	414.5 ± 1.5 (4)	—	0.034 ± 0.011 (4)
Mantle	Male and Female	mito	n.r.s.c.	—	—	—	—
		mic	425.5 (428, 423)	25.1 ± 14.7 (4)	—	—	—
		cyt	—	—	417.7 ± 0.9 (3)	—	0.027 ± 0.018 (3)
Rest	Male and Female	cyt	—	—	415.8 ± 0.8 (4)	—	0.028 ± 0.010 (4)
Foot	Male and Female	cyt	—	—	413.0 (414,412)	—	0.086 (0.098, 0.073)

[a]Cytochrome b_5 and P-450 levels are in pmol mg^{-1} protein; λ_{max} are in nm. Values are means ± SEM or mean plus range where $n = 2$. Unlike the enzyme activity measurements, samples were pooled for these determinations to give sufficient material for analysis.

[b]Pooled data are given for most samples where no sex difference is obvious; the exceptions are indicated (M: male, F: female).

[c]The '415 nm' peak of the P-450 assay has been quantified in terms of the Δ O.D.415 (or λ_{max}) minus 490 nm per gram wet weight from which the fraction was derived.

[d]n.r.s.c. = non-resolvable spectral changes observed.

[e]No spectral change or levels not detected; at least one or more sample of each fraction of each tissue was examined and those not shown in Table 3 had no spectral changes of obvious significance (N.B. many samples showed a decrease in absorbance in the visible region (trough) on addition of NADH but this phenomenon is common and unexplained; Estabrook and Werringloer, 1978).

TABLE 4

MICROSOMAL YIELDS OF PROTEIN, ENZYME ACTIVITIES AND CYTOCHROMES IN THE TISSUES OF *M. edulis*[a]

Tissue	Sex	Protein[b]	NADH-FERRIRED[c]	NADH-CYTCRED[c]	NADPH-CYTCRED[c]	BP hydroxylase[d]	P-450[e]	b_5[e]
Digestive gland	Male	3.85 ± 0.11	3159 + 189	459.3 + 8.8	47.7 ± 5.3	122.2 ± 16.0	224 (0, 448)	1287 (1195, 1379)
	Female	3.09 ± 0.11	2875 ± 188	483.3 ± 30.2	56.4 ± 13.6	273.1 ± 18.8	1212 (1597, 830)	1272 (1244, 1300)
Gills	Male	1.49 ± 0.13	1024 + 75	93.5 ± 3.6	7.6 ± 0.3	20.0 ± 11.3	n.d.	229 (255, 202)
	Female	1.76 ± 0.07	1773 + 109	201.3 + 7.6	7.3 ± 0.4	7.3 + 3.2	n.d.	141 (100, 182)
Mantle	Male	2.71 ± 0.27	1479 + 198	117.3 ± 5.9	7.0 ± 0.9	4.2 ± 2.6	n.d.	77 (153, 0)
	Female	3.00 ± 0.15	2703 ± 781	734.8 ± 36.8	13.7 ± 2.5	19.7 ± 6.7	n.d.	66 (131, 0)
Rest	Male	1.05 ± 0.05	1419 + 96	140 ± 5.0	9.9 ± 4.2	6.5 ± 0.6	n.d.	n.d.
	Female	0.96 ± 0.08	1279 ± 57	135 ± 9.0	11.4 ± 1.6	4.5 ± 2.3	n.d.	n.d.
Foot	Male and Female	0.23 ± 0.04						
Adductor muscle	Male and Female	0.82 ± 0.14						

[a]Enzyme activities are shown only for the fractions in which BP hydroxylase was detected; in the case of BP hydroxylase and cytochromes P-450 and b_5, the levels in the mitochondrial and cytosolic fractions have also been included in the microsomal yield; values are means ± SEM ($n = 4$) or means and range where $n = 2$.
[b]mg g^{-1} fresh weight.
[c]nmol min^{-1} g^{-1} fresh weight.
[d]pmol min^{-1} g^{-1} fresh weight.
[e]pmol g^{-1} fresh weight.

TABLE 5

SPECIFIC ACTIVITIES OF G6PDH, NADH- AND NADPH- FERRICYANIDE AND CYTOCHROME *c* REDUCTASES AND BP HYDROXYLASE IN BLOOD CELLS OF *M. edulis*[a]

Sex	G6PDH	NADH-FERRIRED	NADPH-FERRIRED	NADH-CYTCRED	NADPH-CYTCRED	BP hydroxylase
Male	219.1 ± 10.8	958.8 ± 211.4	79.4 ± 16.6	21.3 ± 4.9	2.98 ± 0.83	8.44 ± 1.83
Female	253.0 ± 27.1	1130 ± 178.0	133.9 ± 66.8	14.4 ± 2.5	4.51 ± 1.80	2.85 ± 1.72

[a] Specific activities are in nmol min^{-1} mg^{-1} protein except for BP hydroxylase which is in pmol min^{-1} mg^{-1}; values are means ± SEM ($n = 4$); no cytochrome b_5 or P-450 was detected.

