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Title: Induction of Hepatic Microsomal Enzymes in Rainbow Trout by Dietary Aroclor 1254 and the Effect of Cyclopropene Fatty Acids

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A dietary level of Aroclor 1254 (100 ppm) was fed to rainbow trout (Salmo gairdneri) for 15 weeks to determine the effects on hepatic microsomal enzyme induction. Fish were also fed combined polychlorinated biphenyl (PCB) (100 ppm) and cyclopropene fatty acids (CPFA) (50 ppm) to determine the effects on mixed function oxidase (MFO) induction.

Dietary PCBs markedly induced the microsomal activities of 7-ethoxyresorufin O-deethylase, 7-ethoxycoumarin O-deethylase, and benzo(a)pyrene monooxygenase. Ethoxyresorufin O-deethylase activity continued to increase to a level 77-fold higher than control at week 15. Ethoxycoumarin O-deethylase and benzo(a)-

pyrene monooxygenase activities increased to 7.1-fold and 48-fold over control at week 9 and then slightly decreased to 6.8-fold and 45-fold over control at week 15, respectively. Cytochrome P450 values remained approximately 2-fold above controls from week 5 through week 15. At weeks 1 and 3, cytochrome P450 levels were not significantly different from control.

Ethoxyresorufin O-deethylase, ethoxycoumarin O-deethylase, and benzo(a)pyrene monooxygenase activities in the combined PCB and CPFA-fed trout were significantly higher than in controls and CPFA-fed fish, and significantly lower than in PCB-fed fish. There was no significant difference in cytochrome P450 levels after week 5.

This is the first time dietary PCBs have been shown to induce the MFO system in PCB-fed rainbow trout.

Induction of Hepatic Microsomal Enzymes in Rainbow Trout by Dietary Aroclor 1254 and the Effect of Cyclopropene Fatty Acids

by

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I. LITERATURE REVIEW

Polychlorinated Biphenyls

Introduction

Polychlorinated biphenyls (PCBs), a class of toxic industrial organic compounds, are chemical mixtures of chlorinated biphenyl isomers and homologs. The biphenyl structure may be substituted with one to ten chlorine atoms, and over 200 compounds are possible (Gustafson, 1970). The atomic structure of PCBs gives them specific chemical characteristics such as extreme stability, nonflammability, nonconductivity, and heat resistance, which are very desireable for certain industrial uses (Nadeau and Davis, 1976). Originally used in transformers, as heat transfer fluids, and as protective coatings for wood, PCBs were also put into paints, inks, pesticides, adhesives, and carbonless copy paper throughout the early 1960's. PCBs, a series of lipophilic compounds even more stable than DDT, were first recognized as environmental pollutants in 1966. Due to

their recent widespread and uncontrolled industrial application, PCBs have become a persistent and ubiquitous environmental contaminant, and also an indirect contaminant of food (HEW/FDA, 1977). During the past 45 years, approximately 635 million kilograms of PCBs were produced in the U.S., of which about one-third now reside in landfills, water, air, soil, and sediments (USEPA, 1976). PCBs have been found in snow melt water indicating fallout from the air may be an important source of contamination. An average of air samples in Florida, Mississippi, and Colorado contained 100 nanograms PCB/ cubic meter of air (HEW/FDA, 1977). PCB compounds possess strong lipophilic properties which combined with their low metabolic and excretion rates, result in an accumulation in adipose tissue and tissues with high lipid content (Platonow et al, 1976). These compounds are transferable and biomagnified within food webs (Nadeau and Davis, 1976).

