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Induction of a putative monooxygenase of crabs (*Carcinus* spp.) by polycyclic aromatic hydrocarbons

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As part of a programme to develop biomarker assays for polycyclic aromatic hydrocarbons (PAHs) in marine invertebrates, two species of crabs, *Carcinus maenas* and *Carcinus aestuarii* were exposed to benzo(*a*)pyrene (B(*a*)P) or crude oil. Microsomes were prepared from the midgut gland (hepatopancreas), examined by gel electrophoresis and Western blotting and assayed for B(*a*)P monooxygenase activity. In early experiments there was evidence of protein degradation and results were inconsistent and inconclusive. However, when steps were taken to minimize this in subsequent experiments, including the inclusion of four protease inhibitors in the homogenization buffer, there was consistent evidence for an increase of proteins of estimated molecular weight 45–60 kDa, and particularly of a distinct band at *c.* 48 kDa, following exposure to PAH at levels down to 0.1 ppm in ambient water. In *C. aestuarii* the increase in this band was found to coincide with an 8–12-fold increase of B(*a*)P monooxygenase activity in midgut gland microsomes. These results suggest that one or more forms of cytochrome P450 may be induced by PAHs in these species. However, Western blotting using antibodies raised to vertebrate P450s, and representing four different gene families, failed to recognize any proteins in either the PAH-treated samples or in the controls. The isolation and characterization of induced protein, and the production of antibodies may provide the basis for a biomarker assay to measure a response to environmental PAHs in crabs.

Keywords: Biomarker, cytochrome: P450, *Carcinus*; Crabs, Benzo(*a*)pyrene), microsomes.

Introduction

In recent years there has been a rapid growth of interest in the use of biomarker strategies to assess the impact of pollutants upon the marine environment (McCarthy and Shugart 1990, Peakall 1992, Depledge and Fossi 1994, Lagadic *et al.* 1994, Walker 1995). Biomarker assays provide measures of biological responses to environmental pollutants at differing levels of organization, and thus allow an interpretation of residue levels found in the marine environment which aids the process of risk assessment (Walker 1996). Historically, a great deal of residue data has been obtained for pollutants in sediments, sea water, and marine organisms which has raised concern about possible harmful effects at the levels of individual organism, population, community and ecosystem. Very seldom, however, has it been possible to establish the biological significance of such data (McCarthy and Shugart 1990, Peakall 1992, Peakall and Shugart 1993). Regarding

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marine organisms, there has been a certain amount of progress in the development of biomarker assays for fish and fish hepatocytes (for recent overview see Walker 1998). In particular, immunochemical assays have been developed for the induction of cytochrome P450 1A1 (Stegeman and Hahn 1994, Goksøyr 1995, Livingstone and Stegeman 1998), and DNA damage caused by environmental carcinogens and mutagens has been measured using P32 postlabelling (Varanasi *et al.* 1992, Mitchelmore *et al.* 1998). Less has been done with marine invertebrates, with the possible exception of the edible mussel (*Mytilus edulis*) (Livingstone 1991, Sole *et al.* 1998). This is surprising given the importance of invertebrates in the marine ecosystem and the current ethical concern about the replacement of vertebrate animals in ecotoxicity testing (Walker *et al.* 1991, Walker and Savva 1998). A number of marine invertebrates have potential as bioindicator organisms in which biomarker assays can be performed. Many species are abundant, of wide distribution, and can be used as sentinel species because they are sessile or have only limited mobility. Notwithstanding these advantages, the development of biomarker assays for them has not proved to be simple or straightforward. In the first place, many assays that have been tried with invertebrates are based upon assays developed for vertebrates. Much of the fundamental work in biochemical toxicology has been done upon vertebrate species rather than invertebrates, this reflecting the far greater investment in medical toxicology than in ecotoxicology. Because of the large biochemical and physiological differences between the two groups, the adaptation of vertebrate biomarker assays for use in invertebrate species has generally been difficult and sometimes impossible. The limited knowledge of the biochemical toxicology of invertebrates represents a serious handicap when attempting to develop biomarker assays for them.

