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A VISUAL TEST FOR HEPATIC EROD ACTIVITY AS A MARKER FOR EXPOSURE TO AROMATIC AND HALOGENATED AROMATIC HYDROCARBONS

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ABSTRACT

The kinetic, spectrophotometric method for determining 7-ethoxyresorufin O-deethylase activity in fish was further developed into a quick-test to be used in situations with poor laboratory facilities e.g. during field experiments. The method, developed for sample screening and monitoring, could precede and indicate whether further analysis is required. The assay can be conducted with microsomes, postmitochondrial supernatant or directly with whole homogenate, and without spectrophotometer.

Key words: 7-ethoxyresorufin O-deethylase, quick-test, whole homogenate, postmitochondrial supernatant, microsomes, fish, spectrophotometer, visual analysis

INTRODUCTION

Interest in the use of specific biological changes linked to pollutant exposure as markers of environmental contamination and effects of chemicals has expanded rapidly in recent years. The biological change most frequently tested as a marker for exposure to xenobiotics is the induction of specific forms of cytochrome P450 in liver or other organs of vertebrates, including mammals, birds and fish (Stegeman *et al.* 1992). Cytochrome P450 genes in subfamily 1A (CYP1A: Nebert *et al.* 1991; Stegeman 1992) are induced by a variety of widely distributed environmental chemicals, inducing polynuclear aromatic hydrocarbons (PAH) and halogenated aromatic hydrocarbons (HAH), particularly the planar polychlorinated biphenyl congeners, and polychlorinated dibenzo-p-dioxins (PCDD) and dibenzofurans (PCDF). These groups of compounds include highly toxic and carcinogenic compounds many of which apparently act by inducing CYP1A through binding to an Ah-receptor (Poland & Knutson 1982). Structural and functional properties indicate a similarity in CYP1A proteins in fish, birds and mammals (Stegeman 1989), and Ah-receptor proteins and inducibility of CYP1A have been detected in

all vertebrate groups examined, from cartilaginous fish to mammals (Hahn *et al.* 1992). Dose response relationships for CYP1A induction show a linearity of response over wide ranges in concentration of PAH and /or HAH, suggesting that this induction might be used as a dosimeter in diverse vertebrates (e.g. Haasch et al. 1992).

The induction of cytochrome P450 monooxygenase activity aryl hydrocarbon hydroxylase (AHH, now known to be catalyzed by CYP1A) was first suggested as a marker for exposure in studies with fish (Payne *et al.* 1984). CYP1A induction in fish is rapidly gaining acceptance as a measure of exposure to inducers in the environment (Stegeman & Lech 1991). Currently, there are several ways in which the induction of CYP1A genes can be detected. These include measurement of CYP1A catalytic activity, measurement of CYP1A protein by immunochemical methods, or by measurement of CYP1A mRNA content (Stegeman *et al.* 1992). Several methods are available for each.

Measurement of catalytic activity of induced protein is the approach most readily available to the largest number of investigators. Two activities, aryl hydrocarbon hydroxylase (AHH) and ethoxyresorufin O-deethylase (EROD) are both catalyzed primarily by cytochrome P4501A in fish liver (Stegeman *et al.* 1985). EROD activity is now more commonly employed, as the substrate (7-ethoxyresorufin) is not hazardous, and there are direct, kinetic assays based on the distinct fluorescence (Burke *et al.* 1974) and absorbance (Klotz *et al.* 1984) characteristics of the product (resorufin). These properties have been adapted for rate measurement by use of multiwell plate readers equipped with fluorometric or spectrophotometric detection (e.g., Galgani & Payne 1991). However, there are many circumstances in which analysis of induction might be desirable, but under which measurement by any of the methods commonly available would be too costly or otherwise impractical. Pohl and Fouts (1980) adapted the fluorometric assay for end-point determination, using stopped-reactions. In this report we describe the adaption of the spectrophotometric method for detecting EROD activity to an end-point colorimetric test that may be applied in the field.

MATERIALS AND METHODS

Chemicals

Ethoxyresorufin and resorufin used in this study were prepared by Kloz *et al.* (1984). Rhodamine b, used as a standard, was obtained from International Dye Stuffs, Rahway, NJ, USA. The absorbtivity of rhodamine b is 12.6 x 10^4 l mol^{-1} cm^{-1}, λmax 554 nm and mw 479.02. Corresponding values for resorufin are 40 x 10^4 l mol^{-1} cm^{-1}, λmax 572 nm and mw 213.19. All other reagents were of highest quality available.