Food Contamination

Contamination of foods with PCBs results primarily from four broad sources: 1) environmental contamination- in fish from contaminated lakes and streams, 2) industrial accidents- leakage or spillage,

3) agricultural contamination- PCB migration to feed from silo coatings and subsequent milk contamination, and 4) food packaging materials- migration into foods (HEW/FDA, 1977). Regulation and surveillance of the food supply is maintained through FDA (Food and Drug Administration) sampling plans. In 1973, Temporary Tolerance Limits for PCBs were established by the FDA for certain food products. These tolerances have recently been lowered due to a decrease in the level of unavoidable contamination. Because of the cumulative effect of PCBs in tissues, the food tolerances are generally higher than the detection limits (HEW/FDA, 1977). According to the Federal Register (June, 1979), the temporary tolerances for unavoidable PCB residues in several classes of foods are: milk and dairy products, 1.5 ppm (fat basis), poultry, 3 ppm, eggs, 0.3 ppm, and fish and shellfish, 2 ppm (HEW/FDA, 1977). Despite these tolerance levels, Hudson River trout and salmon have PCB levels that range as high as 178 ppm, Great Lakes fish have PCB levels as high as 30 ppm (HEW/FDA, 1977), and PCBs have been detected in human milk from many areas of the world ranging from 1.86 to 3.50 ppm (milkfat basis) (Musial et al, 1974).

Human Exposure

An allowable daily dose for PCBs was arrived at after evaluation of toxicological data based on a chronic two year dog study and rat study. A no-effect level for humans based on the dog study was calculated to be 2.5 ug/kg body weight/day, employing the standard 100-to-1 safety factor in relating animal experiments to human health standards, and was calculated to be 3 ug/kg body weight/day based on the rat study (Highland, 1976). Human toxicological data was also used to determine the daily allowable dose derived from investigations of human PCB poisoning that occurred in Yusho, Japan in 1968. The "Kanemi Yusho Incident" was caused by contamination of rice oil with Kaneclor 400, a Japanese brand of PCB containing 48% chlorine. The rice oil contained 2000-3000 ppm PCB and the average daily intake of PCBs was estimated to be 37.5 mg/day (HEW/FDA, 1977). About 1400 persons consumed the contaminated oil (Umeda et al, 1978) and developed nausea, lethargy, acne-like skin lesions (Oishi et al, 1978), increased eye discharge, sweating palms, and pigmentation of the skin and nails (Karatsune et al, 1972), though the disease was not

always accompanied by specific physical symptoms (Umeda et al, 1978). The average cumulative dose causing an effect in the Japanese was reported to be 2000 mg and the lowest total dose causing an effect was 500 mg. Applying a safety factor of 10-to-1, the FDA arrived at an allowable daily dose of 1 to 4 ug/kg body weight/day (Highland, 1976). The validity of these calculations are questionable because they are based on estimates of individual Japanese intakes, an arbitrary safety factor, and a failure to consider long-term effects caused by stored PCB residues (Highland, 1976). Also, these levels are easily exceeded due to the levels of contamination in foods, and the rat and dog have been proven to be unreliable test subjects as primates have been shown to be more sensitive to PCBs than either the rat or the dog (ibid.).

Fetotoxicity

Along with the stillbirths, abortions, and undersized infants attributed to transplacental movement of PCBs in the Japanese mothers who consumed PCB-contaminated rice oil (Karatsune, 1972), adverse reproductive effects have also been reported in animals given

PCBs (Dahlgren, 1972). In mice, small amounts of PCBs enter the fetus through the placenta while larger quantities are transferred to the offspring through the milk (Dahlgren, 1972). PCBs interfere with the reproductive function of male pigs (Platonow et al, 1976), cause reduced fertility in sows (Hansen et al, 1975), and cause reproductive failure in mink (Aulerich et al, 1973), in sea lions (DeLong et al, 1973), and in birds (Risebrough et al, 1968). The percent hatchability, length, and weight of coho salmon are dependent on the concentration of PCBs in the water (Halter and Johnson, 1974). Transplacental passage of PCBs has been reported in rabbits and rats (Villineuve et al, 1971) and in a Jersey cow (Platonow and Chen, 1973). Offspring from female monkeys fed 5 ppm PCB had very high tissue levels of PCB in comparison to rodents, suggesting a large species variation in transplacental movement of PCBs (Masuda et al, 1978). Platonow and Geissinger (1973) reported that low doses of PCB stimulated growth, while high doses of PCB suppressed the growth rate of pigs.