The work reported here was part of a collaborative project funded by the European Commission (EV5V-CT94-0398), the principal aim of which was to develop and evaluate some biomarker assays for selected marine invertebrate species exposed to major types of pollutant which might contribute to environmental risk assessment. For this work, crabs (*Carcinus* spp.) were selected as one of the main indicator species. They are widely distributed in western Europe, and have the advantages of being large enough to provide sufficient material for biomarker assays and of being relatively easy to maintain in the laboratory. At the time of the commencement of the project, some progress had already been made in the development of biochemical (O'Hara *et al.* 1985) and physiological (Depledge and Andersen 1990) assays which could serve the function of biomarkers. Here we describe a study of the biochemical responses of two species of crabs, the shore crab (*Carcinus maenas*) and the Mediterranean crab (*Carcinus aestuarii*) to polycyclic hydrocarbons (PAHs) in the laboratory. PAHs have long been recognized as major pollutants of the marine environment, even in polar regions (Kennicutt *et al.* 1995), and were therefore very appropriate for the present investigation. The objective was to develop certain biochemical biomarker assays in the laboratory, with the intention of using and validating them in the field at a later stage. The close comparison of dose–response curves obtained in the laboratory, with dose–response relationships found in the field was a central concept in this project. The development of physiological biomarker assays which has been reported elsewhere (Bamber and Depledge 1997) was an integral part of this project. The longer term objective was to develop combinations of biomarker assays (biochemical and physiological) which can measure the time-related sequence of

linked responses, at differing levels of biological organization, which represent the underlying process of toxicity (Huggett *et al.* 1992, Peakall 1992, Walker 1998), leading eventually to the appearance of toxic symptoms. The project also included work on other pollutants (PCBs and heavy metals) and investigated some biomarker responses in species other than crabs (*Nereis* spp., and *Mytilus edulis*).

Materials and Methods

Chemicals

In general, chemicals used were of appropriate grade for the procedures described. Unless otherwise indicated, chemicals used for the preparation of buffers and for assay procedures were supplied by Sigma, Poole, UK. A sample of North Sea crude oil (Auk field) was a gift from Dr P. Donkin of Plymouth Marine Laboratory. The nature and source of antibodies used for Western blotting are detailed in table 1. Further details are given, where necessary in the following descriptions of procedures.

Animals

Adult shore crabs (*Carcinus maenas*) were collected at a 'clean' coastal site near Plymouth, UK and Mediterranean crabs (*Carcinus aestuarii*) from 'clean' sites in Tuscany, Italy. *C. maenas* was maintained in filtered sea water of salinity 34 ppt, or in artificial sea water, in both cases at 16 (±2)°C with a 12:12 light:dark regime, both pending and during experimentation. *C. aestuarii* was kept at 20 (±2)°C, with a light period from 6 am to 8 pm.

Preliminary experiments

For preliminary experiments with *C. maenas*, three groups of four crabs were dosed according to the following scheme. Group 1, 1 μl dimethyl formamide (DMF) containing 20 μg of benzo (*a*) pyrene (B(*a*)P); Group 2, 1 μl of DMF alone; Group 3 were treated as controls, and not dosed at all. A further group of crabs were exposed to 2 ml of North Sea crude oil which was added directly to the water in their tank (vol. 201). Injections were performed initially by withdrawing approximately 100 μl of haemolymph with a syringe by penetrating the arthrodal membrane, combining this with 1 μl of DMF or DMF+B(*a*)P, sonicating for 30 s, and then reinjecting the entire volume into the haemolymph. For all treatments, crabs were maintained for 3 days after treatment and then sacrificed (3 days was judged to be long enough for an induction to clearly show after direct dosing). In an initial experiment with *C. aestuarii*, the same protocol was followed as described above, but dimethyl sulphoxide (DMSO) was used as vehicle instead of DMF. In all of these early experiments, the midgut gland was removed, and then homogenized in Buffer 1 (see Table 1).

Later experiments

In one later experiment with *C. aestuarii*, 50 crabs were divided into six groups, and treated as follows. Group 1 ($n = 5$) was a control group and was not dosed. Group 2 ($n = 5$) was kept in water to which had been added Tween 80 (1%). Groups 3–6 ($n = 10$) were kept in water to which Tween 80 had also been added but after incorporation of different concentrations of B(*a*)P, such that they were exposed to the following B(*a*)P concentrations in their ambient water—Group 3, 1 mg l^{-1}; Group 4,

Table 1. Antibodies used for Western blotting.

