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EFFECT OF STORAGE CONDITIONS AND SUBCELLULAR FRACTIONATION OF FISH LIVER ON CYTOCHROME *P*-450-DEPENDENT ENZYMATIC ACTIVITIES USED FOR THE MONITORING OF WATER POLLUTION

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Abstract—Hepatic cytochrome *P*-450-dependent monooxygenase activities of fish, especially 7-ethylresorufin *O*-deethylase activity (EROD activity) have been proposed as possible indicators of water pollution. Nevertheless there is a need for comparisons between different methods of storage and of subcellular fractionation of the liver with regard to absolute activity and responsiveness of EROD activity to pollution. In this report the livers of nase (*Chondrostoma nasus*) were collected in PCB-polluted and reference areas of the River Rhône. They were stored in liquid nitrogen either after slicing or after homogenization in phosphate buffer containing 20% glycerol; subcellular fractionations were performed at the laboratory. Results demonstrate the deleterious effect of freezing slices of liver on EROD activity and point out the protective effect of homogenizing in buffer containing 20% glycerol (a cryoprotector) prior freezing. Besides, we observed a 3-fold higher EROD activity in the microsomal fraction as compared to postmitochondrial fraction, demonstrating the interest of subcellular fractionation to increase the sensitivity of the assay. Finally, we did not notice any difference between methods of storage and of subcellular preparation with regard to the rate of induction of EROD activity between polluted and reference areas. We conclude that in most cases postmitochondrial fraction originating from liver homogenized in buffer containing 20% glycerol prior freezing in liquid nitrogen is a useful preparation to measure EROD activity as a pollution indicator.

Key words—EROD activity, enzymes, cytochrome *P*-450, PCBs, *Chondrostoma nasus*

INTRODUCTION

The chemical pollution of aquatic ecosystems has led to the development of analytical technics able to detect overall pollutants, but during the last decade an increasing interest has been focused on the use of biochemical parameters as indicators of water quality (Haux and Förlin, 1988). It was assumed that a biochemical response should be an early warning signal demonstrating the integration by living organisms of the perturbations of the chemical quality of their environment. In this perspective the study of biotransformation enzymes seems to be promising (Payne *et al.*, 1987).

Biotransformation enzymes are involved in the catabolism of exogenous compounds (pollutants) as well as endogenous substrates (steroids, prostaglandins). Among the several enzymatic systems implicated in biotransformation, there is a considerable interest in cytochrome *P*-450 dependent monooxygenase activities (MO). MO may be modulated (induced or inhibited) by several exogenous molecules. Well known pollutants such as polychlorobiphenyls (PCBs), polyaromatic hydrocarbons (PAHs), polychlorodibenzo-dioxins and -furans are potent inducers of particular MO (7-ethylresorufin *O*-deethylase and "aryl hydrocarbon" hydroxylase). Furthermore, induction can lead to toxicological consequences such as carcinogenesis and disturbance of the metabolism of steroids (Buhler and Williams, 1988; Förlin and Haux, 1985). Therefore, MO could be a valuable warning system allowing the detection of hazardous organic micropollutants at presumably toxic levels. Many field studies have shown the inducibility of fish MO by the above pollutants, demonstrating the usefulness of fish MO as indicators of pollution (Andersson *et al.*, 1988; Monod *et al.*, 1988; Vindimian and Garric, 1989).

Investigations on enzymatic activities of living organisms from the field led to some constrains, especially concerning the storage of the tissues before being back to the laboratory. As cytochrome *P*-450 is a membrane bound enzyme, the conservation procedure may drastically affect its activity; storage into liquid nitrogen is classically used but different methods of preparation of the tissue before freezing are possible. Furthermore, the development of biochemical indicators of pollution must take into

account the cost of the analysis and the practicability of the procedure: enzymatic activities can be measured on more or less purified sample depending on the level of subcellular fractionation, high level of subcellular fractionation ensures generally a high specific activity but is time-consuming and requires ultracentrifugation.

The first objective of the present study was to assess the influence of storage conditions of the liver by comparing 7-ethylresorufin *O*-deethylase (EROD) activity in liver directly frozen in liquid nitrogen with EROD activity in liver frozen after homogenization in a buffer containing a cryoprotector compound. The second objective was to assess the influence of subcellular fractionation of the liver by comparing EROD activity in microsomal fraction with EROD activity in postmitochondrial fraction. Experiments were conducted with liver of nase (*Chondrostoma nasus*) caught in the River Rhône in polluted and reference areas which permitted the comparison of the rates of induction exhibited by the different methods.

