Trout Hepatic Enzyme Activation of Aflatoxin B_1 in a Mutagen Assay System and the Inhibitory Effect of PCBs[1]

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INTRODUCTION

Bacterial mutagen assay systems have frequently been suggested as preliminary screening systems of potentially carcinogenic compounds. One such method, gaining wide acceptance, developed by Ames and co-workers (AMES et al. 1973; 1975), utilizes several histidine mutants of Salmonella typhimurium to detect metabolically activated mutagens. This method usually employs the submitochondrial fraction of rat liver to activate the test compound in a semisolid incubation mixture, but other mammalian tissues have also been utilized (AMES et al. 1975). To the authors' knowledge, nonmammalian tissues, such as fish, have never been used in this mutagen assay system.

It was originally believed that fish lacked the enzymes necessary for xenobiotic metabolism (BRODIE & MAICKEL, 1962), however, numerous studies have since shown a similarity with mammalian mixed function oxidase systems (MFO) (POTTER & O'BRIEN, 1964; CREAVEN et al. 1965; 1967; CHAN et al. 1967; LOTLIKAR et al. 1967; BAKER et al. 1963). Fish MFO appear to be capable of catalyzing many of the same drug metabolic reactions observed in mammalian MFO (BUHLER & RASMUSSON 1968; DEWAIDE 1971; LIDMAN et al. 1976; AHOKAS et al. 1975; BEND et al. 1973). The fish MFO enzymes appear to be more labile than mammalian MFO and have a lower optimum temperature.

Fish MFO drug metabolism has also been shown to be inducible by polychlorinated biphenyl compounds (PCBs) (LIDMAN et al. 1976; HILL et al. 1976; AHOKAS et al. 1975) much as these compounds induce mammalian MFO activity (ALVARES et al. 1973; KONAT & CLAUSEN, 1973; ORBERG & LUNDBERG, 1974; TURNER & GREEN, 1974; IVERSON et al. 1975; LITTERST et al. 1972; 1974). The degree of inducing activity in a series of PCBs, Aroclors, has been shown to be dependent upon the degree of chlorination, the higher chlorinated PCBs having greater activity (SCHMOLDT et al. 1974; ECOBICHON & COMEAU, 1974; ORBERG, 1976; BICKERS et al. 1972). One of these, Aroclor 1254, a 54% chlorinated biphenyl, has been recommended as a pretreatment (i.p. 500 mg/kg) of rats utilized in the Ames mutagen assay (AMES et al. 1975).

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The present work was undertaken to define the optimal conditions for use of the Ames assay with rainbow trout (Salmo gairdneri) liver postmitochondrial fraction. In addition, the effect of several PCBs of varying chlorine contents upon the response of the assay to a metabolically activated carcinogen was investigated. Aflatoxin B_1 (AFB), a potent carcinogen in rainbow trout (SINNHUBER et al. 1968) and a potent mutagen in the Ames assay (AMES et al. 1973; 1975; WONG & HSIEH, 1976) using rat liver postmitochondrial fraction, was utilized as the test compound.

METHODS

Rainbow trout (S. gairdneri), Mt. Shasta strain, average weight ca. 500 g, were used in all experiments. Fish were fed a casein-gelatin based semipurified diet to satiety prior to sacrifice or injection. All fish were held in circular tanks supplied with 3 gallons of well water/min. at 12°C. Fish were stunned by a cranial blow and their livers aseptically removed. Upon recording of liver weight they were perfused with ice-cold Krebs-Ringer solution adjusted for fish (SCHOENHARD et al. 1976) and homogenized in 2 volumes phosphate buffer (pH 7.4) using 4 passes of a pestle in a Potter-Elvejhem apparatus. Liver homogenates were then centrifuged at 20,000 xg for 10 min. and the postmitochondrial fraction (PMF) collected, frozen in dry ice and held at -40°C for no longer than 12 hrs. All operations were carried out at 4°C and 3 replicates of 3 pooled livers were used. Protein content of the PMFs was determined by the Lowry method (LOWRY et al. 1951). PCB experiments were done utilizing fish from the same lot and conditions as outlined above. Anesthetized fish (Tricaine Methane Sulfonate) were injected i.p. with 500 mg/kg of either Aroclor* 1221, 1242, 1254 or 1260 in DMSO. Control fish received 0.3 ml i.p. DMSO. Fish were then fasted and sacrificed 5 days later. Two replicates of 3 pooled fish each for every PCB tested were done.