No.	Description	Supplier
1	Monoclonal antibody, anti-P450 1A1 (cod)	Dr A. Goksøyr (The University of Bergen, Norway)
2	Polyclonal antibody, anti-P450 1A1 (perch)	Prof. Lars Förlin (The University of Göteborg, Sweden)
3	Polyclonal antibody, anti-P450 1A1 (rat)	Dr C. Ioannides (The University of Surrey, UK)
4	Polyclonal antibody, anti-P450 3A (rat)	Dr M. Ingelman-Sundberg (The University of Stockholm, Sweden)
5	Polyclonal antibody, anti-P450 4A (rat)	Prof G. Gibson (The University of Surrey, UK)
6	Polyclonal antibody, anti-P450 2C6 (rat)	Dr C. J. Henderson (Imperial Cancer Research Fund, UK)

100 μg l^{-1}; Group 5, 10 μg l^{-1} and Group 6, 1 μg l^{-1}. (The doses chosen for this study ranged from environmentally realistic levels of PAH to very high levels, and were intended to gain information about the dose–response relationship.) Crabs were arranged in groups of six in glass tanks, each containing 8 l of artificial sea water. B(*a*)P had been incorporated into Tween 80 to improve its dispersion in water, with the intention of facilitating uptake by crabs. After 10 days of exposure, crabs were sacrificed. Later experiments with *C. maenas* also employed the dosing regimens just described for *C. aestuarii*. (A longer period of exposure was used for this method of dosing to allow time for adequate uptake of PAH to occur, there being no available information on kinetics of uptake.) Other later experiments involved dosing *C. maenas* in the manner just described, or as detailed under preliminary experiments. In these later experiments with both species and differing dosing regimens, the following changes were made in the procedures used for sampling tissues and preparing homogenates from those described under preliminary experiments. Crabs were held at 0–4°C, an area of carapace removed, and then killed by dissecting out central ganglia. The midgut gland was bathed in protective Buffer 2 (see Table 2), immediately after the removal of carapace and before dissection of the midgut gland, and homogenates were prepared in this buffer solution. Some samples were also prepared using Buffer 1, to allow comparison between the earlier and the later procedures.

Preparation of microsomes

Homogenates were prepared in Buffer 1 or Buffer 2 (see table 2), and were then spun for 30 min at 10,000 *g* and 0–4°C. The supernatant was withdrawn and spun at 105,000 *g* for 60 min at 0–4°C to bring down the microsomal pellet. The microsomal pellet was resuspended in the buffer that had been used to prepare the homogenate for immediate use, or in 2.6 ml per g resuspension buffer (10 mM Tris, 20% glycerol, pH 7.6) if storing at –70°C. Aliquots of microsomal suspensions were taken for determination of protein by the Lowry method (Lowry *et al.* 1951).

SDS–PAGE and Western blots

Only samples resulting from the procedures described in the 'Later experiments' section were submitted for Western blotting. Microsomal suspensions were added to SDS sample buffer and loaded on to SDS–PAGE gels prepared according to the procedure of Laemmli (1970) (approximately 150 μg of microsomal protein per lane). Rainbow coloured protein molecular weight markers containing myosin (MW 200 kDa), phosphorylase b (MW 97.4 kDa), bovine serum albumin (MW 69 kDa), ovalbumin (MW 46 kDa), carbonic anhydrase (MW 30 kDa), trypsin inhibitor (MW 21.5 kDa), and lysozyme (MW 14.4 kDa) were used as protein standards for gels at approximately 7 μg for each protein per marker lane. After electrophoresis, gels were either stained with Coomassie Brilliant Blue R and scanned on an LKB Ultroscan XL or used for Western blotting.