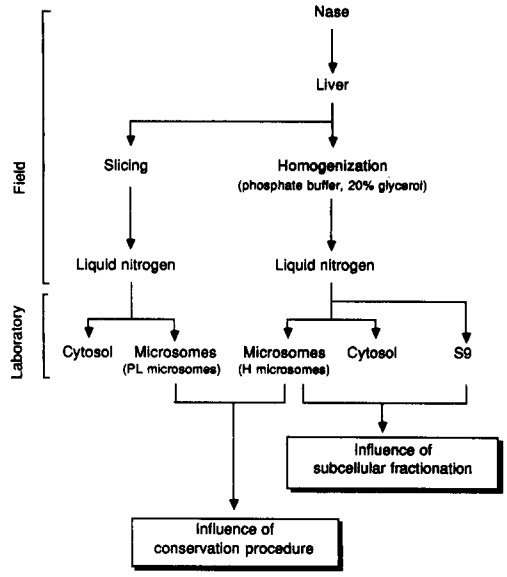

Fig. 1. Protocol for liver preparation and subcellular fractionation.

MATERIAL AND METHODS

Fish

Nase were collected with net by professional fishermen in November 1988. Fish sampling was performed in two locations of the River Rhône presented in a previous paper (Monod *et al.*, 1988). One of the stations (Miribel) was situated in a chemically polluted area (especially PCBs) and the other one (Pont de Lucey) was considered as a reference. Ten males and ten females were caught in each station.

Liver preparation and subcellular fractionation

Protocol for liver preparation and subcellular fractionation was summarized in Fig. 1. Nase were sacrificed by a blow on the head then decapitated for bleeding. Each liver was removed and cut in small pieces; one part was wrapped in aluminium foil and directly frozen in liquid nitrogen, the other part was homogenized in 50 mM phosphate buffer, pH 7.4, containing 0.15 M KCl and 20% glycerol using a Potter–Elvehjem homogenizer driven by a cordless-drill, then collected in 5 ml cryogenic tubes and frozen in liquid nitrogen.

The day after, at the laboratory, samples were thawed and pieces of liver were homogenized in 50 mM phosphate buffer pH 7.4 containing 0.15 M KCl. All homogenates were centrifuged for 20 min (10,000 *g*, 4°C). Supernatant from homogenate (S9) of pieces of liver and one part of supernatant from field-homogenized liver were ultracentrifuged for 60 min at 105,000 *g* at 4°C, the other part of supernatant from field-homogenized liver (S9 fraction) was conserved at −80°C. After ultracentrifugation cytosol was collected and microsomal pellets were resuspended in 50 mM phosphate buffer pH 7.4 containing 1 mM EDTA, 1 mM DTT and 20% glycerol; cytosolic and microsomal fractions were stored at −80°C.

This protocol allowed the comparison of storage conditions using microsomes from field-frozen liver and field-homogenized frozen liver, and the comparison of subcellular fractionations using microsomal and S9 fractions from field-homogenized frozen liver.

Assays

EROD activity was assayed in a final volume of 1 ml containing 0.1 M phosphate buffer, pH 7.4, 0.5 mM NADP,

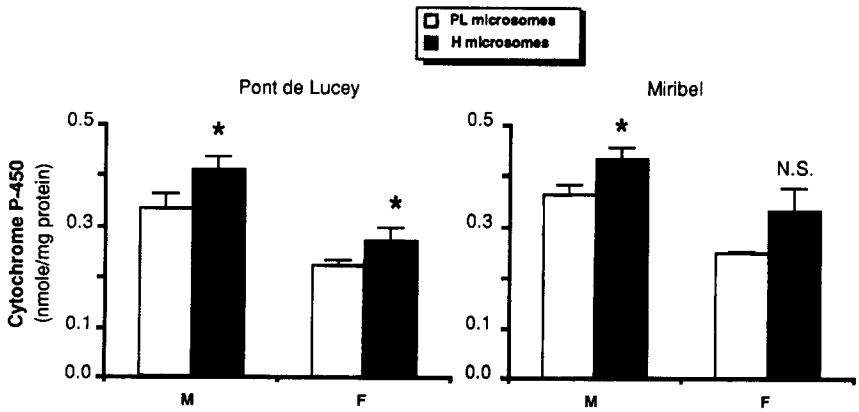

Fig. 2. Cytochrome *P*-450 content in frozen pieces of liver (PL) microsomes and in homogenized liver (H) microsomes of male (M) and female (F) nase caught in reference (Pont de Lucey) and polluted (Miribel) areas. *Significantly different ($P < 0.05$). Each bar represents the mean ± SEM from 10 fish. N.S. indicates not significantly different.

