REGIONAL SEAS

UNITED NATIONS ENVIRONMENT PROGRAMME JULY 1992

Hepatic mixed function oxidase induction in fish as an environmental monitoring technique

Reference Methods For Marine Pollution Studies No. 60

Prepared in co-operation with

IOC IAEA IMO FAO

UNEP 1992

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PREFACE

The Regional Seas Programme was initiated by UNEP in 1974. Since then the Governing Council of UNEP has repeatedly endorsed a regional approach to the control of marine pollution and the management of marine and coastal resources and has requested the development of regional action plans. The Regional Seas Programme at present includes ten regions and has over 120 coastal States participating in it (1),(2).

One of the basic components of the action plans sponsored by UNEP in the framework of the Regional Seas Programme is the assessment of the state of the marine environment and of its resources, and of the sources and trends of the pollution, and the impact of pollution on human health, marine ecosystems and amenities. In order to assist those participating in this activity and to ensure that the data obtained through this assessment can be compared on a world-wide basis and thus contribute to the Global Environment Monitoring System (GEMS) of UNEP, a set of Reference Methods and Guidelines for marine pollution studies is being developed as part of a programme of comprehensive technical support which includes the provision of expert advice, reference methods and materials, training and data quality assurance (3). The Methods are recommended to be adopted by Governments participating in the Regional Seas Programme.

The methods and guidelines are prepared in co-operation with the relevant specialized bodies of the United Nations system as well as other organizations and are tested by a number of experts competent in the field relevant to the methods described.

In the description of the methods and guidelines the style used by the International Organization for Standardization (ISO) is followed as closely as possible.

The methods and guidelines, as published in UNEP's series of Reference Methods for Marine Pollution Studies, are not considered as final. They are planned to be periodically revised taking into account the development of our understanding of the problems, of analytical instrumentation and the actual need of the users. In order to facilitate these revisions the users are invited to convey their comments and suggestions to:

Marine Environmental Studies Laboratory
IAEA Marine Environment Laboratory
19, Avenue des Castellans
MC 98000 MONACO

which is responsible for the technical co-ordination of the development, testing and intercalibration of Reference Methods.

This Reference Method was designed to provide the user with reliable techniques for the determination of certain specific biological effects of chemical contaminants. This is the first in a series of techniques which are a product of the work of the IOC/UNEP/IMO Group of Experts on the Effects of Pollution (GEEP). GEEP has tested each of these techniques in a series of experimental workshops in temperate and tropical conditions. The techniques offer the pollution scientist a sensitive tool for examining the effect of pollutants at sub-acute levels and provide a stronger scientific basis for marine pollution assessments than "traditional" experimental studies of acute toxicity.

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(1) UNEP: Achievements and planned development of the UNEP's Regional Seas Programme and comparable programmes sponsored by other bodies. UNEP Regional Seas Reports and Studies No. 1 UNEP, 1982.

(2) P. HULM: A Strategy for the Seas. The Regional Seas Programme: Past and Future, UNEP 1983.

(3) UNEP/IAEA/IOC: Reference Methods and Materials: A Programme of comprehensive support for regional and global marine pollution assessments. UNEP 1990.

The present document describes the use of hepatic MFO measurements as an environmental monitoring technique. The first section gives a general description and review of hepatic MFOs in fish, and the second provides detailed procedures for measuring hepatic MFO activity using relatively simple equipment and facilities.

This first edition of the Reference Method for Marine Pollution Studies No. 60 was prepared by Dr R. F. Addison of the Department of Fisheries and Oceans, Physical and Chemical Sciences Branch, Scotia-Fundy Region, Bedford Institute of Oceanography, Dartmouth, N.S., Canada. The work was supported partly by the Panel on Energy Research an Development. The author appreciates the support of D. R. Livingstone and D.E. Willis for their comments on the manuscript. The document was edited by UNEP in co-operation with the International Atomic Energy Agency (IAEA) and the Intergovernmental Oceanographic Commission (IOC).