Trace Contaminants

Embryotoxicity of the PCBs has been attributed

to trace contaminants in the commercial products (Vos et al, 1970). Chlorinated dibenzofurans were detected in Aroclors 1248, 1254, and 1260 in the order of 0.1 ug/kg of the PCB (Bowes et al, 1973). Aroclors, a commercial PCB product, are complex mixtures and besides containing approximately 80 different chlorobiphenyls, they also are contaminated with trace amounts of toxic impurities such as chlorinated dibenzofurans (CDF) and chlorinated naphthalenes (Vos et al, 1970; Jensen and Sundstrom, 1974). Little is known of the environmental persistence and bioaccumulation potential of CDF. CDF accumulates in the liver, fat, and spleen in mice (Morita and Oishi, 1977), and it is suggested that they are more rapidly eliminated from the rat than PCBs because they can be detected in the urine (Norstrom et al, 1976). The PCB-contaminated rice oil in Japan was suspected of containing other toxic impurities such as CDF when it was shown to be two times more toxic to mice than the levels of PCBs found (Ikeda, 1972). The Yusho rice oil contained ca 1000 ppm PCB, 5 ppm CDF, and 1000 ppm unknown substances (HEW/FDA, 1977).

Biochemical Effects

There are a variety of biochemical changes in

PCB-exposed animals which are characterized by an increase in drug-metabolizing enzymes (Villineuve et al, 1971; Fujita et al, 1971; Litterst et al, 1972), a proliferation of smooth endoplasmic reticulum (SER) (Vos and Nottenboem-Ram, 1972), hypertrophy and fatty infiltration of the liver (Allen and Abrahamson, 1973; Nishizumi, 1970), increases in microsomal protein and phospholipid (Ishidate and Nakazawa, 1976), enhanced biotransformations of xenobiotics (Bruckner et al, 1974), accumulation of pigments in tissue (Itokawa et al, 1976), decrease in sodium-potassium-dependent ATPase (Davis et al, 1972), and changes in mineral (Yagi et al, 1976) and vitamin metabolism (Yagi et al, 1979; Itokawa, 1975; Baker et al, 1977).

Metabolism

The low chlorine biphenyls tend to have a higher acute toxicity and are less abundant in biological and environmental samples than higher chlorinated biphenyls (Grant et al, 1971; Bailey and Bunyan, 1972), indicating their more rapid metabolism and degradation to more polar compounds (Shulte and Acker, 1974). Bile is the main route of excretion for PCBs (Peterson et al, 1976) although PCB persists in fatty tissues. Peterson et al

(1976) showed that only 20 percent of an injected (iv) dose of hexachlorobiphenyl given to rats was eliminated in the feces, Melancon and Lech (1976) showed that tetrachlorobiphenyl-treated rainbow trout were able to eliminate small amounts of conjugated metabolites in the bile, and Statham et al (1976) showed that low levels of PCB metabolites conjugated with glucuronic acid were excreted in the bile. The chronic toxicity of the higher chlorinated biphenyls is associated with a longer biological half-life, and low susceptibility to metabolic alteration by the liver (Matthews and Anderson, 1975).

Carcinogenicity

PCBs are primary carcinogens and also exhibit both promoting and inhibiting effects on tumor induction by several classes of carcinogens. Allen et al (1973) reported premalignant alteration of gastric mucosa in the Rhesus monkey, and hepatocellular carcinomas were reported in mice by Ito et al (1973) and Makiura et al (1974), and in rats by Kimbrough et al (1975). The anticancer Delaney Clause, that any food additives causing cancer in man or animal may not be used in a food product, does not apply to an environmental con-