Fish material

Scup (*Stenotomus chrysops*) were captured from Vineyard Sound, MA (USA) and kept in tanks at the Environmental Systems Laboratory of the Woods Hole Oceanographic Institution. Some fish were injected intraperitoneally with 3,3',4,4'-tetrachlorobiphenyl (TCB) at 0.1 or 1.0 mg/kg, and sacrificed after 4 days. Samples from untreated and induced scup, showing a broad range of enzyme activities, were used for tissue preparation experiments as well as for other determinations. The weight of the fish ranged from 215 g to 289 g and the weight of the livers from 1.21 g to 2.93 g.

Tissue preparation

The methods for tissue homogenization were tested with several individual scup. The liver of each fish was divided into two pieces. Each was further processed in identical ways, except for the estimate of tissue weight. The first five pieces of tissue were weighed on a Sartorius balance. For the other five

tissue pieces the weight was estimated by volume displacement. In each case samples were centrifuged as before (Stegeman *et al.* 1979). The liver tissue was homogenized in 4 ml of buffer per gram (or ml) of tissue yielding whole homogenate. The homogenate was centrifuged at 755 x g for 10 minutes and further at 12100 x g for 10 minutes. At this point the post mitochondrial supernatant was pipetted out and placed into a new tube. This was then ultracentrifuged at 100,000 x g for 70 minutes. The supernatant was aspirated leaving only the pellet behind. The pellet was resuspended in buffer so that 1 ml of microsomes corresponded to 1 g (1 ml) of fresh tissue. In both cases samples were taken during three stages of the microsomal preparation; whole homogenate (WH), post mitochondrial supernatant (PMS) and microsomes. Half of each sample was stored in liquid nitrogen and half at -80°C.

Analytical techniques

The method developed is based on the common kinetic spectrophotometric method for measuring 7-ethoxyresorufin O-deethylase activity (Klotz *et al.* 1984) as well as on the rapid end-point method of Pohl and Fouts (1980). In these assays we employed buffer composition like that defined by Klotz *et al.* (1984). The assay buffer was 1 M Tris-HCl buffer containing 1 M NaCl, pH 8. The results obtained with the end-point method were compared to the results of the kinetic procedure. Proteins were determined by using the bicinchoninic acid-technique (Smith *et al.* 1985).

RESULTS AND DISCUSSION

Microsomal EROD activity by spectrophotometric endpoint assay

We first evaluated the conditions for spectrophotometric detection of stopped reactions, and compared results to the kinetic assay. The different homogenate preparations, one based on weight/volume and the other on volume/volume measurement of tissue/buffer relationships, did not have statistically significant differences in their EROD activities measured by the kinetic spectrophotometric method, or in protein concentrations (Table I). For microsomes prepared from weighed tissue, the EROD activity was 2.58 nmol/min/mg protein and 3.27 nmol/min/mg protein for the tissue homogenized on a volume/volume basis. The values are the mean activities of 5 samples, each of which was the mean of 3-8 determinations. The corresponding protein contents were 7.47 mg/ml and 6.82 mg/ml.

Table I. A) 7-Ethoxyresorufin O-deethylase activity of microsomal samples prepared by two different means and B) the effect of storage temperature on microsomes, postmitochondrial supernatant (PMS) and total homogenate (WH).

A)		EROD (x ± SD, nmol/min/ml)
microsomes:	W/V[a]	13.38 ± 11.07
	V/V[b]	17.09 ± 21.04

B)	EROD (x ± SD, nmol/min/ml)	
	N_2	-80°C
microsomes	14.43 ± 15.35	15.71 ± 16.16
PMS	4.23 ± 3.93	4.02 ± 4.12
WH	5.70 ± 4.15	5.44 ± 4.27

a) W/V = weight / volume
b) V/V = volume / volume

The microsomal EROD activities (EROD) measured kinetically did not differ statistically after storage in liquid nitrogen (7 days) or at -80°C (10 days) (Table I). Mean values for ten samples were 14.43 and 15.71 nmol/min/ml of microsomes, respectively (Table I). (In other studies of scup microsomes in our laboratory catalytic activity of samples stored in liquid nitrogen were unchanged over 4 years storage.) Activities after four months storage for PMS were 4.23 and 4.02 nmol/min/ml and for whole homogenate 5.70 and 5.44 nmol/min/ml, respectively.