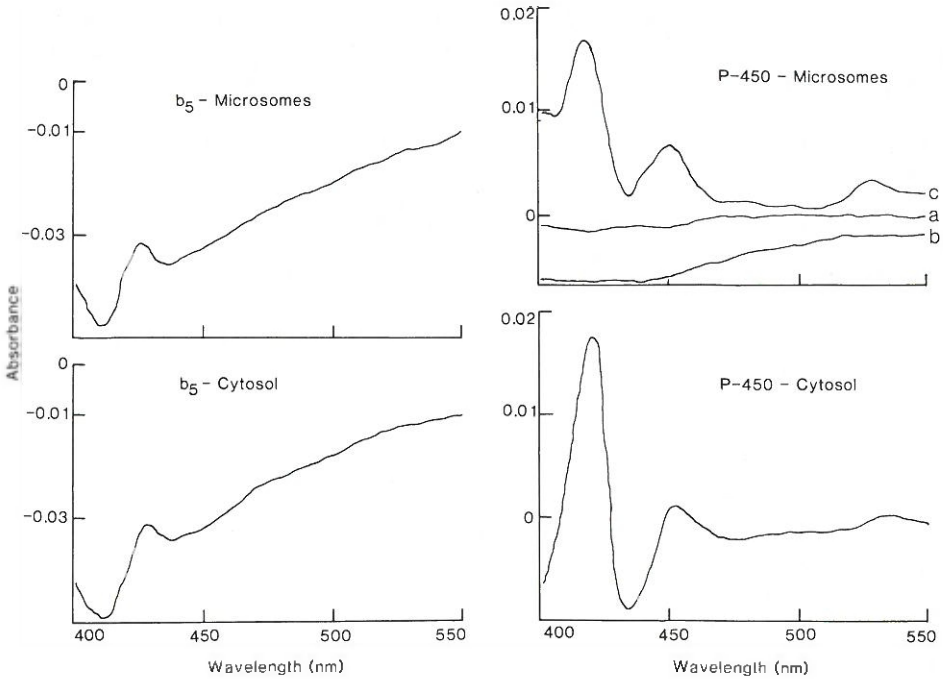

Fig. 3. Cytochrome b_5 (NADH difference spectrum) and P-450 (CO-dithionite/dithionite difference spectrum) spectra of digestive gland microsomal (pooled sex) and cytosolic (female mussels only) fractions. (a) sample and buffer only in both cuvettes; (b) subsequent addition of CO to sample in sample cuvette; (c) subsequent addition (after baseline correction) of dithionite to both cuvettes.

cytochrome P-450 was found only in the digestive gland, mainly in the microsomal fraction but also in the cytosol of female mussels. Maximum values for P-450 were obtained only when CO was added before the dithionite, and the baseline shift then compensated for (Fig. 3. traces a—c). A second major CO-binding reduced-molecule absorbance peak was observed around 413—417 nm and was present in both the microsomal and cytosolic fractions of a number of tissues; the 'concentration' of the peak was highest in the digestive gland cytosol (Table 3). The presence of both cytochrome b_5 and P-450 in the cytosol of the digestive gland, together with the previously discussed results for G6PASE (Fig. 1B), indicate that some material from the endoplasmic reticulum is being solubilized during the tissue extraction process.

Calculating the microsomal yields of protein, enzyme activities and cytochromes clearly indentifies the digestive gland as the main site of the MFO system in *M. edulis*, particularly if the mitochondrial and cytosolic BP hydroxylase and cytochromes are assumed to be microsomal in origin (Table 4); the levels of the various components are 2—10 times higher than in the microsomes of the other tissues and most significantly, P-450 was detectable only in the digestive gland. The sex difference in the digestive gland is

marked with respect to BP hydroxylase and cytochrome P-450 but not with respect to NADPH-CYTCRED. Cytochrome b_5 is clearly present in highest concentration in the digestive gland but also in reasonable concentration in the gills and mantle.

The various enzyme activities were detected in blood cells but no consistent sex differences were seen (Table 5); no cytochrome P-450 or b_5 was detectable. The BP hydroxylase activity of digestive gland microsomes was inhibited by three classical inhibitors of cytochrome P-450 but in no case was the inhibition complete (Table 6).

TABLE 6
INHIBITION STUDIES OF THE BP HYDROXYLASE ACTIVITY OF DIGESTIVE GLAND MICROSOMES OF *M. edulis*[a]

Inhibitor	Activity in presence of inhibitor expressed as a percentage of the control activity		
0.1 m*M* SKF-525A	56.4 ± 9.2		
0.1 m*M* 7,8-benzoflavone	40.9 ± 23.9		
CO[b]			
(a) perfusion	62.8 ± 4.2 (range)		
(b) bubbling	Time:	0	5 15 (min)
	Air:	100	76.3 53.4
	CO:	100	71.4 26.9

[a]Values are means ± SEM ($n = 4$) or means and range where $n = 2$ and are the pooled data for a number of experiments; control activities used in the experiments varied between 6.8 and 17.2 pmol min^{-1} ml^{-1} assay mixture.
[b]CO was either bubbled for varying periods of time or the assay mixture first perfused with CO before the initiation of the reaction (see Materials and Methods for further details).