Microbial mutagen assays were carried out, in general, after the method of Ames et al. (1973; 1975) using the AFB sensitive tester strain S. typhimurium TA 1538**. Twelve hr. Trypticase-Soy Yeast Extract broth cultures were used in the experiments giving an inoculation level of ca. 10^8 cells/assay. Krebs-Ringer solution adjusted for fish was used as the incubation mixture salts solution. Fish PMF equivalent to 50 mg. of wet weight liver and 0.25 µg AFB in 2.5 µl ethanol were also added unless otherwise noted. The G-6-P, NADP(+), PMF, salts, bacteria and toxin were all mixed with top agar and poured upon preset Minimal Davis (Difco) with 2% glucose. Plates were incubated at 25°C for

*Aroclor's were provided by Monsanto Chemical Co., St. Louis, MO.

**Culture was a gift of B. N. Ames, Biochemistry Dept., University of California, Berkeley, California.

9 hrs., then transfered to 37°C for 48 hrs. before counting of the colonies. Each assay was plated in duplicate for the parameters study and in triplicate for the PCB work.

Experiments were run to determine optimal assay conditions with fish PMF. Optimal metabolic incubation time at 25°C for the conversion of AFB to an active mutagen was determined by holding plates for 0, 3, 6, 9, 12, 24 and 48 hrs. prior to 37°C incubation. Varying levels of the PMF were added to each plate to define optimal protein concentration. Two, 4, 6, 8, 10 and 12 mg PMF protein/plate were used. Likewise, AFB concentrations, 0.05, 0.075, 0.1, 0.25, 0.5 and 0.75 µg AFB1/plate were employed to define optimal toxin concentration. Results were plotted on linear graph paper and analyzed by linear regression analysis and Students T-test for line-fit and significance.

The effects of pretreatment of fish with different Aroclors upon mutagen assay response to AFB were determined by varying the amounts of PMF used/assay and comparing the different curves obtained. Results were statistically analyzed as before.

RESULTS AND DISCUSSION

The results of this study clearly indicate the ability of fish liver drug metabolizing enzymes to function in the Ames mutagen assay. By utilizing a different salt solution and incubation regimen, rainbow trout (S. gairdneri) PMF enzymes were able to metabolize AFB to an active mutagen to the test microorganism. An average of 5.2×10^4 revertant bacteria/µg AFB/g wet liver were detected in plates preincubated at 25°C for 9 hrs. prior to transfer to 37°C. This preincubation allowed the trout MFO biotransformation of AFB to its active mutagenic form to occur before raising the temperature for rapid bacterial growth.

Optimal mutagen assay conditions were found to be obtained with 6.4 mg PMF protein and 160 ng AFB/incubation mixture and a metabolic incubation temperature of 25°C. As shown in figure 1, a significant ($P<.10$) increase in the number of revertants was observed when metabolic incubations were carried out at 25°C for 9 hrs. although higher, nonsignificant, responses were obtained at 12, 24 and 48 hours.

An attempt to determine the effects of a series of chlorinated biphenyls, known inducing agents of fish enzymes (AHOKAS et al. 1975; HILL et al. 1976; LIDMAN et al. 1976), upon the mutagen assay response was also made. As shown in figure 2, the result was a significant reduction in response versus control response when fish were pretreated with Aroclor 1242, 1254 and 1260. This result is in contrast with results of assays using rat PMF (AMES et al. 1973; 1975). A pattern of decreasing response with increasing chlorination number was observed, except with Aroclor 1260. Protein levels were not significantly different from control values in agreement with LIDMAN'S et al. (1976)

finding of nonsignificant changes in rainbow trout (S. gairdneri) PMF up to 21 days post oral dosage of 1000 mg PCB/kg. However, these authors reported significant increases in cytochrome P-450 and several drug metabolizing enzyme activities at 3 and 7 days post PCB exposure.