Electrophoretic transfer of proteins from the gel to a PVDF membrane was accomplished using a Sammy-Dry semi-dry blotter from Schleicher and Schuell. Blotting was carried out as described by Bollag *et al.* (1996). When transfer had been completed, the membrane was blocked overnight at 4°C in a phosphate buffer (pH 7.4) containing 1% dried skimmed milk (Marvel™) and 1% Polypep R. The membrane was incubated with primary antibody (see Table 1) for 2 h at room temperature and then washed thoroughly in phosphate buffer containing 1% Triton X100 before incubating for 2 h at room temperature with a secondary antibody (anti-sheep or anti-rabbit) IgG conjugated with horseradish

Table 2. Composition of buffers.

Buffer 1	
50 mM	Potassium phosphate buffer (pH 7.4)
0.4 mM	PMSF
0.1 mM	Phenylthio-urea(PTU)
0.5 mM	EDTA
Buffer 2	
50 mM	Potassium phosphate (pH 7.4)
750 mM	Sucrose
1 mM	Ethylene diamine tetracetic acid (EDTA)
0.5 mM	Dithiothreitol (DTT)
0.4 mM	PMSF
0.01 mM	Leupeptin
0.001 mM	Pepstatin
1 mg l^{-1}	Aprotinin

All reagents for buffers were supplied by Sigma.

peroxidase). The membrane was washed once again, as before, prior to developing the blot using an enhanced chemiluminescence kit supplied by Amersham International plc, Bucks, UK. Development of blots was initially for the time needed to obtain strong development of positive controls (1–10 min), but subsequently for extended periods (<100 min) to ensure that immunoreactive protein was not overlooked. The developed blots were scanned with an LKB Ultrascan XL.

Benzo(a)pyrene monooxygenase (BPMO) assay

BPMO activity was assayed by the method of Kurelec *et al* (1977). Briefly, 0.4 ml microsomal suspension (representing 0.4 g tissues per ml) was added to the following buffer solution; 0.11 M Tris HCl at pH 7.6, 15 mM $MgCl_2$ 0.1 M quinine sulphate, 2 mM B (*a*)P·NADPH was added to, give 1.8 mM final concentration, and incubation proceeded for 1 h at 30°C. Spectrofluometric readings were taken at 396 nm and 522 nm and activity was expressed in fluorometric units, FU mg $protein^{-1}$ h^{-1}.

Results

Initial studies with midgut gland microsomes using Buffer system 1 (i.e. the buffer system without the full complement of proteinase inhibitors), gave variable and unsatisfactory results with both species of *Carcinus*. Inspection of SDS–PAGE gels revealed relatively large quantities of low molecular weight proteins, with very little sign of protein bands in the region 40–60 kDa. Also, BPMO activities were generally low and highly variable. A possible explanation of this was that proteins originally present in the microsomal fraction, including forms of cytochrome P450 which might express BPMO activity, were being degraded to products of lower molecular weight by the action of hydrolases during the course of preparing homogenates and microsomes. A problem here can be the instability of lysosomes, which release hydrolytic enzymes if they are damaged during preparation. Accordingly, the procedures for removing midgut gland and for preparing homogenates and microsomes was changed in an attempt to minimize any effect of this kind. Changes in the procedure were made, taking guidance from an earlier isolation of P450 from the midgut gland of the spiny lobster (*Panulinus argus*) (James 1990 and personal communication). In the first place 750 mM sucrose was included in a new buffer (Buffer 2) to aid stabilization of lysosomes (table 2), and the midgut gland was bathed in this immediately after removal of carapace and before removal of midgut gland. Secondly, three further proteinase inhibitors, leupeptin, pepstatin, and aprotonin, were included, in addition to PMSF, which had been the only one in the original buffer system. Following this modification of procedure there was a marked improvement in the results. After SDS–PAGE, a number of clearly differentiated bands were seen in the region 40–60 kDa, and there was relatively little material of low molecular weight. The BPMO assay gave higher and less variable results in the case of *c. aestuarii.*. This improvement lent support to the idea that the initial assays had been affected by hydrolase activity.