EROD reactions can go to completion during longer incubation times, dependent on the catalytic rate and the volume of microsomes used. This can result from substrate exhaustion and/or substrate or product inhibition (Pohl & Fouts 1980; Klotz *et al.* 1984). However, reactions going to completion could distinguish samples of different activity. Samples of known activity were compared at varying volumes as described in Table II. The reaction was stored overnight at room temperature which permitted the reaction to go to completion. This helped to establish some of the limitations of our potential assay. It demonstrated the necessity of adding enough volume of microsomes, so that those of lower activity would react. Yet when there was sufficient microsomal material added for the less active samples to show color change, then there was no longer any color difference between the highly active and the moderately active since they both ran to completion. This indicated the need to stop the reaction.

Table II. Study of reaction completion (overnight) at varying volumes of different microsomal samples with known activities.

	activity (nmol/min/mg prot)				
microsomes	7.89	2.40	1.45	0.22	0.13
1 µl	N	N	N	X	X
2 µl	C	C	C	N	N
3 µl	C	C	C	N	N
4 µl	C	C	C	N	N
5 µl	C	C	C	N	N

N = not complete
X = no color
C = complete

The reaction cocktail was the same as in the common kinetic method, however, 100 µl methanol was added to stop the reaction. It was seen that stopped samples were affected by unknown (redox ?) reactions occurring in the cuvette (Fig. 1A & B). Neither buffer-substrate alone, nor buffer-substrate-methanol caused any absorbance change. However, when microsomes were added to the buffer-methanol solution, to the cocktail already containing buffer-substrate-methanol or when the ordinary reaction was stopped with methanol, a continuous elevation of absorbance was detected with microsomes of low or high EROD activities. With the cofactor NADPH the elevation observed was the same as without NADPH (not illustrated). In some cases microsomes in buffer-substrate solution alone produced such change. When the mixture contained microsomes inactivated by heating, no elevation was seen (Fig. 1A & B). The apparent increase in absorbance was more or less the same in samples with high or low EROD activity or sometimes even higher in low activity samples (Fig. 1A & B). This indicates that methanol is efficient in stopping the 7- ethoxyresorufin O-deethylation but gives rise to some other reaction. The apparent increase in absorbance was not, however, accompanied by any increase in pink color. The requirements of this reaction, still to be recognized, are at least the presence of microsomes and methanol in buffer solution.

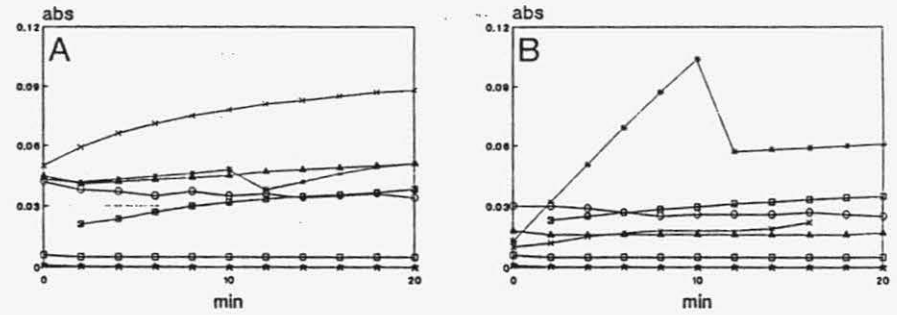

Fig. 1. The effect of different components of the 7-ethoxyresorufin O-deethylase assay mixture on the recorded absorbance in kinetic assay when microsomes of A) lower and B) higher activities were used. (- - absorbance of buffer and substrate; -□- buffer, substrate and methanol; -▲- buffer, substrate and microsomes; -⊠- buffer, microsomes and methanol; -x- buffer, substrate, microsomes and methanol; -*- normal reaction stopped by methanol at 10 min; -○- buffer, substrate, boiled microsomes and methanol).