CONTENTS

I. THE USE OF MIXED FUNCTION OXIDASES (MONO-OXYGENASES) AS AN ENVIRONMENTAL MONITORING TECHNIQUE

1. Introduction

Our present interest in using MFO induction as an environmental monitoring technique arises from work undertaken since the early 1950s to understand detoxification processes, usually in mammals, and usually focussing on drug metabolism. By the early 1970s the following points had been established:

1. There existed in vertebrate (usually mammalian) liver a group of enzymes which catalysed the metabolism of a wide range of lipophilic substrates (both endogenous and foreign) to more polar (hence more readily excreted) products.
2. These enzymes were associated with the smooth endoplasmic reticulum and could be centrifuged as "microsomes" at 100,000 x g; they required oxygen, NADPH and involved one or more isozymes of a cytochrome P450 (so-called from the absorption maximum of its CO complex).
3. These "mixed function oxidases" were inducible: under normal conditions, their activity was relatively low, but if the organism were exposed to certain compounds, their activity would increase, apparently to accelerate the degradation and clearance of the inducing compound.

This last point attracted the interest of environmental toxicologists, because many of the more powerful inducers were compounds such as polynuclear aromatic hydrocarbons (PAH) or some polychlorinated biphenyls (PCB), which were persistent environmental contaminants. Hepatic MFO activity in organisms taken from the wild might therefore indicate exposure to ambient levels of some environmental contaminants, in other words, it may serve as a field sub-lethal bioassay.

Since the mid-1970s, research on hepatic MFOs can be considered (for the purposes of this article) to have taken two directions. Research on mammalian MFOs has focussed on the basic biochemistry of the system. This aspect of the subject has been reviewed recently (Ortiz de Montellano, 1986) and will not be discussed further here. The other objective of MFO research has been to describe in detail MFO systems in aquatic biota, and to apply measurements of MFO activity, particularly in fish, as an environmental monitoring tool.

2. Hepatic MFOs in fish

Hepatic MFO systems in fish are generally similar to those in mammals in terms of their sub-cellular localisation (centrifuged as microsomes at 100,000 x g) and co-factor requirements (O_2 and NADPH). Structurally, the fish hepatic P450s of most interest (those which correspond to the mouse P4501A1: Nebert et al., 1987) are similar in terms

of amino acid sequence, structure of active site, and MW (48,000 - 56,000). Substrate specificity and the structure of products formed during P450-catalysed reactions are also generally similar in fish and in mammals, but may differ in detail (e.g., Goksøyr et al., 1986). There are differences in the rates of P450-catalysed reactions, those in fish proceeding more slowly reflecting different temperature adaptations. Since the P450s in rat and fish are structurally similar, these rate differences probably result from the organisation of the P450 + NADPH-P450-reductase + phospholipid complex (Williams et al., 1983).

The main difference between fish and mammalian hepatic MFOs lies in their inducibility. In mammals, at least five representative compounds usually induce distinct P450 isozyme groups (although these P450s may catalyse the conversion of a wide range of substrates): these inducers are (i) ß naphthoflavone (BNF), (ii) phenobarbitone (PB), (iii) pregnenolone-16-α-carbonitrile (PCN), (iv) isosafrole (ISF) and (v) clofibrate. In fish, only BNF and related compounds (which include PAH, and planar chlorobiphenyls, chlorinated dibenzodioxins and dibenzofurans) have consistently induced hepatic MFOs in a wide range of species (Kleinow et al., 1987). PCN appears not to induce, or does so to only a very limited extent (Hansson et al., 1980; Vodicnik and Lech, 1983). PB and related compounds have been reported occasionally, and usually in the older literature, to induce fish hepatic MFOs, but more recent work using sensitive assays of PB induction, suggests that PB is not an inducer (Addison et al., 1987; Ankley et al., 1987). ISF has been reported (on one occasion) to induce MFO enzymatic activity in trout (Vodicnik et al., 1981). No information exists about induction in fish by clofibrate. It is worth emphasising that most experimental work on MFO induction in fish has been done with salmonids (mainly rainbow trout) and inducers other than BNF may be effective in other species. Furthermore, most of the evidence for the absence of induction by (e.g.) PB rests on measurements of enzymatic activity, rather than on immunochemical measurement of specific isozymes.

Dose-response relationships have been established between exposure to BNF inducers and MFO activity in fish. In brook trout, hepatic ethoxycoumarin O-de-ethylase (ECOD) activity was related (by a power function) to PCB residue concentrations in the fillet after experimental feeding of Aroclor 1254 (Addison et al., 1981); the form of the equation suggested that ECOD should be sensitive to PCB concentrations encountered in "naturally" contaminated environments. A similar relationship existed between ethoxyresorufin O-de-ethylase (EROD) activity or aryl hydrocarbon hydroxylase (AHH) activity and Aroclor 1242 injected intraperitoneally to rainbow trout (Elcombe and Lech, 1978). Flounder exposed to petroleum-contaminated sediment showed increases in hepatic EROD activity related to sediment hydrocarbon concentration in the range 0.3 - 91 ppm (Payne et al., 1988). In the tropical fish Haemulon sciurus hepatic EROD and specific P450 isozymes were related to BNF injections in the range 0 - 10 mg $\cdot kg^{-1}$ body weight (Stegeman et al., 1990). Overall then,

there is evidence from several species of fish exposed to various chemicals to show that MFO activity can be predictably related to petroleum or PCB exposure, though the nature of the relationship may vary from species to species.