DISCUSSION

Despite early reports of the absence of aryl hydrocarbon hydroxylase and other activities of the MFO system in bivalve molluscs including *M. edulis* (see Lee, 1981; Stegeman, 1981a), in recent years a number of such activities have been detected in vitro but at low levels compared with vertebrates or even invertebrates of other phyla, viz. BP hydroxylase in *M. edulis* (Stegeman, 1980, 1981a, b; Mix et al., 1981; Payne et al., 1983) and *Crassostrea virginica* (Anderson, 1978a, b), biphenyl hydroxylation in *M. edulis* (Willis and Addison, 1974), aldrin epoxidation in *M. edulis* (Moore et al., 1980), *Anodonta* sp. (Khan et al., 1972) and *M. californianus* (Krieger et al., 1979, 1981) antipyrine hydroxylation in *M. californianus* (Krieger et al., 1979) and *p*-nitroanisole *O*-demethylation in *M. californianus* (Trautman et al., 1979). Studies on mutagenicity (Parry et al., 1981; Dixon et al., 1983), in vivo transformation (Krieger et al., 1979) and the apparent inducibility of the MFO-associated NADPH-neotetrazolium reductase

TABLE 7

COMPARISON OF MICROSOMAL ENZYME ACTIVITIES AND LEVELS AND THE ACTIVITIES OF SOME REGULATORY ENZYMES OF INTERMEDIARY METABOLISM IN SELECTED SPECIES FROM DIFFERENT PHYLA[a]

Phyla and species	NADH-FERRIRED[b]	NADH-CYTCRED[b]	NADPH CYTCRED[b]	BPH[c]	P-450[d]	b_5[d]	Ref.	Species	PFK[e]	Ph[e]	HK[e]	Ref.
Chordata												
Rat	980	258	60—470	400	720—950	450—1060	(1, 6, 7)	Rat	47	50	1.9	(5)
Trout (*Salmo trutta*) or Scup (*Stenotomus chrysops*)	—	179	106—110	100—685	350—610	60—70	(6, 7, 9, 11)	Trout (*Salmo gaidneri*)	58.3	48	1.6	(5)
Arthropoda												
Blue crab (*Callinectes sapidus*)	—	—	5.2	42	180	—	(3, 8)	Edible crab (*Cancer pagarus*)	9.6	8.9	0.8	(12)
Acorn barnacle (*Balanus eburneus*)	—	112	28.6	43.2	110	20	(10)	Barnacle (*Lepas anatifera*)	5.4	1.9	2.2	(12
Annelida												
Nereis virens	—	—	0.9—2.34	24	110	50	(4)	*N. virens*	10.3	11.5	0.7	(12)
Mollusca												
Mytilus edulis	930	157	18.4	31.0	134	76	This paper	*M. edulis*	2.3	0.7	0.1	(2)

[a]Microsomal enzyme activities are from liver, hepatopancreas or digestive tissue and regulatory enzyme activities from white muscular tissue; most assays were at 25°C with the exception of the microsomal enzyme activities for rat which was at 37°C.

[b]nmol min^{-1} mg^{-1} protein.

[c]pmol min^{-1} mg^{-1} protein (3-hydroxybenzo[*a*]pyrene or total metabolites).

[d]pmol mg^{-1} protein.

[e]Regulatory enzymes — PFK: phosphofructokinase (E.C.2.7.1.11), Ph: phosphorylase (E.C. 2.4.1.1.) and HK: hexokinase (E.C. 2.7.1.1); activities in μmol min^{-1} g^{-1} fresh weight.

References 1. Benedetto et al., (1981)
2. Ebberink and De Zwaan (1980)
3. James et al. (1979)
4. Lee et al. (1981)
5. Newsholme and Start (1973)
6. Schwen and Mannering (1982a)
7. Schwen and Mannering (1982b)
8. Singer and Lee (1977)
9. Stegeman and Binder (1979)
10. Stegeman and Kaplan (1981)
11. Stegeman et al. (1979)
12. Zammit and Newsholme (1976)

activity (Moore et al., 1980) have also indicated the existence of a bivalve MFO system. The results of this study confirm the earlier observations and, with the work of Ade et al. (1982), clearly establish that a cytochrome P-450-mediated MFO system is present in *Mytilus* in activities comparable to those of other marine invertebrates (Table 7). Furthermore, this MFO system is primarily or totally membrane-bound in the endoplasmic reticulum, as was concluded by Stegeman (1981b), and is localised predominantly in the digestive gland, which is in agreement with the indications of earlier enzyme studies (Anderson, 1978a; Moore et al., 1980) and the observation that this tissue is an important site in the uptake of hydrocarbons (Lee et al., 1972; Palmork and Solbakken, 1981; Riley et al., 1981; Widdows et al., 1983).

In a number of respects the mussel MFO system is indicated to be similar to that typically described for mammals (see Sato and Omura, 1978) i.e. from the presence of the cytochromes and enzyme activities, the relationship between NADPH-cytochrome *c* reductase and BP hydroxylase activities, and the inhibition studies. In other respects differences are seen: the inhibitory effect of α-naphthoflavone is more akin to that seen for other marine invertebrates (see below) and some fish (Stegeman et al., 1979) and the NADH-reductases activities are relatively higher than in mammals (Table 7 — compare microsomal components of rat and *M. edulis*); it has been suggested that reductive enzymes may be of a more primitive nature than oxidative enzymes in drug metabolism (see Carlson, 1972).