Acetone as well as ethanol were also tested in stopping the reaction, however, they had the same effect as methanol in producing an increase in absorbance after stopping the deethylation reaction. However, the effect was usually less than what was seen with methanol, and in some cases the effect was absent (Fig. 2A & B). An additional problem in using ethanol was the difficulty of mixing the two solute phases. When the samples were placed into the cuvette, sealing the cuvette and inversion at least 3-5 times was necessary for mixing.

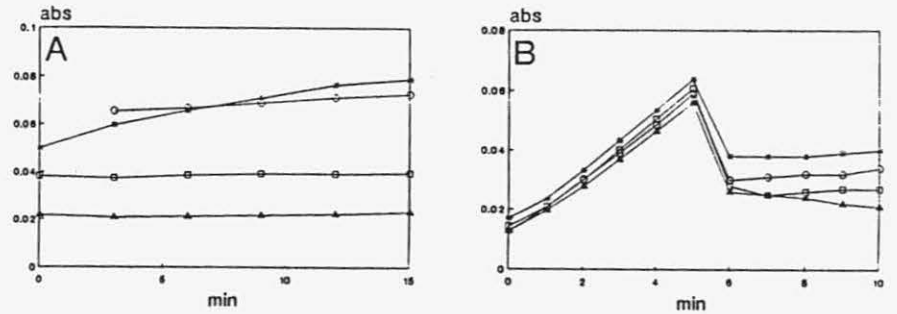

Fig. 2. The effect of different compounds (-*- methanol; -□- ethanol; -○- acetone; -▲- sodium hydroxide) in stopping the 7-ethoxyresorufin O-deethylation reaction.(A) microsomes with low activity stopped at 0 min; B) reaction of microsomes with high activity stopped at 5 min)

Sodium hydroxide (1 N), on the other hand, decreased the absorbance in course of time indicating that the product formed is not stable in those conditions. During longer time at different concentrations of NaOH (1 N, 0.5 N, 0.1 N) all samples lost their color (data not shown).

If the unspecified elevation of both sample and blank absorbances is the same and continues at the same rate, as it seems according to results (Fig. 3A), then samples and their corresponding blanks have to be measured exactly at the same time or within the same time delay when spectrophotometric determination is used. Analysis of absorbances after stopping EROD reactions with methanol showed that the elevation continued at least three hours, being highest during the first 40 minutes (Fig. 3A). After 21 hours the absorbance was decreased to less than the absorbance measured just after stopping the reaction.

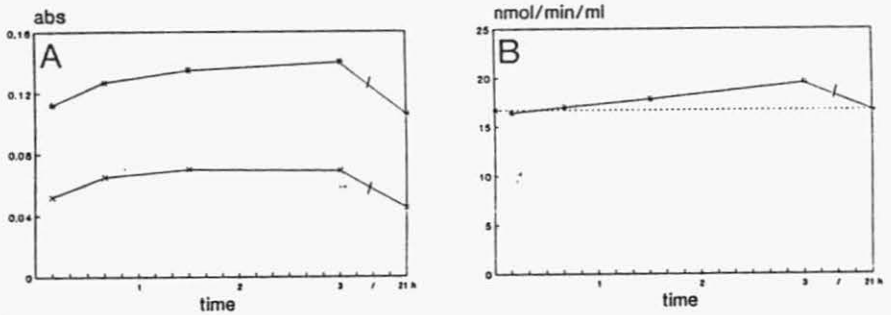

Fig. 3. The effect of delay between stopping the reaction with methanol and measuring the absorbance after 20 minutes incubation. (A) absorbance of -*- real and -x- blank reaction; B) calculated activity -*- compared to the activity by the corresponding kinetic method --*--.

It seemed that ten, or more preferably twenty minutes should be the proper time for incubation. The activities measured, compared to those with kinetic procedure, indicated that greater accuracy is reached in 20 minutes incubation (e.g. Table III).

A protocol for a spectrophotometric end-point measurement of 7-ethoxyresorufin O-deethylase activity in microsomal samples of fish was based on the above studies, using methanol to stop reactions. The sample studied (e.g. 2.5 µl of uninduced microsomes), is added into complete and blank reaction tubes at least in duplicates. Immediately after pipetting the samples the substrate-buffer solution (487.5 µl, final concentration of ethoxyresorufin 2 µM) is added following the addition of NADPH (10 µl, 25 mg/ml), to start the reaction. In all additions an exact timetable must be maintained. After 20 minutes incubation 500 µl of methanol is added to stop the reaction and the absorbance at 572 nm is read against air one hour after stopping the reaction (within 30 min - 1 hour 30 min; Fig. 3B). The samples must be read in exact order as incubated and all at the same time to avoid differences in storage time between samples and their corresponding blanks. With unknown samples a preliminary test to evaluate the need for sample dilution is recommended.