taminant, such as PCBs, that are unavoidably present in foods (HEW/FDA, 1977). PCBs promoted tumor induction in mice treated with the carcinogen benzene hexachloride (Nagasaki et al, 1972), while they inhibited tumor incidence in rats treated with diethylnitrosamine (DEN) or N-2-fluorenylacetamide (FAA) (Makiura et al, 1974), and dietary Aroclor 1254 inhibited Walker 256 carcinosarcoma growth in rats (Kerkuliet and Kimeldorf, 1977). Kimura et al (1976) reported that oral administration of PCBs to rats after administration of 3-methyl-4-dimethylaminoazobenzene (3-Me-DAB) resulted in a high incidence of hepatocarcinoma, however, administration of the PCBs before or together with 3-Me-DAB did not induce tumors. PCBs, fed simultaneously with aflatoxin B_1 (AFB_1) to rainbow trout, markedly reduced the hepatocarcinogenicity of AFB_1 (Hendricks et al, 1977). But in recent studies, Hendricks et al (in press a) showed that exposure of rainbow trout embryos to AFB_1 followed by feeding the fry Aroclor 1254 failed to have an effect on tumor growth and incidence, while exposure of rainbow trout embryos to Aroclor 1254 prior to AFB_1 challenge significantly increased the carcinogenicity of AFB_1 (Hendricks et al, in press b). Aflatoxin B_1 is a liver carcinogen

that requires microsomal metabolism to the ultimate carcinogen and therefore may be influenced by liver enzyme inducers such as PCBs (Schoenhard et al, 1974). Makiura et al (1974) theorized that PCBs exert their inhibitory effect on the carcinogenicity of 3-Me-DAB, FAA, and DEN in rats through induction of enzymes that metabolize these carcinogens to noncarcinogenic metabolites.

Mixed Function Oxidase System

In addition to causing liver injury, it is well-documented that PCBs are potent inducers of hepatic mixed function oxidase (MFO) enzymes in mammals (Bickers et al, 1972). It was originally believed that fish lacked the enzymes necessary for xenobiotic metabolism, however studies have since shown that hepatic preparations from fish are usually able to metabolize xenobiotics by all the biotransformation pathways known to occur in the mammalian system (James et al, 1979). Evidence that induction of this system may be relevant in the natural environment has been provided by studies showing increased levels of benzo-(a)pyrene monooxygenase activity in the liver of fish sampled from oil-polluted water (Payne and Penrose,

1975). Being primarily concerned with lipophile-hydrophile conversions rather than detoxification per se, MFO enzymes can transform substances that are initially relatively innocuous to toxic, mutagenic, and/or carcinogenic compounds (Kurelec et al, 1979).

Low oral doses of PCBs to rats markedly induced cytochrome P450, aminopyrine N-demethylase, and benzo-(a)pyrene monooxygenase (Shimada and Ugawa, 1978). Swine and sheep exhibited significant increases in MFO activity in response to Aroclor 1254 (Hansen et al, 1977). Elcombe and Lech (1978) reported that PCBs and PBBs (polybrominated biphenyls) induce hepatic benzo(a)-pyrene monooxygenase, ethoxycoumarin O-deethylase, and ethoxyresorufin O-deethylase reactions in rainbow trout after a single intraperitoneal injection. Lidman et al (1976) showed induced cytochrome P450 levels and p-nitroanisole O-demethylation activity in rainbow trout following stomach tube feeding of capsules containing PCBs. Addison et al (1978) also demonstrated MFO enzyme induction in brook trout by giving several large oral doses of Aroclor 1254. High tissue levels of PCBs persist following the discontinuation of PCB exposure and liver enzymes also remain stimulated (Allen and

Abrahamson, 1979), although it has been shown that at least a portion of the PCBs are metabolized through an arene oxide intermediate (Hsu et al, 1975).