An endogenous function for the system is possibly suggested by the differences between male and female mussels; sex differences in MFO activities and components are well known in other organisms, e.g. in rat (Kremers et al., 1981), fish (Stegeman, 1981a) and crabs (Lee et al., 1977; O'Hara et al., 1982). The primary localisation of the MFO system in one or several tissue-types is well-established for vertebrates (the liver) and is also the case for the other marine invertebrates so far studied (see Lee, 1981; Stegeman and Kaplan, 1981; O'Hara et al., 1982). Similarly, the wider tissue distribution of cytochrome b_5 is often observed (e.g. Benedetto et al., 1981). The inhibition effects (see Table 6) were similar to those observed for other marine invertebrates, viz. percentage inhibitions were, 100 μM SKF-525A: 35% (the crab *Carcinus maenas*; O'Hara et al., 1982) to 48% (the crab *Callinectes sapidus*; Singer et al., 1980); 100 μM α-naphthoflavone 21% (the ragworm *Nereis virens*; Lee and Singer, 1980) to 84% (*C. sapidus*) and CO (various bubbling times): 55% (*N. virens*), 70% (the barnacle *Balanus eburneus*; Stegeman and Kaplan, 1981) and 100% (*C. maenas*); the reaction was also inhibited by CO in the digestive gland of the oyster *Crassostrea virginica* (Anderson, 1978a). The significance of the incomplete inhibition by CO is unknown but may indicate that some of the BP polar metabolites are produced by enzyme systems not involving P-450 (e.g. see Reed and Marnett, 1982). It is possible to calculate P-450 turnover numbers for BP hydroxylase activity, but these can only be approximate for *M. edulis*, as they are determined in terms of total metabolites. However, the values are

similar to those for other marine invertebrates, viz. in nmol product min^{-1} $nmol^{-1}$ P-450 (calculated from Table 7), *M. edulis*, 0.23; *C. sapidus*, 0.23; *B. eburneus*, 0.39; *N. virens*, 0.22.

Comparing the data with that available for the other bivalves, *M. galloprovincialis* (Ade et al., 1982) and *M. californianus* (Krieger et al., 1979), both similarities and differences are seen. The difference absorbance maximum of cytochrome b_5 was lower than that for *M. galloprovincialis* (425 nm c.f. 428 nm) and microsomal b_5 levels were similar to those recorded for *M. californianus* but somewhat less than those for *M. galloprovincialis*. Cytochrome P-450 levels were higher than those recorded for *M. galloprovincialis*. The drop in baseline on addition of carbon monoxide to the 'oxidised' sample (see Fig. 3, traces a—c) may be due to cytochrome oxidase (E.C.1.9.3.1) (Ade et al., 1982) but if this is so then the enzyme must be already partially reduced and presumably also somewhat denatured as no difference absorbance maximum was observed at 430 nm (Vanneste, 1965). The other major CO-binding reduced-molecule absorbance peak occurred at a lower wavelength than for *M. galloprovincialis* (413—417 nm c.f. 424 nm) and was accompanied by distinct α- (568.3 ± 1.7 nm, $n = 3$) and β- (534.5 ± 0.9 nm, $n = 6$) bands indicating the haemoprotein nature of the molecule; the molecule may be a breakdown product of cytochrome P-450 but given its wide distribution is more likely to be some other haemoprotein such as a cytochrome or a peroxidase (e.g. Lindenmeyer and Smith, 1964; Appleby, 1969). The blood cell BP hydroxylase activities were somewhat greater than previously recorded aldrin expoxidation activities (Moore et al., 1980) and the failure to detect cytochrome P-450 leaves the nature of these reactions a matter for future study.

The MFO activities of bivalves are often regarded as being low but this is in comparison to the activities for vertebrates. In relation to their own metabolism, the activities are those to be expected. This point is illustrated in Table 7 where a vertebrate/invertebrate comparison is made between microsomal MFO activities and cytochromes and the specific activities of some regulatory enzymes of intermediary metabolism which represent the maximum potential fluxes through various important pathways (Newsholme and Start, 1973), viz. phosphofructokinase (glycolysis), phosphorylase (glycogenolysis) and hexokinase (glucose utilization). Comparing rat and *M. edulis* it can be seen that the activities of the regulatory enzymes of intermediary metabolism are an order of magnitude less in the bivalve and similarly, with the exception of the NADH-reductases, the same relationship is observed for the microsomal enzyme activities and components.

