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Induction of Benzo (a)Pyrene Monooxygenase in Fish and the Salmonella Test as a Tool for Detecting Mutagenic/Carcinogenic Xenobiotics in the Aquatic Environment

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PAYNE and PENROSE (1975), AHOKAS et al.(1976), and GRUGER et al. (1977) have shown that benzo (a) pyrene monooxygenase (BPMO) can be induced in fish exposed to petroleum. BPMO measurements in local fish appear to be a practical biological indicator for monitoring marine petroleum pollution (PAYNE 1976). BPMO has been shown to be induced in Blennius pavo, a species with a restricted territorial range, in response to exposure to Diesel No. 2 oil (KURELEC et al., 1976). This has made possible the recognition of polluted marine areas and even a follow-up study of the consequences of an oil-pollution incident by measuring the increased BPMO levels in the livers of the local Blenniide (KURELEC et al. 1977). Such findings have confirmed that BPMO measurement in the livers of non-migrant fish could serve as a useful biochemical parameter and a diagnostic tool for environmental monitoring and evaluation of the effects of acute or long-term oil pollution at a given site.

From the ecological point of view and on account of the interest in the environmental assessment of biological effects of xenobiotics on water organisms, it would be highly desirable to detect their presence by an equally revealing biochemical method in water samples. Considering the induction of BPMO as a means of detoxification in fish, we studied the consequences of i/p application of polluted water extracts from different marine sites to young carp. Aliquots of these extracts served in Salmonella/microsome mutagenicity tests using liver homogenates of pollution-induced fish as activating systems. The results we obtained so far are presented in this paper.

MATERIAL AND METHODS

Chemicals: Hexane, fluorescent grade, was from E. Merck, Darmstadt, Germany; commercial corn oil from Oil Factory, Zagreb, Yugoslavia; aflatoxin, NADP, NADPH, glucose-6-phosphate, and dimethyl sulphoxide from Serva, Heidelberg, Germany; benzo (a) pyrene from Sigma, St. Louis, U.S.A. All other chemicals were of analytical grade.

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Sea-water samples were collected at the boarder of the "mixing-zone" area of the outlet of a fish cannery in the North Adriatic at distances of 50 and 500 m off shore. Two 1l samples were collected with a sampler at a depth of 1m. 40ml of hexane were added immediately to each sample in a separatory funnel and shaken for 1 min. The phase separation was performed for 15 min. The hexane extracts were stored in glass vials at 0°C. Further processing of the sample included the reduction of the volume to 0.2 ml with a Rotavapour (Büchi, Switzerland) at 35°C. 0.2 ml of corn oil were added to the hexane extract, and the evaporation was continued to a constant volume. This was used for i/p injection. The efficacy of the extraction was tested with 0.2 ml of crude oil from Libya and with Diesel No. 2 oil, which were added to 1l of charcoal filtered sea-water and extracted with hexane. Fluorescence of the hexane aliquots using a Perkin-Elmer fluorometer and monochromators for activation at 395 μm and emission at 520 μm showed that the extraction was complete.

Animals. Specimens from a large population of Mugil cephalus, which are supposed to spend a large part of their time in the "mixing zone" of the cannery run-off, were caught by amateur fishermen from January to April 1977. Livers from the specimens, weighing 190-240 g, were processed within 10 min for the assay of BPMO activity. One-year old specimens of wintering carp from one hatchery-school, weighing 210-225 g, were kept for two months in 180 cm x 80 cm x 50 cm acclimatisation basins, with 1 m^3 of dechlorinated, well-aerated water, at a density of 200 specimens per m^3, at a flow of five total changes per day at 13°C, for use in the experiments. Animals were allowed no food during the acclimatisation period nor during the experimental period.

Preparation of homogenates, subcellular fractionation, and enzyme assay. Postmitochondrial fractions were prepared according to PAYNE and PENROSE (1975) with minor modifications described previously (KURELEC et al., 1977). 100 μl aliquots were used in the BPMO assay according to NERBERT and GELBOIN (1968). Protein in the aliquots was determined by the method of LOWRY et al. (1951). Aliquots of the postmitochondrial fractions from Mugil and from carp were used either immediately after preparation or after a storage at -80°C in the Salmonella - activated mutatest.

Mutagenicity testing. The mutagenicity of water extracts was tested using the "plate incorporation assay" (AMES et al., 1975) with the histidine auxotroph S. typhimurium strain TA 100, kindly provided by B.N. Ames, University of California, Berkeley, U.S.A. The strain was grown overnight in nutrient broth. Postmitochondrial fish-liver fractions, the cofactors NADP and glucose-6-phosphate, bacteria, and the extracts or standard substances to be tested were

mixed into a soft agar layer and plated onto histidinedeficient agar media according to AMES et al. (1975). The number of his^+ revertants was determined after 48 h. The increase in number of revertants with respect to the number of control revertants was taken as a measure for the mutagenic activity of the extracts.