For these latter experiments, comparisons were made between SDS–PAGE banding patterns for controls, and those for individuals exposed to B(*a*)P or crude oil. Inspection of gels and scans of gels revealed that a band, estimated to be at approximately 48 kDa by comparison with molecular markers, appeared or was greatly increased relative to other major peaks after exposure to PAH. Figure 1 gives an example of an SDS–PAGE gel for midgut gland microsomes of *C. aestuarii* from individuals exposed to 10 μg l^{-1} 100 μg l^{-1} or 1 mg l^{-1} of B(*a*)P in ambient water (Tween vehicle), compared with control. When comparing treated samples with controls, there was a large increase in the broad band at 47–48 kDa in all treated samples relative to other bands resolved on the gel. In this experiment

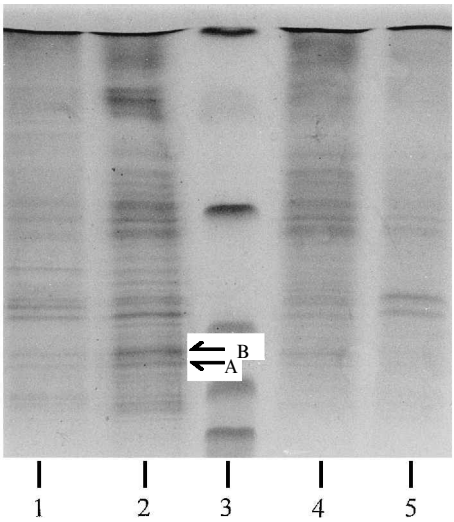

Figure 1. Photograph of an SDS–PAGE gradient (8 to 18%) gel stained with Coomassie Brilliant Blue R. The top of the photograph is the top of the gel (high molecular weight proteins). Lanes 1, 2 and 4 represent crabs (*C. aestuarii*) exposed to 10 μg l^{-1}, 100 μg l^{-1} and 1 mg l^{-1} of B(*a*)P respectively. Lane 3 contains markers of 30, 46, 69 and 97 kDa, and lane 5 represents controls exposed to Tween vehicle only. On the gel, A is a protein band of 47–48 kDa and B is a protein band of 53–59 kDa. Pooled microsomes were used for this separation (10 individuals for each treated sample and five individuals for the control).

the microsomal protein yield for control and treated samples was 0.85±0.05 and 1.05±0.04 mg^{-1} midgut gland respectively. Thus, protein yield was increased after dosing, and the yield of 47–48 kDa was estimated to be five-fold or more greater in all treated samples in comparison with controls. On closer inspection of the original gel, using a magnifying glass, a narrow band at 48 kDa was distinguishable from the main band in all treated samples (most strongly in lane 2) which was not visible in the control. This detail cannot be seen in the photograph as reproduced here, but was clearly visible in gel scans. Figure 2 shows a scan of an SDS–PAGE gel of midgut gland microsomes from *C. maenas* injected with 20 μg B(*a*)P, again compared with control, where there is a substantial peak at 48 kDa in treated microsomes but only a very small one in the same position in the control. In both cases there is a marked increase in 48 kDa in treated individuals in comparison with controls. This observation has now been made in four consecutive experiments with *C. maenas* or *C. aestuarii* exposed to either B(*a*)P or crude oil (the smallest number of individuals in any group, in any experiment, was four). There was also evidence of an increase of one or more proteins in the general region 53–59 kDa (see area B in Figure 1). However, these bands were not well resolved, making interpretation difficult.

Microsomal preparations from later experiments which showed this difference between treated samples and controls were examined by Western blotting (table 1). Six different antibodies were tried, representing four different gene families (P450s 1, 2, 3, and 4); all had been raised to vertebrate forms of P450, four of them to mammalian forms and two to piscine forms. In no case was there any evidence of induced proteins in the region 40–60 kDa following treatment of crabs with PAH. Microsomes from rats and red-legged partridges (*Alectoris rufa* cross), which had