The BP hydroxylase activities in this study and in the study of Ade et al. (1982) were higher than those of previous studies i.e. 20—30 pmol product min^{-1} mg^{-1} protein compared with about 1—5. Furthermore, from the tissue survey data and the percentage tissue composition of the mussels, whole animal BP hydroxylase activites of 18.8 (male) and 39.7 (female) pmol min^{-1} g^{-1} wet weight can be calculated. This poses the question as to why in vivo oxidative transformations have been difficult to detect in mussels

and other bivalves (e.g. Lee et al., 1972; Widdows et al., 1983). Contributary factors to low in vitro activity may have included assay technique difficulties, the possible presence of endogenous inhibitors (Lee, 1981), and activity loss brought about by early sedimentation of microsomes with the mitochondrial fraction, solubilization of the endoplasmic reticulum and high protease activity. In relation to both in vitro and in vivo activities, three factors are seasonality, conjugation reactions and animal condition. Seasonal variations in general enzyme activities are well known for *M. edulis* (Livingstone and Clarke, 1983) and have been indicated for MFO activities (Parry et al., 1981; Stegeman, 1981b). In our own studies the digestive gland BP hydroxylase activities measured in the tissue survey were the highest we have recorded and at other times of the year activities have been very low and microsomal preparations visibly of poorer quality. Conjugation reactions have been suggested as a major fate for polar metabolites in *M. edulis* (Ernst, 1979; Brown et al., 1983) and it is possible that they could function to efficiently remove the products of the MFO reaction. Finally, the activities of drug-metabolizing enzymes are influenced by nutritional status (Fielding and Hughes, 1976) and presumably animal health; in *M. edulis*, declines in general enzyme activities can be observed even in apparently well-maintained laboratory animals (Livingstone and Bayne, 1974) and it is possible that the MFO system may be susceptible to general stress effects.

In conclusion, although much more work is required on the characterization of the MFO system in *M. edulis*, particularly in relation to in vivo metabolism, the cytochemical NADPH-neotetrazolium reductase enzyme activity and the indications of inducibility of the system (Anderson 1978b; Moore et al., 1980; Dixon et al., 1983; Guillaume et al., 1983; Payne et al., 1983), the debate as to its existence can no longer be considered necessary. The likelihood is that in many respects it will be similar to the mammalian MFO system, but equally, differences may exist as indicated by the unusual BP metabolite profile of *C. virginica* (Anderson, 1978b). The potential for its development as a specific index of organic pollution and biological impact therefore exists and future research can now be directed to this aim.

ACKNOWLEDGEMENTS

The work of R.K. Pipe in sectioning and taking the electron micrographs of the subcellular fractions and of A. Nunn for the NAGASE assays is gratefully acknowledged. Dr. B.L. Bayne and Dr. M.N. Moore are thanked for critically reading the manuscript. This work forms part of the estuarine and nearshore research programme of the Institute for Marine Enviromental Research. It was supported in part by the Department of the Environment (Contract No. DGR/480/47).

REFERENCES

Ade, P., M.G. Banchelli Soldaini, M.G. Castelli, E. Chiersara, F. Clementi, R. Fanelli, E. Funari, G. Ignesti, A. Marabini, M. Oronesu, S. Palmero, R. Pirisino, A. Ramundo

Orlando, V. Silano, A. Viarengo and L. Vittozzi, 1982. Comparative biochemical and morphological characterizations of microsomal preparations from rat, quail, trout, mussel and *Daphnia magna*. In: E. Hietanen, M. Laitinen and O. Hänninen (Eds.), Cytochrome P-450. Biochemistry, Biophysics and Environmental Implications. Elsevier Biomedical, Amsterdam, pp. 387—390.

Anderson, R.S., 1978a. Developing an invertebrate model for chemical carcinogenesis: metabolic activation of carcinogens. Comp. Pathobiol., 4: 11—24.

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

Appleby, C.A., 1969. Electron transport systems of *Rhizobium japonicum*. I. Haemoprotein P-450, other CO-reactive pigments, cytochromes and oxidases in bacteroids from N_2-fixing root nodules. Biochim. Biophys. Acta, 172: 71—87.

Baginski, E.S., P.P. Foà and B. Zak, 1974. Glucose-6-phosphatase. In: H.U. Bergmeyer, (Ed.), Methods of Enzymatic Analysis. Academic Press, New York, pp. 876—880.

Barrett, A.J. and M.F. Heath, 1977. Lysosomal enzymes. In: J.T. Dingle (Ed.), Lysosomes, a Laboratory Handbook. North-Holland Publishing Co., Amsterdam, pp. 19—145.

Bayne, B.L., M.N. Moore, J. Widdows, D.R. Livingstone and P.N. Salkeld, 1979. Measurement of the responses of individuals to environmental stress and pollution: studies with bivalve molluscs. Phil. Trans. R. Soc. Lond. Ser. B, 286: 562—581.

Benedetto, C., M.U. Dianzani, M. Ahmed, K. Cheeseman, C. Connelly and T.F. Slater, 1981. Activation of carbon tetrachloride, and distribution of NADPH-cytochrome *c* reductase, cytochrome P-450, and other microsomal enzyme activities in rat tissues. Biochim. Biophys. Acta, 677: 363—372.

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

Brown, D.A., R.W. Gossett, S.R. McHugh and K.D. Jenkins, 1983. Metabolism and detoxification — toxification of xenobiotic organics in the marine environment. In: 4th International Ocean Disposal Symposium, Plymouth, U.K., in press.

Cairns, J., Jr. and W.H. van der Schalie, 1980. Biological monitoring. Part I — Early warning systems. Water Res., 14: 1179—1196.

Carlson, G.P., 1972. Detoxification of foreign organic compounds by the quahaug, *Mercenaria mercenaria*. Comp. Biochem. Physiol., 43B: 295—302.