Table III. Within-run precision of kinetic and end-point measurements of 7-ethoxy-resorufin O-deethylase activity. Scup microsomes were assayed by both methods. Posmitochondrial supernatants and total homogenates were assayed by end-point measurement.

A) Kinetic	Microsomes	
	just thawed	after 2 hours
EROD[a]	14.02±7.77%	12.80±3.80%
n[b]	(6)	(7)

B) End-point	Microsomes		PMS	WH
	10 min	20 min	20 min	20 min
EROD	10.19±5.61%	11.15±8.61%	7.29±3.50%	9.24±2.95%
n	(20)	(19)	(20)	(20)

a = x (nmol/min/ml) ± SD (%)
b = number of samples

Postmitochondrial supernatant and whole homogenate

Our objective was to develop a quicker and simpler test for detecting the influence of xenobiotics on fish. Preparation of microsomes requires ultracentrifugation. Therefore we adapted the above protocol for use with the whole liver homogenate (WH) or post mitochondrial supernatant (PMS). In this adaptation we analyzed the linearity with time and enzyme activity, as well as the parity amongst whole homogenate, post mitochondrial supernatant and microsomal fractions.

Microsomes, PMS and WH representing equivalent amounts of liver were used in triplicate in reactions stopped at 0, 5, 10, 15 and 20 minutes, and in unstopped kinetic EROD assays. A highly active sample from scup that had been PCB-induced was compared to a sample of uninduced control scup. Since a considerably smaller volume of microsomes was needed than WH or PMS, it was necessary to dilute the microsomes in resuspension buffer to achieve an equivalent volume. The volumes used were 6 µl, 6 µl and 1 µl (+5 µl buffer) of WH, PMS and microsomes, respectively, for the highly active sample, and 15 µl, 15 µl and 2,5 µl (+12,5 µl buffer), respectively, for control scup. According to Lubet *et al.* (1985) 10µM dicumarol was included in the reaction mixture in case of PMS or WH to avoid the further metabolism of the resorufin product by quinone oxidoreductase (DT-diaphorase).

The nmoles of product for the WH and PMS were nearly identical in the stopped and unstopped assays for the highly active sample (Fig. 4). The amounts of product in the stopped reactions after two hours were still quite near the others (not illustrated).

As with microsomes, reaction mixtures with whole homogenate or post mitochondrial supernatant showed an increase in the absorbance after addition of methanol. The greater amounts of protein added also caused increasing turbidity in spectrophotometric assay because of protein precipitation. To remove the interfering protein after the reaction was stopped, the reaction mixtures were centrifuged (480 x g for 10 min) which allowed the reading without the hindrance of turbidity. A linear relationship between time and product formation was then established, even though the samples had low activity.

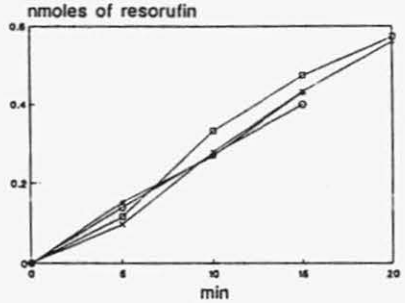

Fig. 4. The linearity of kinetic assay of WH (-□-) and PMS (-x-) reactions, compared to stopped assay of WH (-o-) and PMS (-*-) reactions.

The precision and accuracy of the measurement were tested with pooled samples. The activity in the kinetic spectrophotometric method for the pool of microsomes thawed just before the measurement was 14.02 nmol/min/ml ± 7.77 % (x ± SD%). The same microsomal preparation held for 2 hours in an ice-bath had 12.8 nmol/min/ml ± 3.8% (Table III). The end-point determination of activity for the same pool of microsomes gave 10.19 nmol/min/ml ± 5.61% at 10 minutes and 11.15 nmol/min/ml ± 8.61% at 20 minutes (Table III). With PMS the results were 7.29 nmol/min/ml ± 3.50 % and for WH 9.24 nmol/min/ml ± 2.95% for end-point reactions at 20 minutes (Table III). No corrections or exclusions of extreme data were done. The activities of microsomes, PMS and WH are not directly comparable, because they were from slightly different sample pools. However, the results indicate generally comparable activities per g liver for the three different fractions.