RESULTS

Induction of BPMO activity in carp by i/p application

A single i/p injection of an adequate substance can induce BPMO activity in carp within one or two days (Fig. 1). Adequate inducers used in our experiments were as follows: benzo(a)pyrene, 25 and 50 µg; aflatoxin, 25 µg; crude oil, 50 µl; hexane extract of charocoal filtered sea-water with an added equivalent of 50 µl of crude oil, and sea-water extracts from the "mixing zone" 50 m off a cannery outlet. Hexane extracts of sea-water (HESW) from the sites 200 m, 500 m, and 3 km, or HESW with an addition of 50 µl Diesel No. 2 oil, or 0.1 ml of hexane with 0.1 ml of corn oil, or corn oil alone did not induce BPMO activity.

BPMO activity in Mugilus specimens

Mugilus caught in the "mixing zone" revealed an extremely high BPMO activity of 1600 ± 200 units (10 measurements), whereas three specimens caught in "clean" areas had activities of 65, 71, and 52 units. These activities are comparable with values of 69, 22, 73, 17, 29, and 18 units found previously (KURELEC et al.,1976) in sardines from different sites in the North Adriatic, or with values of a mean value of < 100 units found in Blenniide at "clean" sites in the vicinity of Rovinj (KURELEC et al. 1977). Obviously, BPMO in the specimens caught in the mixing zone was induced by substances present in the effluents from the fish cannery.

Mutagenicity testing

It is evident from Table 1 that neither the HESW extract nor aflatoxin nor benzo(a)pyrene were mutagenic in the absence of liver postmitochondrial fraction. Standard substances, benzo(a)pyrene and aflatoxin, which require metabolic transformation to an active compound, produced revertants when the liver fraction from untreated carp was added. HESW 50, as different from HESW 500, induced BPMO in carp and was mutagenic for Salmonella when the liver fraction from HESW 50 - treated carp was added. The maximum mutagenic effect of HESW 50 was observed in the presence of the liver fraction from Mugil caught in the mixing zone, but the same liver fraction

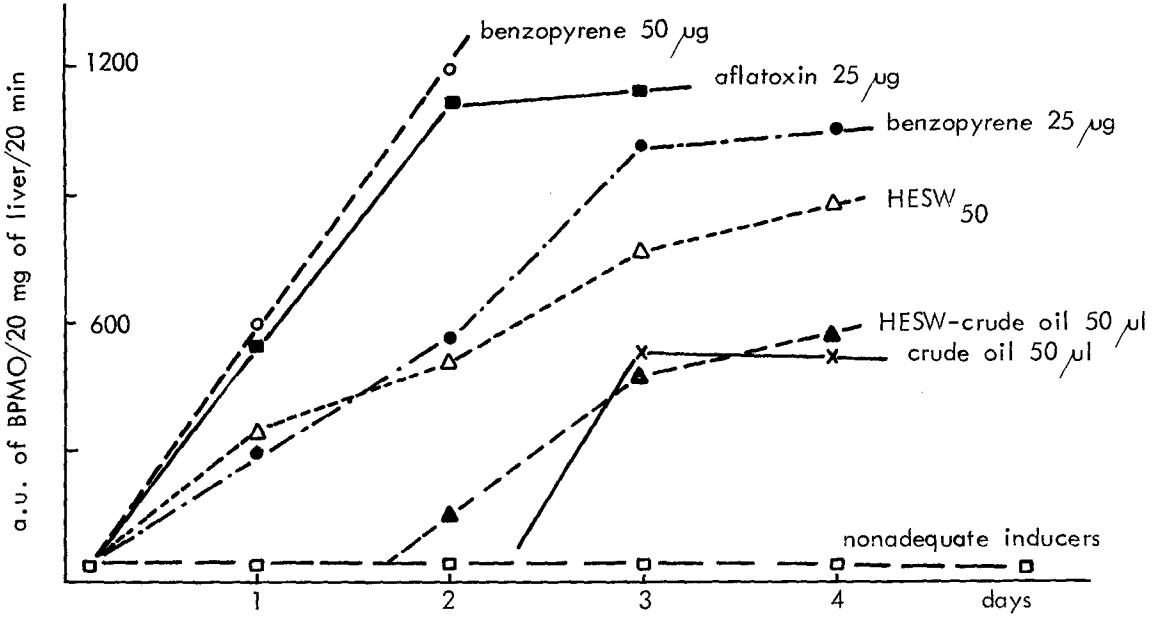

Figure 1. Induction of BPMO in carp after i/p application of xenobiotica

Nonadequate inducers were: $HESW_{500}$, $HESW_{200}$, $HESW_{3000}$, HESW with added 50 µl Diesel No 2 oi 0.1 ml of hexane and 0.1 ml of corn oil

TABLE 1
Induction of *his*$^+$ revertants in *S. typhimurium* strain TA 100 in plate incorporation testing with liver postmitochondrial fraction

Liver postmitochondrial fraction	Carp HESW 50 m treated	Carp BP treated	Carp untreated	Mugil from mixing zone	Liver postmitochondrial fraction omitted
BPMO a.u.***	790	1600	180	1300	
Substrate					
$HESW_{50}$**	++*	+	+	++	-
$HESW_{500}$**	-	-	-	++	-
BP	+	+	+	++++	
Aflatoxin	+	-	++	+++	
Control (no substrate)	-	-	-	++	-

* one + corresponds to a 30% increase in number of revertants over control plates;

** HESW = hexane extract of sea-water at a distance of 50 m or 500 m from the cannery outlet;

*** a.u. = arbitrary units.

produced revertants even without substrate.