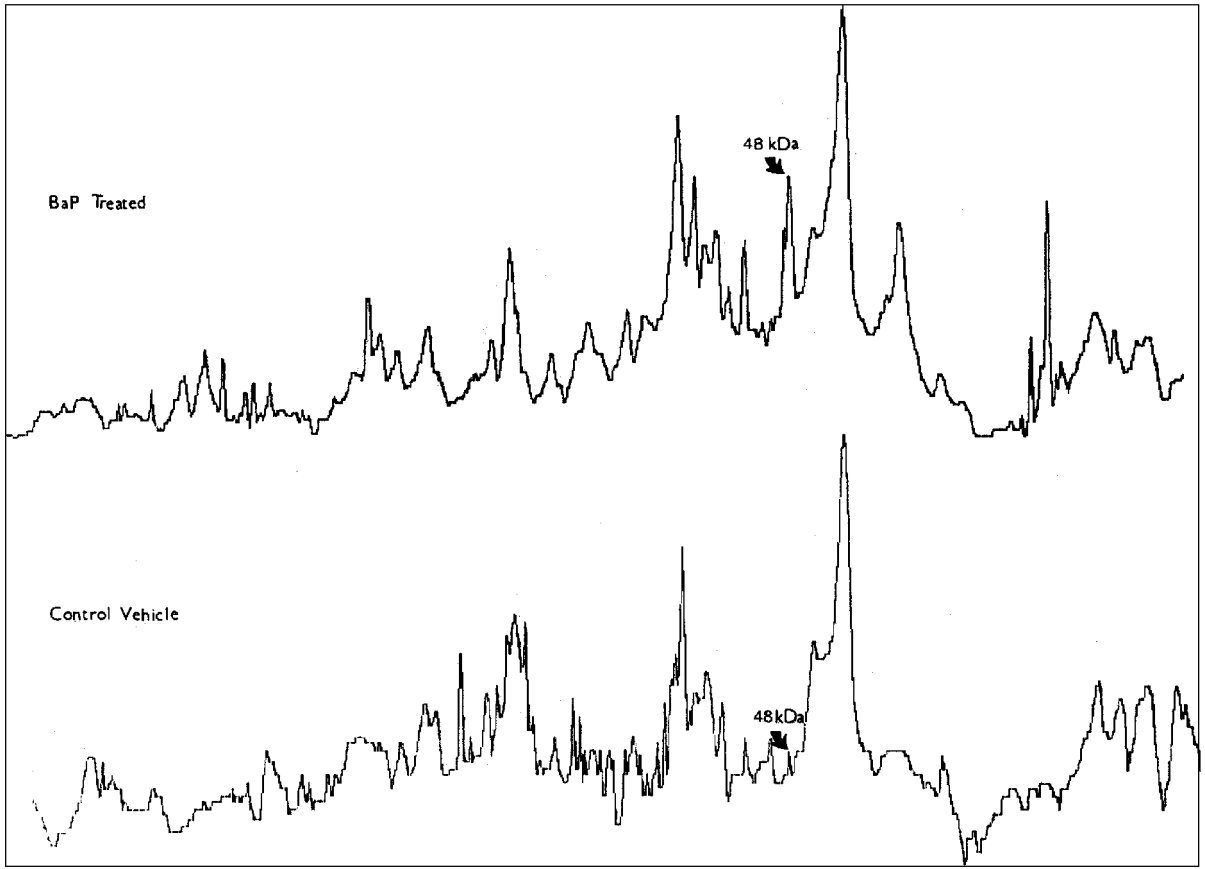

Figure 2. Scan of an SDS–PAGE gradient (8 to 18%) gel stained with Coomassie Brilliant Blue R. The top of the gel (high molecular weight proteins) is on the left of the scan. *C. maenas* was dosed by injecting 20 μg of B(*a*)P dissolved in 1 μl of DMF; controls received 1 μl of DMF only. Each separation was on a pooled sample of microsomes from four individuals.

been treated with inducing agents, were used as positive controls on the same gels. All antibodies, except the monoclonal antibody raised to cod P450 1A1, clearly recognized induced forms of P450 in the expected position on gels (the monoclonal antibody to cod P450 1A1 proved to be very specific, and did not, in any instance, recognize induced P450 1A1 of birds or mammals in subsequent studies).

When buffer 2 was used for preparing microsomes, there was clear evidence for the induction of BPMO activity in midgut microsomes of *C. aestuarii* following dosing with B(*a*)P (table 3 and Fossi *et al.* (1996)). Thus in midgut gland microsomes of individuals exposed to 0.1 and 1 ppm ($\mu g\ ml^{-1}$) of B(*a*)P in water, BPMO activities were respectively 12- and 8-fold greater than controls. In the same experiment, there was evidence for a 45-fold induction of BPMO activity in gills, after exposure to 1 ppm of B(*a*)P (Fossi *et al.* 1996).