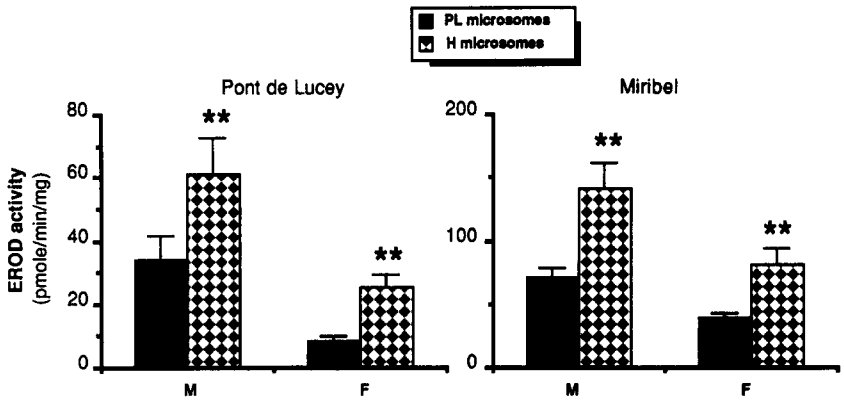

Fig. 3. EROD activity in PL and H microsomes. See Fig. 2 for further details. **Significantly different ($P < 0.01$).

5 mM G 6-P, 2 unit/ml G 6-PDH, microsomal or S9 protein and 1 μM 7-ethylresorufin (added in 10 μl DMSO). Reaction was stopped by 2 ml acetone and samples were centrifuged in order to remove the protein precipitate. Fluorimetric analysis was performed on the supernatant at excitation wavelength 537 nm and emission wavelength 583 nm after standardization by resorufin. The extinction coefficient of resorufin was 73 mM^{-1} (Klotz *et al.*, 1984). Cytochrome *P*-450 content was measured according to Matsubara *et al.* (1976). Glutathione *S*-transferase (GSH-t) was determined following the method of Habig *et al.* (1974) with 1-chloro-2,4-dinitrobenzene as a substrate. Protein content was analyzed by the method of Hartree (1972).

Statistics

As the protocol of liver preparation and subcellular fractionation was performed on each liver, the data obtained from different methods were not independent. Thus, two groups of paired Student's *t*-tests were performed to compare methods of storage and methods of subcellular fractionation.

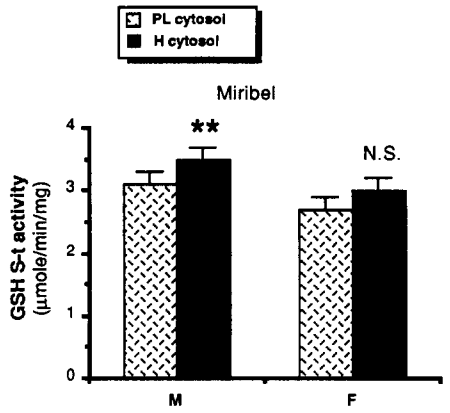

Fig. 4. Glutathione *S*-transferase activity in PL and H cytosol. See Fig. 2 for further details. **Significantly different ($P < 0.01$). N.S. indicates not significantly different.

RESULTS

As hepatic EROD activity of nase is sex dependent (Masfaraud *et al.*, 1990) the experiments were conducted on each sex.

In Fig. 2 it appears that cytochrome *P*-450 content was slightly but statistically significantly higher in microsomes from field homogenized liver ("H microsomes") than in microsomes from frozen pieces of liver ("PL microsomes").

Much more pronounced differences were observed considering EROD activity, with the highest activity in "H microsomes" (Fig. 3).

Figure 4 shows that, in males from Miribel but not in females, GSH-t was slightly lower in cytosol from field-frozen pieces of liver than in cytosol from field-homogenized frozen liver. No analyses were performed on fish from Pont de Lucey.