Cytochrome P450

Most inducers of drug-metabolizing enzymes can be classified as either phenobarbital (PB) (barbituate)-type inducers which induce cytochrome P450, or 3-methylcholanthrene (3-MC) (aromatic hydrocarbon)-type inducers which induce cytochrome P448. Aroclor 1254 possesses inducing properties of both these classes of inducers (Hansen et al, 1975; Alvares and Kappas, 1977). Cytochrome P450, a heme enzyme that functions as an electron carrier (Metzler, 1976), is the terminal oxidase in the liver that metabolizes drugs, carcinogens, and other foreign compounds. Several forms of mammalian liver cytochrome P450 are known and all are tightly bound to membranes of the SER (Metzler, 1976). Drugs and xenobiotics which are substrates for hepatic cytochrome P450 bind with the hemeprotein to produce difference spectra of two general types, type I and type II (Alvares and Kappas, 1977). The liver microsomal enzyme system which catalyzes the hydroxylation of a variety of substrates, requires NADPH, molecular oxygen,

cytochrome P450, NADPH cytochrome P450 reductase, and a phospholipid (Coon et al, 1976). Oxygen activation of cytochrome P450 is believed to involve the stepwise addition of two electrons to molecular oxygen, the loss of water, and formation of an iron-bound oxenoid species capable of attacking a substrate carbon radical intermediate (ibid.).

Cyclopropene Fatty Acids

Effects of dietary cyclopropene fatty acids (CPFA) fed in conjunction with PCBs on induction of the MFO system has not been reported. CPFA (sterculic and malvalic acids) are naturally-occurring fatty acids in cottonseed oil, kapok oil, and okra. CPFA are important because they exert a marked synergistic effect on AFB_1 carcinogenesis (Lee et al, 1968; Lee et al, 1971) and are also primary carcinogens in rainbow trout (Sinnhuber et al, 1976). Eisle et al (1978) reported that CPFA-fed trout had reduced levels of cytochrome P450, cytochrome b_5, and decreased cytochrome c reductase activity, but increased benzo(a)pyrene monooxygenase activity. CPFA inhibit the liver fatty acid desaturase system, decrease the hepatic MFO activity, and cause cytologic damage of liver parenchymal cells

(Lee et al, 1968). It has been proposed that CPFA exert their effect by altering lipid composition and metabolism, causing subsequent changes in hepatocyte membrane and mitochondrial function (Eisle et al, 1978).

II. RESEARCH PAPER

Introduction

Although industrial production and use of polychlorinated biphenyls (PCBs) has been discontinued in the U.S., significant body burdens have accumulated and persist in many species, including man (Bruckner et al, 1977). PCBs, a series of lipophilic compounds even more stable than DDT, were first recognized as environmental pollutants in 1966. Total environmental levels have increased substantially in recent years (Maugh, 1975).

It is well documented that PCBs are potent inducers of hepatic microsomal enzymes, providing both barbituate and polycyclic aromatic hydrocarbon-type induction in several mammalian species (Alvares and Kappas, 1977). In addition, Elcombe and Lech (1978) reported that PCBs and PBBs (polybrominated biphenyls) induce hepatic benzo(a)pyrene monooxygenase, ethoxycoumarin O-deethylase, and ethoxyresorufin O-deethylase reactions in rainbow trout (Salmo gairdneri) after a single intraperitoneal injection. Lidman et al (1976) showed that the hepatic microsomal enzyme system of

rainbow trout was induced following stomach tube feeding of capsules containing PCBs. Addison et al (1978) also demonstrated mixed function oxidase (MFO) enzyme induction in brook trout by giving several large oral doses of Aroclor 1254. Feeding dietary levels of PCBs as a means of exposure has not been reported in trout.

PCBs have also been related to carcinogenesis, as primary carcinogens in rats, as well as promoters or inhibitors of several classes on carcinogens in rodents and fish. Hendricks et al (1977) reported that simultaneous feeding of Aroclor 1254 (100 ppm) and aflatoxin B_1 (AFB_1) (6 ppb) to rainbow trout reduced the hepatocarcinogenicity of AFB_1. Exposure of rainbow trout embryos to AFB_1 followed by feeding the fry Aroclor 1254 (100 ppm), however, failed to inhibit or promote tumor growth and incidence (Hendricks et al, in press a). But exposure of rainbow trout embryos to Aroclor 1254, via gravid females, prior to AFB_1 challenge significantly increased the carcinogenicity of AFB_1 (Hendricks et al, in press b). It was theorized that the various effects of Aroclor 1254 upon the activity of AFB_1 were mediated through the induction of AFB_1-epoxide detoxifying enzymes, alternate meta-