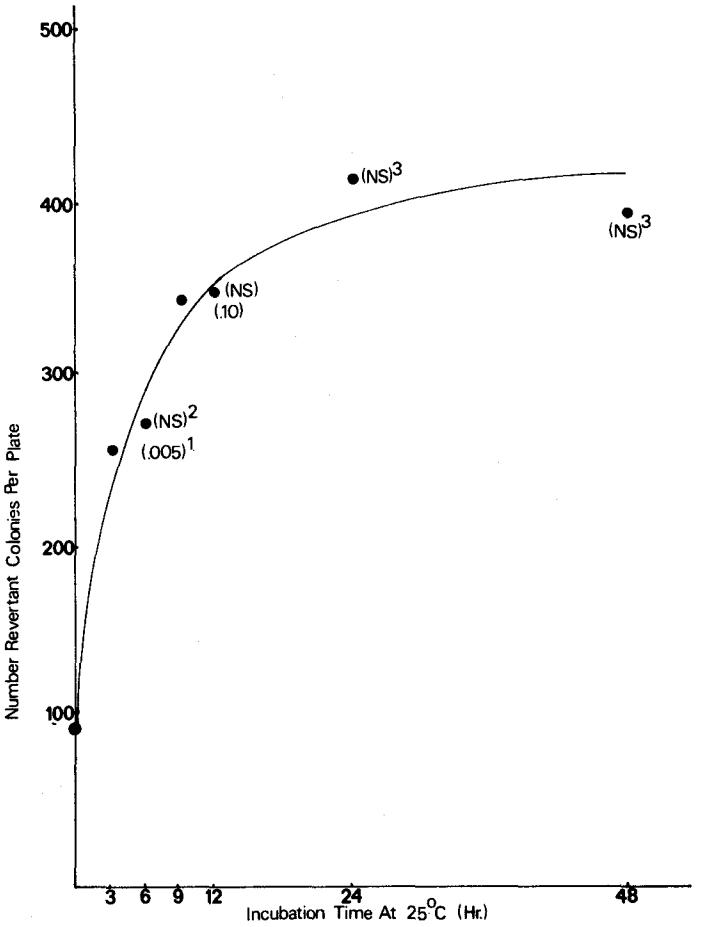

Fig. 1. Effect of length of metabolic incubation at 25°C upon mutagenic response of S. typhimurium TA 1538.

[1]Probability obtained by comparison with preceding point.
[2]NS = Nonsignificant difference.
[3]NS compared with 9 hr. data.

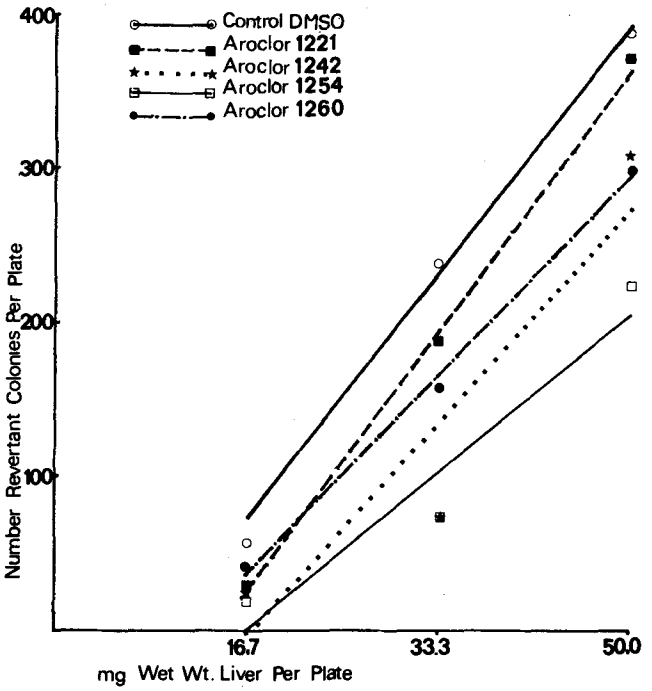

Fig. 2. Effect of PCB pretreatments of fish 5 days prior to mutagen assay with S. typhimurium TA 1538.

A possible explanation of the apparent conflict between reported PCB induction of trout MFO and of decreased mutagen assay responses in PCB treated fish may be that mutagen detoxifying enzyme systems were induced to a greater extent than the AFB activating MFO enzyme system. It has been reported that one such potential enzyme, epoxide hydrase, is inducible in the

rat (BELLWARD et al. 1975; LU et al. 1975). The decreasing assay response with increasing chlorination of the PCB may reflect the PCBs ability as an inducer of these enzyme systems in the rainbow trout. This finding appears to substantiate recent findings in rainbow trout feeding trials of Aroclor 1254 and AFB in which PCB treated fish had a lower AFB induced incidence of cancer (HENDRICKS, communication, our laboratory).

In summary, it was demonstrated that the trout PMF may be successfully employed in the Ames mutagen assay method by utilization of proper salts solution and a metabolic preincubation period at 25°C. It has also been observed that, unlike the rat, pretreatment of rainbow trout with various PCBs decrease the mutagen assay response to AFB.

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