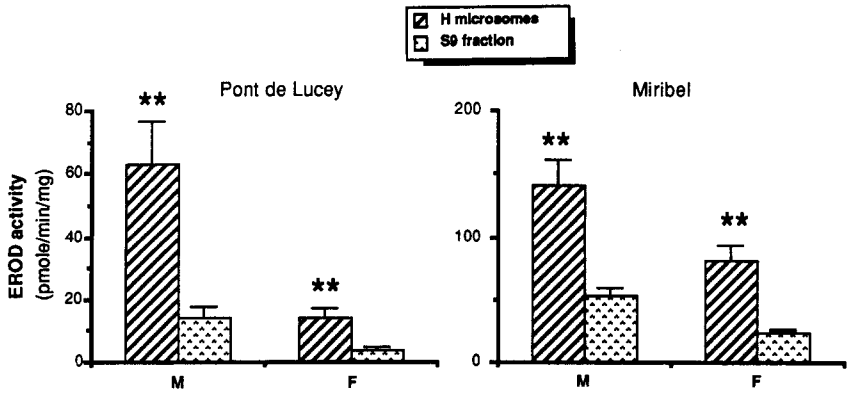

Fig. 5. EROD activity in H microsomes and S9 fraction. See Fig. 2 for further details. **Significantly different ($P < 0.01$).

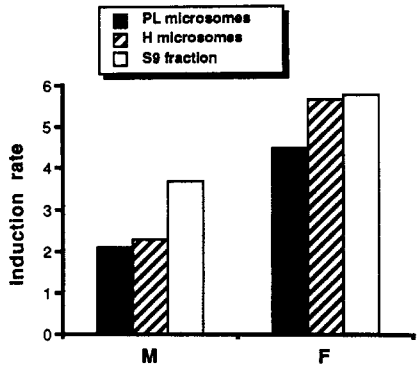

Fig. 6. Induction rate of EROD activity (Miribel activity/Pont de Lucey activity) measured with PL microsomes, H microsomes and S9 fraction. See Fig. 2 for further details.

Figure 5 clearly shows that an EROD activity 3–4 times higher in H microsomes than in S9 fraction was observed in both stations.

Figure 6 points out the fact that the induction rates are of common values whatever the procedure of preparation is.

DISCUSSION

A significant loss of "biochemical integrity" was observed (with male or female fish from polluted or reference areas) using microsomes from frozen liver instead of microsomes from frozen homogenates containing 20% glycerol (a cryoprotector). The difference between loss in cytochrome *P*-450 content (about 20%) and loss in EROD activity (about 50%) might be due to the type of "biochemical integrity" needed. The EROD activity needs the conservation of the catalytic properties of the enzyme whereas the cytochrome *P*-450 content dosage is only dependent on the integrity of the prosthetic heme structure of the cytochrome. As cytochrome *P*-450 is a membrane bound enzyme the alteration of the membrane structure due to freezing without cryoprotector can inhibit the catalytic activity with minor variations in the spectroscopic properties of the heme. The fact that the cytosolic glutathione *S*-transferase (a soluble enzyme) activity is only slightly affected by the direct liver freezing is an other argument for the explanation through a direct membrane alteration. Consequently, the "on field" homogenization of the liver before freezing should be recommended as it prevents the drastic decrease (50%) of the EROD activity observed following direct freezing of pieces of liver.

From the comparison of the S9 and the microsomal enzymatic activities we can conclude that the purification of microsomes increases 3 times the sensitivity of the assay. This increase is not sex dependent and is not significantly affected by the enzymatic induction due to exposure to pollutants.

This study clearly points out the great importance of the conservation and purification procedures used for field studies using cytochrome *P*-450 dependent activities. The EROD activity assay being one of the most widely used whenever fish are concerned, the standardization of the procedure is needed for inter-laboratory work. The loss of activity due to liver freezing compared to post homogenization freezing is one of the major points. Furthermore Förlin and Andersson (1985) demonstrated that the freezing of the rainbow trout liver was only efficient for 3 days which is a serious time-limit for the investigation of field areas far from the laboratory. Therefore, so long as homogenization can be quickly and efficiently carried out immediately after the capture of fish using a cordless motor driven homogenizer, this method can be recommended.

The fact that the S9 EROD activity showed the same rate of induction as the microsomal EROD activities (with only a scale factor) is a good argument for the use of S9 for EROD assay. The purification of microsomes led to the highest activities and should be done when the increase of sensitivity is the major problem.

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