bolic pathways for AFB_1, or competitive inhibition of AFB_1 metabolism (Hendricks et al, in press b) but data on MFO induction due to dietary exposure to PCBs was lacking. Thus, the present study was designed to determine whether dietary Aroclor 1254 (100 ppm) causes induction of hepatic microsomal enzymes in rainbow trout, a feature which may aid in explaining the different effects of dietary Aroclor 1254 on AFB_1 carcinogenesis in rainbow trout.

The present study also investigates the effects of dietary cyclopropene fatty acids (CPFA) fed in conjunction with PCBs on the induction of the MFO system in trout. CPFA are important because they exert a marked synergistic effect on AFB_1 carcinogenesis (Lee et al, 1968; Lee et al, 1971) and are also primary liver carcinogens in rainbow trout (Sinnhuber et al, 1976). Eisle et al (1978) reported that CPFA-fed trout had reduced levels of cytochrome P450 and cytochrome b_5. The activity of benzo(a)pyrene monooxygenase increased in the same trout. It is therefore of interest to determine the combined effects that these two types of apparently antagonistic compounds (CPFA and PCB) have on trout MFO activity.

Materials and Methods

Chemicals and Animals

Aroclor 1254 was obtained from Monsanto Chemical Company (St. Louis, MO). [^{3}H]Benzo(a)pyrene was purchases from Amersham/Searle (Des Plains, IL). Resorufin was purchased from Matheson, Coleman, and Bell (East Rutherford, NJ) and purified 7-ethoxyresorufin was purchased from Pierce Chemical Company (Rockford, IL). Umbelliferone (7-hydroxycoumarin) was obtained from Sigma Chemical Company (St. Louis, MO) and purified by recrystallization from ethanol; 7-ethoxycoumarin was synthesized by the method of Ullrich and Weber (1972) by Dr. Norman E. Pawlowski (Food Science Department, Oregon State University). NADP and NADPH were obtained from Sigma.

Shasta strain rainbow trout (Salmo gairdneri) were spawned and hatched at the Food Toxicology and Nutrition Laboratory at Oregon State University. The fry were fed a semipurified diet described by Sinnhuber et al (1977) for 10 months prior to initiating this experiment and weighed 30-35 grams each. Fish were kept in circular fiberglass tanks supplied

with 12°C well water. A 12-hour (6 AM-6 PM) light cycle was used.

Exposure and Microsomal Preparation

Duplicate lots of fish were put into tanks (100 per tank) and fed either the control diet or the control diet containing one of the following: 100 ppm Aroclor 1254, 50 ppm CPFA, or combined PCB (100 ppm) and CPFA (50 ppm) for 15 weeks. Sampling was done at 1,3,5,9,11, and 15 weeks.

Fish were killed by a blow on the head following 48 hour starvation and the livers removed, weighed, and perfused with 0.9% NaCl. Livers from enough fish were pooled to obtain a total of 6 grams per sample; triplicate samples were obtained from each diet. The livers were minced and then homogenized in 4 vol of 0.15 M KCl in 0.01 M potassium-phosphate buffer, pH 7.4 using a motor-driven Potter-Elvejhem glass-Teflon homogenizer (8 complete strokes). The homogenate was centrifuged at 2000 xg for 30 min using a Sorvall type SS-34 rotor and Sorvall RC2-B Superspeed centrifuge. The supernatant was centrifuged at 10,000 xg for 30 min. The post-mitochondrial supernatant was centrifuged at 105,000 xg for 60 min using a Sorvall AH 627 rotor and

Sorvall OTD-65 ultracentrifuge. The microsomal pellet was rinsed and resuspended in 10-15 ml Tris/HCl buffer (0.1 M, pH 7.6) to obtain 2-5 mg/ml protein. All procedures were performed at 0-4°C.