The time-course of induction has been determined in several studies. Various species of temperate-water fish show clear induction (either enzymatic activity or P450 isozyme induction) after 3 - 5 days (Lidman et al., 1976; Addison et al., 1981; Fingerman et al., 1983). Changes in the mRNA which codes for P450 isozyme synthesis occur even sooner (Haasch et al., 1988). In tropical fish acclimated to higher temperatures, induction of enzymatic activity may occur within three days (Stegeman et al., 1990). Since MFO induction is related to tissue concentrations of certain chemicals (reflecting exposure) and since these chemicals are usually cleared only fairly slowly, MFO activity usually remains elevated for some time even after a single dose (Elcombe and Lech, 1978; Addison et al. 1981; Stegeman et al., 1990).

Several natural factors influence hepatic MFO activity in fish. The most obvious of these is sex. In sexually mature fish, there are differences in MFO enzymatic activity (Stegeman and Chevion, 1980; Förlin, 1980) though these are usually not so obvious in immature fish. Not only are there sex differences in MFO activity, but also in the inducibility of MFO activity (Förlin, 1980). In addition, there were pronounced seasonal variations in MFO activity which were related to the reproductive cycle in apparently uninduced fish from a clean environment (Edwards et al, 1988). In summary, seasonal (or other) variations in the reproductive cycle appear to have a major influence on hepatic MFO activity, and should be eliminated during sampling of fish for monitoring studies. This may be achieved most easily by sampling reproductively immature specimens.

3. Selection of fish species for MFO monitoring

A fish species suitable for MFO monitoring is likely to have the following characteristics:

(i) It should be common and easily available, and its basic biology (habitat, feeding behaviour, migration and reproductive cycle) should be well defined; its migration should be short compared to the distances over which spatial comparisons are being made.

(ii) It should be robust enough to be kept in the laboratory so that its MFO system can be characterised and examined experimentally.

(iii) Reproductively immature specimens should be large enough to yield about 1 g liver for study.

(iv) It should probably (though not necessarily) be a bottom dweller, since many of the contaminants which affect MFO systems are likely to be sediment (or at least particle) bound.

In temperate marine waters, these criteria are often met by flatfish such as members of the flounder family: thus, winter flounder (Pseudopleuronectes americanus) or Pacific dabs (Citharichthys spp.) have been used successfully in North American monitoring programmes (Payne et al., 1984; Spies et al., 1980; 1982) and the European flounder (Platichthys flesus) has been used in Norwegian waters (Addison and Edwards 1988; Stegeman et al., 1988). It should be emphasised, however, that the last of the criteria listed above need not be applied rigidly: many fish which are not demersal have been used in successful monitoring programmes; these include blenny (Kurelec et al., 1977) and perch (Förlin et al., 1985).

It is worth noting that the fish hepatic MFO induction measurements which have been most useful in indicating the effects of contamination have involved comparisons of fish from suspect habitats with those from clean or reference sites. Absolute measurements of MFO induction depend to a considerable extent on technique and on factors such as the choice of buffer pH or ionic strength, enzyme : substrate ratios, etc.. As a result, comparative studies, in which samples from different sites are analysed using any standard techniques, are preferable.

4. MFOs in aquatic invertebrates

Sessile aquatic invertebrates would appear to be better candidate organisms for environmental monitoring than would fish, because of their immobility. Unfortunately, their MFO systems appear to be much less sensitive to organic pollutants than those in fish. There was some controversy about whether aquatic invertebrates in general have inducible MFO systems at all; however, the more recent literature (which is based on more sensitive and specific methods for detecting components of such systems) has shown that MFO systems are indeed present in aquatic invertebrates (Livingstone et al. 1989; James, 1989; Livingstone, 1990). It is clear, however, that invertebrate, particularly molluscan, MFO systems are in general less well characterised than are vertebrate systems, and are not yet ready to be applied as a routine environmental monitoring tool (Livingstone, 1988).