Croxton, F.E., 1953. Elementary Statistics with Applications in Medicine and the Biological Sciences. Dover Publications Inc., New York, 376 pp.

Dallner, G., 1963. Studies on the structural and enzymic organization of the membranous elements of liver microsomes. Acta Pathol. Microbiol. Scand. Suppl., 166: 94 pp.

DePierre, J.W., M.S. Moron, K.A.M. Johannesen and L. Ernster, 1975. A reliable, sensitive and convenient radioactive assay for benzopyrene monooxygenase. Anal. Biochem., 63: 470—484.

Dixon, D.R., I.M. Jones and F.H. Harrison, 1983. Cytogenetic evidence of inducible processes linked with xenobiotic metabolism in larval and adult *Mytilus edulis*. Mar. Environ. Res., in press.

Ebberink, R.H.M. and A. de Zwaan, 1980. Control of glycolysis in the posterior adductor muscle of the sea mussel *Mytilus edulis*. J. Comp. Physiol., 137: 165—171.

Ernst, W., 1979. Metabolic transformation of 1-(1-^{14}C) naphthol in bioconcentration studies with the common mussel *Mytilus edulis*. Veröff. Inst. Meeresforsch. Bremerhaven, 17: 233—240.

Estabrook, R.W., J. Peterson, J. Baron and A. Hildebrandt, 1972. The spectrophotometric measurement of turbid suspensions of cytochromes associated with drug metabolism. In: C.F. Chignell (Ed.), Methods in Pharmacology, Vol. 2, Physical Methods. Appleton-Century-Crofts, Educational Division, Meredith Corp., New York, p. 303.

Estabrook, R.W. and J. Werringloer, 1978. The measurement of difference spectra:

application to the cytochromes of microsomes. In: S. Fleischer and L. Packer (Eds.), Methods of Enzymology, Vol. LII. Biomembranes: Part C: Biological Oxidations, Microsomal, Cytochrome P-450, and other Hemoprotein Systems. Academic Press, New York, pp. 212—220.

Fielding, A.M. and R.E. Hughes, 1976. Changes in liver microsomal cytochrome P-450 induced by dietary proteins and lipid material. Biochem. Pharmacol., 25: 1916—1917.

Guillaume, J.R., D. Carles, M. Leveau, J.C. Bertrand and M. Gilewicz, 1983. Induction of cytochrome P-450 by hydrocarbons in a mollusc bivalve: *Mytilus galloprovincialis*. In: Proceedings of the 5th Conference of the European Society for Comparative Physiology and Biochemistry; Physiological and Biochemical Aspects of Marine Biology, Springer-Verlag, in press.

Ichikawa, Y., T. Yamano and H. Fujishima, 1969. Relationship between interconversion of cytochrome P-450 and P-420 and its activities in hydroxylations and demethylations by P-450 oxidase systems. Biochim. Biophys. Acta, 171: 32—46.

James, G.T., 1978. Inactivation of the protease inhibitor phenylmethylsulfonyl fluoride in buffers. Analyt. Biochem., 86: 574—579.

James, M.O., M.A.Q. Khan and J.R. Bend, 1979. Hepatic microsomal mixed-function oxidase activities in several marine species common to coastal Florida. Comp. Biochem. Physiol., 62C: 155—164.

Johannesen, K., J.W. DePierre, A. Bergstrand, G. Dallner and L. Ernster, 1977. Preparation and characterization of total, rough and smooth microsomes from the lung of control and methylcholanthrene-treated rats. Biochim. Biophys. Acta, 496: 115—135.

Khan, M.A.Q., A. Kamal, R.J. Wolin and J. Runnels, 1972. In vivo and in vitro *epoxidation of aldrin* by aquatic food chain organisms. Bull. Environ. Contam. Toxicol., 8: 219—228.

Kmetec, E., 1966. Spectrophotometric method for the enzymic microdetermination of succinic acid. Analyt. Biochem., 16: 474—480.

Kremers, P., P. Beaune, T. Cresteil, J. de Graeve, S. Columelli, J. Leroux and J.E. Gielen, 1981. Cytochrome P-450 monooxygenase activities in human and rat liver microsomes. Eur. J. Biochem., 118: 599—606.

Krieger, R.I., S.J. Gee, L.O. Lim, J.H. Ross and A. Wilson, 1979. Disposition of toxic substances in mussels (*Mytilus Californianus*): Preliminary metabolic and histologic studies. In: M.A.Q. Khan, J.J. Lech and J.J. Menn (Eds.), Pesticide and Xenobiotic Metabolism in Aquatic Organisms. Amer. Chem. Soc. Symp. Ser., 99: 259—277.

Krieger, R.I., S.J. Gee and L.O. Lim, 1981. Marine bivalves, particularly mussels, *Mytilus* sp., for assessment of environmental quality. Ecotoxicol. Environ. Safety, 5: 72—86.

Lee, R.F., 1981. Mixed function oxygenases (MFO) in marine invertebrates. Mar. Biol. Lett., 2: 87—105.