Microsomal protein content was determined by the colorimetric method of Lowry et al (1951) using a bovine serum albumin standard.

Enzyme Assays

7-ethoxycoumarin O-deethylation was measured by the direct fluorimetric procedure of Ullrich and Weber (1972). Final quantities of reactants in the cuvette were 10 ul NADPH (12.6 mM), 5 ul 7-ethoxycoumarin (0.1 M in DMSO), 2-4 mg microsomal protein, 1 ml Tris/HCl buffer, pH 7.6 (0.1 M) to a final volume of 2.515 ml.

7-ethoxyresorufin O-deethylation was measured by the method of Burke and Mayer (1974, 1977). Final quantities of reactants in the cuvette were 10 ul NADPH (50 mM), 20 ul 7-ethoxyresorufin (50 uM in MeOH), 0.04-4 mg microsomal protein, 2 ml sodium-potassium phosphate buffer, pH 7.8 (0.12 M) to a final volume of 2.43 ml.

Fluorimetric reactions were performed on an

Aminco SPF-125 Spectrofluorimeter. Samples were brought to 30°C and then fluorescence was read for two minutes at room temperature. Sensitivity of the fluorimeter was set with 5 ul umbelliferone (1 mM) or 0.15 ml resorufin (0.01 mM). The fluorimetric assays were performed on the day of microsomal preparation.

The monooxygenation of [^{3}H]benzo(a)pyrene was determined by the method of De Pierre et al (1975).

Spectral Measurements

Cytochrome P450 was measured on a Beckman Acta CIII Spectrophotometer by the method of Estabrook et al (1972) using an extinction coefficient of 91 $mM^{-1}cm^{-1}$. Cytochrome P450 and benzo(a)pyrene monooxygenase assays were performed on microsomes stored for 12-14 hr at -40°C.

Statistics

Statistical comparisons were made using analysis of variance. A level of $p<0.05$ was considered significant.

Results

Dietary PCBs caused a marked increase in microsomal enzyme activity. Figures 1-4 summarize the results for each individual assay over the 15-week study. There was a steady increase of 7-ethoxyresorufin O-deethylase activity throughout the experiment. At week 15, activity was 77-fold higher than control. The activities of 7-ethoxycoumarin O-deethylase and benzo(a)pyrene monooxygenase continued to increase through week 9 and then decreased. However, since controls also decreased between 9 and 15 weeks, activity remained approximately the same for weeks 9 and 15; 7-ethoxycoumarin activity was 7.1-fold 6.8-fold over controls and benzo(a)pyrene monooxygenase activity was 48-fold and 45-fold over controls at weeks 9 and 15, respectively ($p<0.05$). Cytochrome P450 did not significantly increase until week 5 and then levels remained approximately 2-fold higher than controls through to week 15 ($p<0.01$).

In this study, dietary CPFA did not induce benzo(a)pyrene monooxygenase (Figure 4). Cytochrome P450 levels were significantly lower ($p<0.05$) than control at weeks 3 and 15. Ethoxyresorufin O-deethylase acti-

vity was significantly lower ($p<0.05$) than control at weeks 3 and 5, but no significant difference occurred at weeks 1 or 15. Throughout the experiment, there was no significant difference over control in ethoxycoumarin O-deethylase activity.

Fish fed the combination of dietary PCB and CPFA showed an increase in microsomal enzyme activity for the two O-deethylations and for benzo(a)pyrene monooxygenation, though levels were below those caused by PCBs alone. The microsomal activity of the combined diet PCB and CPFA-fed trout was significantly higher than CPFA-fed and control-fed fish microsomal activity ($p<0.05$), and significantly lower than PCB-fed fish MFO activity ($p<0.05$) for weeks 1 and 3 and also at week 15 for ethoxyresorufin O-deethylase and benzo(a)pyrene monooxygenase. Levels of cytochrome P450 were not significantly different from controls after week 5.