Discussion

In the early stages of the present study a familiar problem was encountered—the instability of proteins and associated enzyme activities when preparing homogenates and microsomes from the tissues of crustaceans (see, for example, James 1990). This difficulty has also been encountered in the preparation of microsomes from insect tissues (Wilkinson 1979). A major factor found to cause instability in these earlier studies was the release of hydrolases, which can degrade proteins to lower molecular weight peptides during the preparation of homogenates. In the case of the midgut gland of crustaceans, hydrolases will be released if lysosomes are disrupted. In the present study satisfactory results with SDS–PAGE separations and BPMO activity assays were only obtained after the incorporation of four different proteinase inhibitors in the buffer system used for preparing homogenates by a modified procedure. This was similar to the system proposed by James (1990 and personal communication) for isolation of P450 of the spiny lobster. In this modified procedure, the midgut gland was bathed in the buffer before its removal to prepare homogenates. A relatively high sucrose content (0.75 M) was intended to prevent osmotic disruption of lysosomes. The improved separation of microsomal proteins of 40–60 kDa by SDS–PAGE, and the substantial reduction in the content of proteins of low molecular weight support the suggestion that the action of hydrolases was the major problem in the initial studies with homogenates and microsomes.

The elevation of BPMO activity in midgut microsomes of *C. aestuarii* following dosing with B(*a*)P (table 3 and Fossi *et al.* (1996)) was accompanied by a large increase in a band at *c.* 48 kDa after SDS–PAGE. The apparent induction of a protein running in this position was seen in four separate experiments in both species

Table 3. BPMO activities in microsomes of midgut gland.

Treatment (B(*a*)P)	Activities of treated samples expressed as a -fold increase compared with tween controls (± SE)
0.1 ppm	11.6 (±5)
1.0 ppm	7.6 (±2)

Activities originally calculated in fluorescence units, then expressed as a -fold increase of Tween controls ($n = 10$).

See also Fossi *et al.* (1996).

of *Carcinus*, after dosing with B(*a*)P or crude oil. In some cases there was also evidence of an increase in one or more other proteins, in the general region 53–59 kDa, although resolution was not good in this area, there being several overlapping peaks. Taken together, these results strongly suggest that there was induction of one or more forms of cytochrome P450 following dosing with PAH. There was also evidence suggesting a similar induction in gill microsomes (Fossi *et al*. 1996). The failure to detect an induced form of P450 in these samples by Western blotting, using antibodies raised against vertebrates, is not altogether surprising. Considerable differences in the forms of cytochrome P450 (and other detoxifying enzymes) are to be expected when comparing marine invertebrates with vertebrates (Livingstone and Stegeman 1998). In a development of this study the 48 kDa protein is being purified with the intention of raising antibodies to it, and developing a biomarker for environmental risk assessment (Walker and Savva 1998). The successful use of assays for P450 1A1 in field studies of fish from polluted waters illustrates the potential of this approach (Livingstone and Goldfarb 1998). As discussed earlier, there is much to gain by developing similar assays for marine invertebrates.

The possible induction of cytochrome P450 of crabs by PAHs is related to the question of genotoxic effects. In mammals, 1A1 and other forms of cytochrome P450 are known to have an important role in converting these compounds to reactive metabolites such as diol epoxides, which can form adducts with DNA and lead to mutation. Thus, the induction of P450 can, in theory, lead to an increased rate of activation of PAH, and consequently to an increase in DNA damage. Little is known about this process in crabs or other marine invertebrates. However, it is interesting to note that in the present study there was preliminary evidence for DNA damage, as well as induction of P450, in *Carcinus* spp. dosed with PAH (Fossi *et al*. 1997, Walker and Savva 1998). The alkaline unwinding assay indicated an increase in DNA strand breaks, and an ELISA assay indicated an increase in B(*a*)P/DNA adducts following dosing with PAH. This raises the interesting possibility of measuring two linked stages in the mechanism of genotoxicity using biomarker assays. Such combinations of assays linking stages of the intoxication process which differ in space and in time, can provide a more powerful tool for environmental risk assessment than individual biomarker assays (Peakall 1992, Peakall and Shugart 1993, Walker 1995).

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