II. PRACTICAL MEASUREMENT OF MFO ACTIVITY

1. Scope and field of application

When vertebrates (and some invertebrates) are exposed to chemicals like petroleum hydrocarbons or some polychlorinated biphenyls, their hepatic microsomal mixed function oxidases (MFO, or mono-oxygenases) are induced. Increased hepatic MFO activity in wild organisms may therefore indicate contamination of their habitat; in other words, MFO induction may be a sub-lethal bioassay of environmental contamination by certain chemicals.

2. Principle

The methods summarised here are based on conventional laboratory procedures for estimating MFO activity. These have been modified for use in less well-equipped laboratories or in the field, usually by choosing robust and simple versions of more elaborate apparatus. (Obviously, if advanced instruments are available, they can be used.) The basic facilities required include some bench space and a source of electrical power (in North America, 110V 60 Hz single phase, maximum 10A). The methods and equipment described have been used in such diverse environments as aboard ship (CSS Hudson Arctic cruises), in a tent at Resolute Bay, (North West Territories, Canada), using power supplied from a gasoline generator and on Sable Is., Nova Scotia.

It is convenient, particularly in field work, to prepare in advance as many reagents and solutions as possible. Most, like those required for protein determinations, are stable and will withstand freezing and thawing if kept in plastic bottles. It is usually not possible to prepare nucleotide co-enzyme solutions in advance, however, and since (usually) small amounts of these are needed and as they are relatively expensive, it is desirable to pre-weigh appropriate amounts of these, and keep them (cooled and desiccated) in small vials. The instruments described are simple and robust examples of their type and which have simple maintenance or support requirements, but which are adequately sensitive or precise.

Weighing presents special problems, especially aboard ship unless a weighing table on gimbals is available. A partial solution to the problem is to use an "averaging" balance of the sort that integrates over a period of several seconds; even this, however, is not satisfactory for weights below 1g. (If liver size must be estimated, its volume can usually be measured accurately by displacement in a small measuring cylinder.) Fortunately, most variables of interest in MFO assays can be expressed in terms of protein contents, which in turn can be measured aboard ship using only volumetric

manipulations.

The following pages describe methods for preparing MFO suspensions, for two MFO assays (ethoxyresorufin O-de-ethylase: EROD and benzo(a)pyrene hydroxylase, also known as aryl hydrocarbon hydroxylase: AHH), and for protein estimation. These procedures have been chosen since they have been well tested both in the field and the laboratory as monitors of contamination, mainly by PAH, PCB and chlorodibenzodioxins. The general procedures for both MFO assays can be applied to different substrates. The procedure for EROD, for example, can be applied to pentoxy- or benzyloxy-resorufin O-de-alkylase (PROD and BROD) to indicate induction of other P450 isozymes (Burke and Mayer 1983; Lubet et al., 1985). Similarly, the EROD assay can be modified by changing only the fluorimeter excitation and emission wavelengths to detect 3-cyano-7-ethoxycoumarin O-de-ethylation (CN-ECOD), which may also indicate induction of other P450 isozymes (White, 1988). The excitation and emission wavelengths used for benzo(a)pyrene hydroxylation can be modified to allow the use of diphenyloxazole as a safer substrate. It must be emphasised, however, that CN-ECOD, PROD and BROD have not been "field tested" as indicators of contaminant-induced stress.

Although the assays described here are "classical" indicators of MFO induction, other indicators are being developed continuously. Protein determinations can be carried out by various methods: the bicinchoninic acid (BCA) reagent has been introduced recently, for example, and promises to be a convenient one-step process (Anon., 1989). However, as it has not yet been evaluated extensively, the more conventional Lowry method is described here. Immunochemical probes for P450 isozymes are now available (e.g., Stegeman et al., 1988) and the mRNAs which code for some of the P450s can now be detected (e.g., Haasch et al., 1988). However, such approaches are still at the research stage and as they require the use of reagents not available commercially, they are not described in detail.

Finally, it should be emphasised that the methods described here only illustrate the procedures for measuring MFO induction. They should not be regarded as immutable; indeed, it is desirable that investigators should modify the methods for enzymatic activity measurements, e.g., by varying pH, substrate concentration etc., to optimise incubation conditions for their sample types.