Lee, R.F., J.M. Davies, H.C. Freeman, A. Ivanovici, M.N. Moore, J. Stegeman and J.F. Uthe, 1980. Biochemical techniques for monitoring biological effects of pollution in the sea. In: A.D. McIntyre and J.B. Pearce (Eds.) Biological Effects of Marine Pollution and the Problems of Monitoring. Rapp. P. V. Reun. Cons. Int. Explor. Mer, 179: 48—55.

Lee, R.F., E. Furlong and S. Singer, 1977. Metabolism of hydrocarbons in marine invertebrates: aryl hydrocarbon hydroxylase from the tissues of the blue crab, *Callinectes sapidus* and the polychaete worm, *Nereis* sp. In: C.S. Giam (Ed.), Pollutant Effects on Marine Organisms, Lexington Books, Lexington, pp. 111—124.

Lee, R.F., R. Sauerhebel and A.A. Benson, 1972. Petroleum hydrocarbons: uptake and discharge by the marine mussel *Mytilus edulis*. Science, 177: 344—346.

Lee, R.F. and S.C. Singer, 1980. Detoxifying enzymes system in marine polychaetes: increases in activity after exposure to aromatic hydrocarbons. In: A.D. McIntyre and J.B. Pearce (Eds.), Biological Effects of Marine Pollution and the Problems of Monitoring. Rapp. R. V. Reun. Cons. Int. Explor. Mer, 179: 29—32.

Lee, R.F., S.C. Singer and D.S. Page, 1981. Responses of cytochrome P-450 systems in marine crab polychaetes to organic pollutants. Aquat. Toxicol., 1: 355—365.

Lind, C. and L. Ernster, 1974. A possible relationship between DT diaphorase and the aryl hydrocarbon hydroxylase system. Biochem. Biophys. Res. Commun., 56: 392—400.

Lindenmeyer, A. and L. Smith, 1964. Cytochromes and other pigments of Baker's Yeast grown aerobically and anaerobically. Biochim. Biophys. Acta, 93: 445—461.

Livingstone, D.R., 1981. Induction of enzymes as a mechanism for the seasonal control of metabolism in marine invertebrates: glucose-6-phosphate dehydrogenases from the mantle and hepatopancreas of the common mussel *Mytilus edulis* L. Comp. Biochem. Physiol., 69B: 147—156.

Livingstone, D.R. and B.L. Bayne, 1974. Pyruvate kinase from the mantle tissue of *Mytilus edulis* L. Comp. Biochem. Physiol., 48B: 481—497.

Livingstone, D.R. and K.R. Clarke, 1983. Seasonal changes in hexokinase from the mantle tissue of the common mussel *Mytilus edulis* L. Comp. Biochem. Physiol., 74B: 691—702.

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

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

Moore, M.N., D.R. Livingstone, P. Donkin, B.L. Bayne, J. Widdows and D.M. Lowe, 1980. Mixed function oxygenases and xenobiotic detoxication/toxication systems in bivalve molluscs. Helgol. Wiss. Meeresunters., 33: 278—291.

Moore, M.N., 1982. Personal communication.

Neff, J.M., 1979. Polycyclic Aromatic Hydrocarbons in the Aquatic Environment: Sources, Fates and Biological Effects. Applied Science Publishers, London, 262 pp.

Newsholme, E.A. and C. Start, 1973. Regulation in Metabolism. Wileys, London, Chichester, 349 pp.

Nordlie, R.C., 1974. Metabolic regulation by multifunctional glucose-6-phosphatase. In: B.L. Horecker and E.R. Stadtman (Eds.), Current Topics in Cellular Regulation. Vol. 8. Academic Press, New York, pp. 33—117.

O'Hara, S.C.M., E.D.S. Corner, T.E.V. Forsberg and M.N. Moore, 1982. Studies on benzo[*a*]pyrene monooxygenase in the shore crab *Carcinus maenas*. J. Mar. Biol. Assoc. U.K., 62: 339—357.

Omura, T. and R. Sato, 1964. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem., 239: 2370—2378.

Palmork, K.H. and J.E. Solbakken, 1981. Distribution and elimination of [9-^{14}C] phenanthrene in the horse mussel (*Modiolus modiolus*). Bull. Environ. Contam. Toxicol., 26: 196—201.

Parry, J.M., M. Kadhim, W. Barnes and N. Danford, 1981. Assays of marine organisms for the presence of mutagenic and/or carcinogenic chemicals. In: C.J. Dawe, J.C. Harshbarger, S. Kondo, T. Sugimura and S. Takayama (Eds.), Phyletic Approaches to Cancer. Japan Scientific Societies Press, Tokyo, pp. 141—166.

Payne, J.F., A. Rahimtula and S. Lim, 1983. Metabolism of petroleum hydrocarbons by marine organisms: the potential of bivalve molluscs. In: 26th Annual Meeting of the Canadian Federation of Biological Societies, Vol. 26, no. PA-235.

Pohl, U. and R. Wiermann, 1981. NADH/NADPH-cytochrome *c* reductase assay: interference by non-enzymatic cytochrome *c* reducing activity in plant extracts. Anal. Biochem., 116: 425—432.

Raw, I. and H.R. Mahler, 1959. Studies of electron transport enzymes. III. Cytochrome b_5 of pig liver mitochondria. J. Biol. Chem., 234: 1867—1873.