Assay without spectrophotometer

The above comparisons were based on spectrophotometric analysis of product. It was our objective to modify the EROD assay so that a spectrophotometer was not necessary. This was approached by developing a colorimetric test with a set of standards to which unknown samples could be compared.

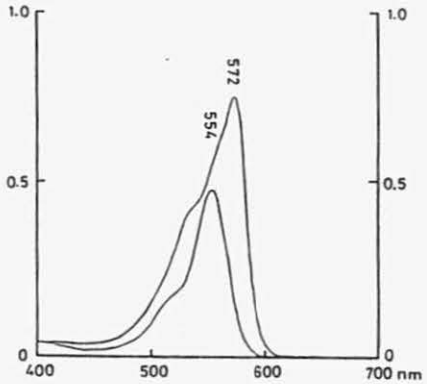

Fig. 5. Rhodamine b and resorufin spectra.

Several dyes were evaluated, and rhodamine b was selected as having a similar visual appearance to resorufin in solution, although spectral analysis shows a λmax approximately 20 nm below that of resorufin (Fig. 5). A standard dilution series was made from the standard dye rhodamine b (Fig. 5, 6A). It seemed to be highly stabile, compared to resorufin, in water solution at room temperature (Fig. 7). Resorufin dilution series of known concentrations were compared to rhodamine b standards at corresponding absorbance maximum wavelengths (572 nm and 554 nm, respectively; Fig. 6A and B). The set of rhodamine b standards used in the assay were prepared according to the absorbance of resorufin standards. The difference in absorbance maximum between these two reagents was so small that it did not interfere with the colorimetric ranking of the samples.

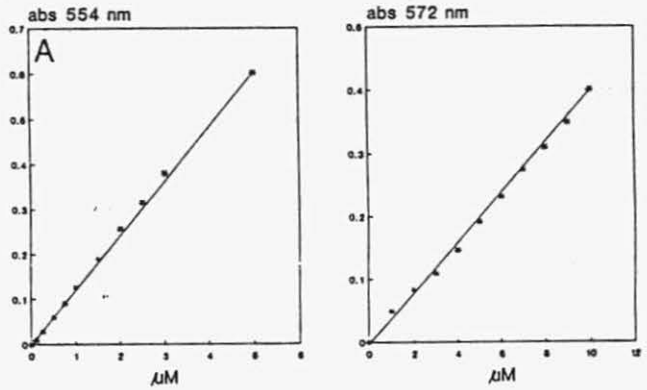

Fig. 6. Rhodamine b (A) and resorufin (B) standard curves.

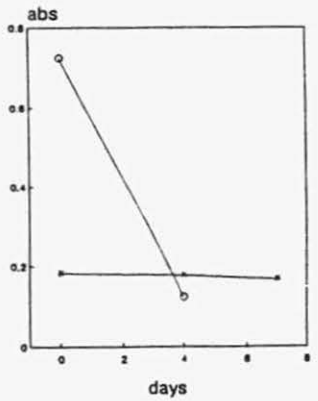

Fig. 7. The effect of storing rhodamine b for 7 days at room temperatue and light (-*-) compared to resorufin (-o-; 4 days).

Microsomes, PMS and WH of known activities were incubated, reactions stopped with MeOH as above, and pink color compared to the rhodamine b standards. After stopping the reaction, test tubes were placed in a rack with a set of standards in identical tubes. Unknown samples were visually compared to the standards to see what the range they fell in. These comparisons were made several times by several 'untrained' people. The estimations of all samples were checked against the absorbances and activity measured by the spectrophotometer. The unknown samples were accurately compared and their activity estimated visually by comparison to the series of dye dilutions that had been previously prepared and calibrated according to the real resorufin standards (Table IV).

Table IV. A) Rhodamine b standards and corresponding resorufin concentrations as well as B) visual analysis of four microsomal samples made by four untrained people and run by the end-point EROD assay.