3. IMPORTANT NOTE ON SAFETY

Safety deserves special attention, especially if these techniques are to be used in the field where medical help is not available. Most of the chemicals and equipment recommended below are relatively harmless, provided they are not abused. It is simply good laboratory practice to treat any chemical or equipment item with respect, even if it is known to be harmless; furthermore, the toxicology of some chemicals (such as

*ethoxyresorufin) has not been investigated. Disposable items should be used wherever possible, as should safety items such as "propipettes", gloves, bench-top soakers etc. The benzo(a)pyrene hydroxylase assay presents special problems, since the substrate is carcinogenic: the assay is best carried out in an area dedicated to the work (which is desirable in any case, as the assay should be done under safe lights since the products are photo-sensitive); protective clothing **MUST** be worn, pipetting by mouth **MUST** be avoided, and the working area **MUST** be protected with soakers which **MUST** be combined with carcinogenic waste for special disposal.*

4. Preparation of samples for analysis

The procedures for isolating MFOs are shown schematically in Fig. 1. This section describes the steps for preparing a sample prior to the measurement of MFOs.

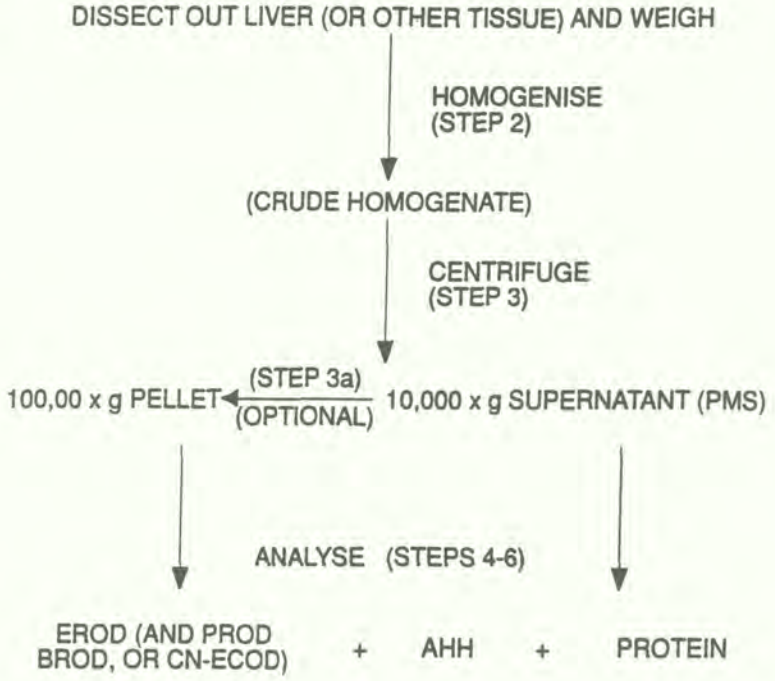

Figure 1. Scheme for preparation of mixed function oxidases from fish tissue. Numbers identify steps described in the text.

4.1. Reagents

4.1.1. Homogenisation solution. 1.15% KCl prepared in advance and stored frozen or cooled in plastic bottles.

4.1.2. Ice (for cooling tissues).

4.2. Equipment

4.2.1. Top-pan balance weighing to 0.1g.
4.2.2. Conventional dissection instruments.
4.2.3. Bench space with adequate lighting.
4.2.4. Ice bucket.
4.2.5. Range of small beakers.
4.2.6. Electric drill capable of 1750 rpm.
4.2.7. Potter-Elvehjem teflon-glass homogeniser (5 or 15 ml).
4.2.8. Measuring cylinder (10 or 25 ml capacity).
4.2.9. Scissors.
4.2.10. Beckman "Microfuge" or equivalent (capable of achieving at least 10,000 x g) and tubes. This instrument is not self-cooled, but it may be run in a domestic freezer. (If a conventional refrigerated centrifuge is available it should be used: sample volumes should be modified as required.).
4.2.11. Refrigerated ultracentrifuge, 100,000 x g, if available.
4.2.12. Graduated tubes, 5-15 ml.

4.3. Procedure

4.3.1. Dissection and isolation of tissue

Kill fish, usually by a blow to the head and/or severing spinal cord; weigh fish with appropriate accuracy (± 1%). Dissect out tissue (usually liver) avoiding rupturing the gall bladder, since bile may contain MFO inhibitors. Weigh the liver (± 1%) and place in a beaker on ice, pending homogenisation.

4.3.2. Homogenisation of tissue (Step 2, Fig.1)

Mince weighed tissue (ideally 1g minimum, weighed to ± 0.1g) with scissors and place in homogeniser tube on ice; add KCl solution in ratio 4:1, v:w. Homogenise with 6 vertical strokes at 1750 rpm, keeping tube cooled in ice. This yields the "crude homogenate".