Reed, G.A. and L.J. Marnett, 1982. Metabolism and activation of 7,8-dihydrobenzo[*a*] pyrene during protaglandin biosynthesis. J. Biol. Chem., 257: 11368—11376.

Reynolds, E.S., 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol., 17: 208—212.

Riley, R.T., M.C. Mix, R.L. Schaffer and D.L. Bunting, 1981. Uptake and accumulation of naphthalene by the oyster *Ostrea edulis* in a flow-through system. Mar. Biol., 61: 267—276.

Sato, R. and T. Omura, 1978. Cytochrome P-450. Kodansha Ltd., Tokyo, Academic Press, New York, 233 pp.

Schwen, R.J. and G.J. Mannering, 1982a. Hepatic cytochrome P-450-dependent monooxygenase systems of the trout, frog and snake. I. Components. Comp. Biochem. Physiol., 71B: 431—436.

Schwen, R.J. and G.J. Mannering, 1982b. Hepatic cytochrome P-450-dependent monooxygenase systems of the trout, frog and snake. II. Monooxygenase activities. Comp. Biochem. Physiol., 71B 437—443.

Shimakata, T., V. Mihara and R. Sato, 1972. Reconstitution of hepatic microsomal stearoyl-coenzyme A desaturase system from solubilized components. J. Biochem., 72: 1163—1174.

Singer, S.C. and R.F. Lee, 1977. Mixed function oxygenase activity in blue crab, *Callinectes sapidus*: tissue distribution and correlation with changes during molting and development. Biol. Bull., 153: 377—386.

Singer, S.C., P.E. March Jr., F. Gonsoulin and R.F. Lee, 1980. Mixed function oxygenase activity in the blue crab, *Callinectes sapidus*: characterization of enzyme activity from stomach tissue. Comp. Biochem. Physiol., 65C: 129—134.

Stegeman, J.J., 1980. Mixed-function oxygenase studies in monitoring for effects of organic pollution. In: A.D. McIntyre and J.B. Pearce (Eds.), Biological Effects of Marine Pollution and the Problems of Monitoring. Rapp. P. V. Reun. Cons. Int. Explor. Mer, 179: 33—38.

Stegeman, J.J., 1981a Polynuclear aromatic hydrocarbons and their metabolism in the marine environment. In: H.V. Gelboin and P.O.P. TS'O (Eds.), Polycyclic Hydrocarbons and Cancer, Vol. 3. Academic Press, New York, pp. 1—60.

Stegeman, J.J., 1981b. Metabolism of aromatic hydrocarbons by the bivalve mollusc *Mytilus edulis*. In: 1980—81 Annual Sea Grant Report, Woods Hole Oceanogr. Inst. Annu. Rep: 15.

Stegeman, J.J. and R.L. Binder, 1979. High benzo[*a*]pyrene hydroxylase activity in the marine fish *Stenotomus versicolor*. Biochem. Pharmacol., 28: 1686—1688.

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

Stegeman, J.J. and H.B. Kaplan, 1981. Mixed-function oxygenase activity and benzo[*a*] pyrene metabolism in the barnacle *Balanus eburneus* (Crustacea: Cirripedia). Comp. Biochem. Physiol., 68C: 55—61.

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

Trelstad, R.L., K.R. Lawley and L.B. Holmes, 1981. Non-enzymatic hydroxylation of proline and lysine by reduced oxygen derivatives. Nature, 289: 310—312.

Van Cantfort, J., J. de Graeve and J.E. Gielen, 1977. Radioactive assay for aryl hydrocarbon hydroxylase. Improved method and biological importance. Biochem. Biophys. Res. Commun., 79: 505—512.

Vanneste, W.H., 1965. The a_3: total heme *a* ratio in cytochrome aa_3 of heart muscle. Biochem. Biophys. Res. Commun., 18: 563—568.

Widdows, J., S.L. Moore, K.R. Clarke and P. Donkin, 1983. Uptake, tissue distribution and elimination of [1-^{14}C] naphthalene in the mussel, *Mytilus edulis*. Mar. Biol., 76: 109—114.

Willis, D.E. and R.F. Addison, 1974. Hydroxylation of biphenyl in vitro by tissue preparations of some marine organisms. Comp. Gen. Pharmacol., 5: 77—81.

Yip, C.C., 1964. The hydroxylation of proline by horseradish peroxidase. Biochim. Biophys. Acta, 92: 395—396.

Zammit, V.A. and E.A. Newsholme, 1976. The maximum activities of hexokinase, phosphorylase, phosphofructokinase, glycerol phosphate dehydrogenases, lactate dehydrogenase, octopine dehydrogenase, phosphoenolpyruvate carboxykinase, nucleoside diphosphatekinase, glutamate-oxaloacetate transaminase and arginine kinase in relation to carbohydrate utilization in muscles from marine invertebrates. Biochem. J., 160: 447—462.

Zs.-Nagy, I., 1977. Cytosomes (yellow pigment granules) of molluscs as cell organelles of anoxic energy production. In: G.H. Bourne, J.F. Danielli and K.W. Jeon (Eds.), International Review of Cytology. Academic Press, New York, pp. 331—377.