DISCUSSION

Using the Salmonella test and the BPMO induction, it has been shown that the bioactivation products of hexane-extracted xenobiotics in carp are similar to the bioactivation products of the action of mixed-function oxidases (MFO) in the sea-water fish Mugilus cephalus in a polluted marine environment. This indicates that hexane extracts from sea-water contain just those xenobiotics which induce enzymes in fish living in a particular marine environment. In addition, mixed function oxidases induced using i/p application are of the same species as those induced in natural exposure (gills, ingestion). Further, freshwater fish respond to xenobiotics in a similar manner as sea-fish.

From the results presented it may be concluded that the detection of the presence of xenobiotics in a given sample of water is possible using the techniq of i/p application of water-hexane extracts to carp, followed by the measurement of BPMO induction.

One-year old carp were chosen as the most convenient test animals for the following reasons: 1. They are representatives of the aquatic environment; 2. they do possesss inducible mixed-function oxidases; 3. it is possible to produce clones by artificial spawning; 4. it is easy to keep large schools under the same environmental conditions. To the advantages of the material used, which is well equilibrated by both the genetic criteria (NEBERT and FELTON 1976) and the criteria of environmental factors (GILLETTE 1976), some practical criteria should be added: simple keeping, manipulation, and inexpensiveness. These arguments suggest the use of the described technique as a pre-screen tool for prescribing an appropriate cost-effective analytical method for detecting pesticides, polynuclear aromatic hydrocarbons, and other mutagenic and/or cancerogenic pollutants.

The induction of BPMO at the same time represents a badly needed sublethal effect which could be detected by a biochemical, i.e. reproducible, standardized, and quantitative method (GESAMP/UNEP 1976). Besides this diagnostic value, the BPMO induction shows some further characteristics important from the ecological point of view. Mixed function oxidase systems of animals are not "tool proof" (BRATTSTEN and WILKINSON 1977). Being programmed primarily to effect lipophile-hydrophile conversions rather than detoxification per se, mixed function oxidase enzymes sometimes transform substances that are initially relatively innocuous into actually toxic, mutagenic and/or cancerogenic compounds. Enzyme systems have turned out to be suicidal to the host organism. The syntheses catalysed by these

enzymes have been termed also "lethal synteheses" (PEDERSEN et al. 1975: PETERS 1963). Thus we may present a rather severe interpretation of the ecological significance of the presence and induction of mixed function oxidases. Whenever they are induced by xenobiotics in the aquatic environment, the capacity of the biological system to "bioactivate", i.e., to produce mutagens and/or cancerogens, is increased. Recent studies in this field have established that more than 90% of cancerogens stem from the environment (McCANN and AMES 1976), that most of them have to be bioactivated (MAGEE 1975), that the induction is highly correlated to tumor incidence (BARTSCH et al. 1976), and that the initiation of tumors is proportional to the level of enzyme induction (BUTY et al. 1976; NEBERT et al. 1977). Thus, the induction of BPMO seems to have a predictive value in the assessment of the environmental hazard from mutagenic and/or cancerogenic substances. This seems to be true of our experiments with mutatesting of water extracts. The components of sea-water extracts from a "mixed-type"-pollution site do not cause reversion in the Ames test with the Salmonella strain T 100. The same extracts, however, applied i/p to carp, induce BPMO and produce revertants in the presence of the carp liver postmitochondrial fraction. At the same time, Mugil specimens contaminated with the same pollutants, were induced with respect to BPMO activity. Addition of the liver fraction from naturally induced Mugil specimens to sea-water extracts tested using the Salmonella test, resulted in the highest reversion rate of Salmonella mutants. It may be concluded that the sea-water from the "mixing zone"does contain substances that can be qualified as premutagens and/or precarcinogens, since they can be biotransformed with microsomal enzymes into mutagens and carcinogens.

The assessment and prediction of the mutagenic and/or carcinogenic hazard from man-made substances for the aquatic ecosystem would depend strongly not only on the induction of MFO, but also on (a) the capacity of their DNA-repair mechanism (HART et al. 1977), (b) on the activity of the biotransformation pathway which converts epoxides into diols, including microsomal epoxide hydrase (OESCH 1974) and (c) on glutathione S-transferases (JACOBY and KEEN 1977). The latter enzymes catalyse the formation of glutahione conjugate with electrophile (cancerogen), just preventing the damage of DNA at the price of an expandable protein. However, the first step in any study aimed to assess the mutagenic and/or carcinogenic hazard is detection of the presence of these substances in the environment. It seems that the i/p application of polluted water extracts to carp, followed by the determination of BPMO induction and combined with the Salmonella test could be used as a relevant method for detecting mutagenic and/or cancerogenic xenobiotics in aquatic environment.

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