4.3.3. Preparation of 10,000 x g homogenate (Step 3, Fig.1).

Place 1.5 ml aliquots of crude homogenate in "Microfuge" tubes and spin for 15 min. The supernatant is nominally 10,000 x g supernatant ("post-mitochondrial supernatant", PMS) or 12,500 x g supernatant, depending on instrument. Retain the supernatant for analysis, or process further as described in paragraph 4.3.4. (Note: protein concentrations in supernatants prepared in this way do not differ significantly from those prepared by conventional centrifuging (5 min at 500 x g followed by 15 min at 10,000 x g) in refrigerated centrifuges.)

A single "Microfuge" tube usually provides sufficient supernatant (>1 ml) for benzo(a)pyrene hydroxylase, ethoxyresorufin O-de-ethylase and protein determinations. If activity or protein concentrations are low, or if subsequent

samples are required for, say, electrophoretic studies of P450 isozymes, further aliquots of the crude homogenates can be centrifuged.

4.3.4. [*Optional*] Preparation of 100,000 x g pellet (microsomes) (Step 3a, Fig.1).

This is optional, if 5 - 10 ml 10,000 x g supernatant have been prepared, and ultracentrifuge is available. Transfer the 10,000 x g supernatant (or an aliquot of it) to an ultracentrifuge tube. Spin at 100,000 x g for 40 min. at 4°C. Discard the supernatant ("cytosol"). Loosen the microsomal pellet with a glass rod, and transfer it quantitatively to a tissue homogeniser. Resuspend the pellet in 3 - 5 ml 1.15% KCl solution, keeping the homogeniser cooled in ice. Rinse the homogenate into a graduated tube and record its volume. Hold on ice.

5. Protein determination (Lowry et al., 1951) (Step 4, Fig.1).

The reader is referred to the bibliography for full details of this widely-employed method which will only be described briefly here.

5.1. Reagents

5.1.1. Distilled water.

5.1.2. 0.5% aq. copper sulphate ($CuSO_4.5H_2O$) in 1% aq. sodium or potassium tartrate solution

5.1.3. 2% aq. sodium carbonate in 0.1 N sodium hydroxide.

5.1.4. Mixed reagent "A" , prepared not more than one day in advance, as described by Lowry et al. (1951). Add 1 ml reagent 5.1.2. to 50 ml reagent 5.1.3.

5.1.5. Folin-Ciocalteu phenol reagent, diluted to 1 N. (The reagent available commercially from(e.g.) Sigma is 2 N and must be diluted 1:1 v/v with distilled water.

5.1.6. Protein standard, e.g., bovine serum albumin, diluted (accurately) to approx. 100 - 200 $\mu g \cdot ml^{-1}$ (so that appropriate volumes can be pipetted conveniently to prepare standard curves).

5.2 Equipment

5.2.1. 15 ml test-tubes and rack.

5.2.2. Micropipettes with disposable tips to deliver 10, 25 or 500 μl,.

5.2.3. Graduated glass pipettes to deliver 2 ml and 10 ml.

5.2.4. 250 ml glass beakers.

5.2.5. Vortex mixer.

5.2.6. Spectronic 20 or similar simple robust single beam spectrophotometer with wavelength range to 660 nm and cuvettes.

5.3. Procedure

Prepare a standard curve for each set of determinations. Use four points (plus a blank) in the range 20 - 200 μg protein. Make up to final volume 1 ml with distilled water. To duplicate 10 μl (PMS) or 25 μl (microsomal) samples in test tubes add 1 ml distilled water. Prepare reagent "A" as above and add 5 ml reagent "A" to each standard and sample tube. Mix on vortex mixer and allow to stand about 20 mins. Then, while each tube is being mixed on the vortex mixer, add 0.5 ml diluted Folin-Ciocalteu phenol reagent and allow to stand a further 20 min. Read at 660 nm v. distilled water.

6. Ethoxyresorufin O-de-ethylase (EROD) determination (Step 5, Fig.1).

This is based on the method of Burke and Mayer (1974) in which the substrate (ethoxyresorufin) is incubated in a fluorimeter cuvette with enzyme preparation and cofactor (NADPH) in appropriate buffer, and the fluorescence increase due to resorufin production is recorded.