A	[rhodamine b] μM	[equivalent resorufin] μM
	0	0
	0.1	0.13
	1.25	0.31
	0.5	0.87
	0.75	1.36
	1.0	1.77
	1.5	2.92
	2.0	4.51
	2.5	5.89

B	true resorufin in stopped reaction (μM)[a]	visual examination (converted to resorufin μM)[b] I	II	III	IV
1.	0.950	1.36-1.77[c]	1.36-1.77	0.87-1.36	1.77
2.	0.545	0.31-0.87	0.87	0.87	0.87
3.	0.059	0.13-0.31	0.31-0.87	0.13	0.31
4.	0.039	0-0.13	0.31	0.13	0.13

[a] measured spectrophotometrically
[b] I - IV refer to different untrained observers
[c] the range where the sample fell when compared to rhodamine b standards and converted to resorufin (see above)

General discussion

Hepatic EROD activity is increasingly recognized as robust in identifying individuals of many vertebrate species that have induced P4501A. Interlaboratory comparisons (e.g., Rutten *et al.* 1992) show that fluorometric or spectrophotometric assay, under a variety of conditions, allow investigators to reliably distinguish groups that have experienced greater or lesser exposure to inducers, and even to reliably rank the individuals in terms of activity. The method we describe here takes advantage of the spectral properties of substrate and product, and indicates that visual inspection and comparison to a standard can allow the untrained eye to distinguish samples of greater or lesser EROD activity, and even to estimate that activity. This method could be used as the first screening method, particularly with appropriate control samples for comparison. It gives a way to proceed with EROD determination in laboratories, or situations, e.g. in the field, with less facilities available.

Resorufin in stopped reactions can be analyzed fluorometrically or spectrophotometrically. A simple colorimeter can yield results quite similar to those obtained with more sophisticated instruments. As we show here, visual inspection of reaction mixtures and comparison with rhodamine b standards allows an accurate estimation of EROD activity. The use of a simple colorimeter in the field would provide objective confirmation of that estimate.

There are circumstances under which analysis of catalytic activity alone may not reveal the degree of induction. A number of inducers, such as 3,3',4,4'-tetrachlorobiphenyl, can strongly inhibit catalytic activity of P4501A (Gooch *et al.* 1989). Alkyltins can inactivate P4501A, and possibly other isoforms (Fent & Stegeman 1991). Heavy metals (e.g. cadmium) can induce heme oxygenase and reduce the heme available for formation of holoenzyme (Maines *et al.* 1979). Measurement of P4501A protein or P4501A mRNA can circumvent those problems. However, analysis of field samples indicates that activity measurement of protein or mRNA can be complementary in evaluating induction. Regardless of the method used, however, there is need to determine the significance of a certain level of induction, and what action should be taken. For example, responses in the lower 10th or 20th percentile of a species capacity to respond might be of little concern. Even though catalytic inhibition does occur in some extremely contaminated environments (e.g., Monosson & Stegeman 1991; Elskus *et al.* 1989), in most cases the activity would still be sufficiently elevated to indicate whether there was or was not cause for concern. Analysis of induction by measuring mRNA, protein, or the visual estimation of EROD activity suggested here could be sufficiently accurate to distinguish such responses. However, the evaluation of this visual method in the field remains to be done.

In some freshwater systems downstream from pulp mill effluents there is inhibition in EROD activities measured from exposed fish samples (e.g. Lindström-Seppä & Oikari 1989, 1990a). Observing an increase in EROD activity with increasing distance could signal such inhibition. There could also be some fish species with a P4501A homologue that does not catalyze ethoxyresorufin. Therefore the use of caged fish, which usually are hatched and studied species, is recommended. The use of homogenous material with known prehistory, decreases the variability usually interfering when feral fish are used (Lindström-Seppä & Oikari 1989, 1990b).

The calibration of signals in the field would require knowledge of the maximal capacity of the species being investigated. As yet, few fish species have been thoroughly investigated. Much work has to be done to get some kind of average knowledge about the basic biotransformation abilities among fish species and among fish in different kinds of habitats. The information about the more and the less sensitive species would be of most importance. When using this method, as well as the more precise ones, the investigator would require some knowledge of the basic enzyme activity levels within the species studied as well as their induction potential.

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