6.1 Reagents

6.1.1. 0.1M phosphate buffer in range 7.5 - 8.5.
6.1.2. Ethoxyresorufin (substrate) 100 μM in 2.5% aqueous Tween 80.
6.1.3. Resorufin (product) in range 10 μM in ethanol (both substrate and product should be protected from light).
6.1.4. NADPH, 5 mg in pre-weighed vial.

6.2. Equipment

6.2.1. Micropipettes with disposable tips to deliver 10, 25, 50, 100, 200 μl.
6.2.2. Glass pipette to deliver 2 ml.
6.2.3. Fluorimeter functioning in range excitation 510 nm and emission 585 nm with 15 nm bandwidth in each beam (Turner 430 or similar is suitable), and connected to strip chart recorder or other recording device (temperature control of the cuvette is optional).

6.3 Procedure

6.3.1. Ethoxyresorufin O-de-ethylase (EROD)

Place 2 ml buffer in fluorimeter cuvette (maintained at an appropriate temperature if possible) and add NADPH to a final concentration of 200 μM; add appropriate amount of enzyme (10,000 x g supernatant, or microsomal preparation) and mix. Record fluorescence with excitation at 510 nm and emission at 585 nm. (There should be no increase over time.) After 1 min add ethoxyresorufin to a final concentration of 1.25 mM and record increase in

fluorescence for 2 min. Finally add a known concentration of resorufin and note increase in fluorescence; express gradient of enzymatic fluorescence increase in terms of product concentration. A typical run is shown in Fig. 2.

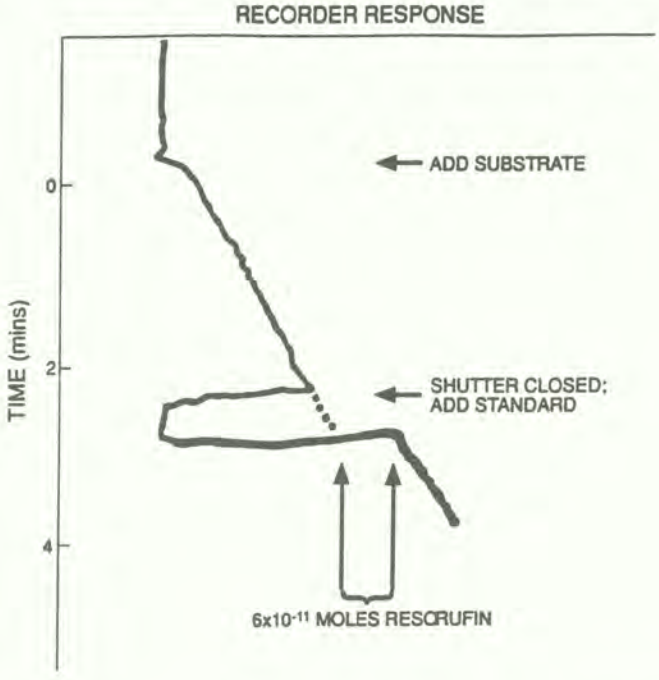

Figure 2. Strip chart record of ethoxyresorufin O-de-ethylase (EROD) activity in a flounder (Pseudopleuronectes americanus) liver preparation, illustrating (a) constant fluorescence due to buffer, enzyme preparation and co-factor; (b) increase in fluorescence due to resorufin production after addition of substrate, and (c) increase in fluorescence after addition of a known amount of product for calibration.

6.3.2. Pentoxy- and benzyloxy-resorufin O-de-ethylase (PROD and BROD).

PROD and BROD are sensitive indicators of the P450 isozymes which are induced by PB in some species (Burke and Mayer, 1983; Lubet et al., 1985). Although there is some doubt as to whether fish hepatic MFOs are inducible by PB (see Part I) the only species in which these enzyme activities have been measured are trout (Addison et al., 1987; Ankley et al., 1987) and flounder (Addison et al., 1991). The assays are described in the event that they may be useful in other species.

These assays are carried out exactly as described for EROD (since the same product, resorufin, is measured) except that pentoxyresorufin or benzyloxyresorufin is substituted for ethoxyresorufin.

6.3.3. 3-Cyano-7-ethoxycoumarin O-de-ethylase (CN-ECOD) determination.

7-ethoxycoumarin O-de-ethylase (ECOD) is a popular and sensitive indicator of induction of (probably) a fairly wide range of P450 isozymes. Since

the product of the reaction, umbelliferone, does not fluoresce at physiological pH it is difficult to determine by "kinetic" methods. White (1988) has described the preparation of the 3-cyano derivative which is easily detectable in the pH range 6 - 9, and has shown that CN-ECOD, like ECOD, is a sensitive indicator of MFO induction though probably less specific than the resorufin derivatives.

The assay is carried out exactly as described for EROD except that the wavelengths are 400 nm (excitation) and 455 nm (emission).

7. Benzo(a)pyrene hydroxylase determination (Step 6, Fig.1)

This is based on the procedure of Nebert and Gelboin (1968) in which the substrate (benzo(a)pyrene) is incubated with an enzyme preparation and base-extractable phenolic products are isolated and determined fluorimetrically.

READ THE PARAGRAPH ON SAFETY. BENZO(A)PYRENE IS CARCINOGENIC. IF SUITABLE FACILITIES FOR HANDLING CARCINOGENS ARE NOT AVAILABLE, USE AN ALTERNATIVE SUBSTRATE SUCH AS DIPHENYLOXAZOLE (PPO) TO ASSESS AROMATIC HYDROCARBON HYDROXYLASE (AHH) ACTIVITY.

7.1. Reagents

7.1.1. Buffer solution 0.1M phosphate pH 7.5.
7.1.2. 1.15% KCl solution.
7.1.3. Acetone.
7.1.4. Hexane, spectroscopic grade (redistilled from $KMnO_4$ if necessary).
4.1.5. Benzo(a)pyrene, 19 mM in acetone.
4.1.6. NADPH, 10 mg $\cdot$ ml^{-1} in phosphate buffer pH 7.5.
4.1.7. Quinine sulphate (fluorescence standard) in range 0-100 $\mu g \cdot ml^{-1}$

7.2. Equipment

7.2.1. Disposable polypropylene test-tubes (16 ml) (Falcon #2006 is suitable).
7.2.2. Micropipettes with disposable tips, 10 μl to 100 μl.
7.2.3. Waste pail dedicated to carcinogenic waste and lined with plastic bag.
7.2.4. Clinical centrifuge, preferably with disposable liners in buckets.
7.2.5. Vortex mixer.
7.2.6. Fluorimeter capable of functioning at 395 nm (excitation) and 520 nm (emission) (corresponding wavelengths for assay based on PPO are 345 and 520 nm).
7.2.7. Water bath set at 27°C.
7.2.8. Safe light (25W red lamp).
7.2.9. Test-tube racks.
7.2.10. Pasteur pipettes.

7.2.11. Disposable tissue, gloves and bench-top soaker.
7.2.12. Timer.
7.2.13. Long wave UV lamp (for monitoring spills of benzo(a)pyrene)

7.3. Procedure

To a 16 ml polypropylene tube add 500 μl phosphate buffer, 100 μl NADPH solution, 10 μl 19 mM benzo(a)pyrene, and bring to 27°C. Add 10 - 100 μl 10,000 x g homogenate or microsomal preparation. Prepare a "zero-time" blank with pooled enzyme preparations and stop the reaction immediately with 0.5 ml acetone.

Carry out the following steps under safelights: incubate all samples and blank with gentle shaking for 15 min. Stop reaction by addition of 0.5 ml acetone and add 2 ml hexane to each tube. Mix 30 sec. on Vortex mixer and centrifuge 3 min. (Wrap the tubes and use liners to prevent leakage at these steps.) Transfer hexane phase (Pasteur pipette) as completely as possible to second tube and back extract with 2 ml 1N NaOH. Mix 30 sec. and centrifuge 3 min. Remove hexane layer to incubation tube to minimise waste. Measure fluorescence of the NaOH phase against a curve prepared with quinine sulphate in the range 100 $\mu g \cdot ml^{-1}$. This secondary standard is standardised against 3-hydroxybenzo(a)pyrene (stock 100 μM in acetone, diluted to 10 - 200 nM in NaOH).

Seal all disposable tubes and add to waste. Monitor area with long wave UV lamp and clean with solvent and/or NaOH-soaked tissues. Incinerate all waste.

8. Calculations.

It is conventional to calculate the following:

(i) Liver % body weight: $\frac{\text{Liver wt (g) x 100}}{\text{Fish wt. (g)}}$

(ii) PMS or microsomal protein content: mg protein $\cdot$ g liver^{-1}

(iii) Enzymatic activities as:

(a) moles product formed $\cdot$ min^{-1} $\cdot$ (mg protein)$^{-1}$

(b) moles product formed $\cdot$ min^{-1} $\cdot$ (g fresh tissue)$^{-1}$

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