Discussion

The results of the present study show significant induction of ethoxyresorufin and ethoxycoumarin O-deethylases and benzo(a)pyrene monooxygenase from dietary PCB. After an initial lag, cytochrome P450 content also increased. These results show that feeding dietary levels of PCBs to rainbow trout achieves comparable MFO induction as previously reported for injection (ip) or stomach tube feeding of PCBs to trout (Elcombe and Lech, 1978; Lidman et al, 1976).

A theory has been proposed that the more highly chlorinated PCB isomers commonly present in the crude, commercial product, Aroclor 1254, would accumulate in the body upon continued ingestion, producing greater induction of drug metabolism (Bruckner et al, 1977). However, a time study by Bruckner et al (1977) of PCB-fed rats, and Hendricks et al (1977) of PCB-fed trout showed that tissue levels of chlorinated hydrocarbons stabilize or plateau upon prolonged ingestion of low dietary levels. In the present study of PCB-fed rainbow trout, ethoxyresorufin O-deethylase enzyme activity continued to increase for 15 weeks, P450 stabilized after 5 weeks to approximately 2-fold over controls,

while ethoxycoumarin O-deethylase and benzo(a)pyrene monooxygenase activities began to decrease after 9 weeks. But activity only decreased from 7.1-fold to 6.8-fold over control for ethoxycoumarin O-deethylase and from 48-fold to 45-fold over control for benzo(a)pyrene monooxygenase for weeks 9 and 15, respectively. Thus, as tissue levels stabilize, enzyme induction appears to stabilize or slightly decrease upon prolonged PCB feeding, with the exception of ethoxyresorufin O-deethylase activity. The activity of ethoxyresorufin O-deethylase may stabilize after a longer period on the diet.

The mixed function oxidase system resides on the lipid-rich membrane of the smooth endoplasmic reticulum. It has been proposed that cyclopropenes exert their effects by altering lipid composition and metabolism, causing alterations in hepatocyte membranes and mitochondrial function (Eisle et al, 1978). Eisle et al (1978) found that dietary CPFA in trout resulted in decreased amounts of cytochromes P450 and b_5, and decreased activity of NADPH cytochrome c reductase. After 22 days, benzo(a)pyrene monooxygenase activity was 40% higher than controls in these same fish. It

is evident from figures 1-4, and in agreement with previous studies, that enzyme activities and cytochrome P450 content of CPFA-fed fish were significantly lower than control values for all assays except benzo(a)-pyrene monooxygenase. No significant difference was observed in benzo(a)pyrene monooxygenase activity, which may result from the use of postmitochondrial fraction in Eisle's study and the use of microsomes in this study.

By combining dietary PCB and CPFA, we saw a compromise in the two effects as opposed to one dominating the other. The antagonistic relationship of PCB and CPFA is indicated by PCB and CPFA-fed fish having significantly higher hepatic microsomal enzyme activities than either CPFA-fed fish or control-fed fish, and significantly lower enzyme activities than PCB-fed fish ($p<0.05$). At the levels used in this study, CPFA may inhibit PCB-inducing ability by alteration of membranes.

This study clearly demonstrates that the 100 ppm dietary dose of Aroclor 1254 that we have given to trout in previous experiments (Hendricks et al, 1977; Hendricks et al, in press a; Hendricks et al, in press b) is capable of significantly inducing the hepatic MFO

system. This finding may aid in explaining the varied responses that we have observed with PCB and AFB_1 in rainbow trout. The best interpretation of the varied responses are as follows: 1) exposure to PCB prior to AFB_1 challenge causes MFO enzyme induction, increased AFB_1-epoxide formation, and increased carcinogenesis; 2) simultaneous exposure to PCB and AFB_1 results in reduced AFB_1-epoxide formation and carcinogenesis due to competitive inhibition since PCB and AFB_1 are both substrates for the same microsomal enzyme system; and 3) exposure to PCB after AFB_1 tumor induction has no effect even though the MFO system of the liver would be induced.

Table 1. Effects of Dietary PCB (100 ppm) and CPFA (50 ppm) on Ethoxyresorufin O-deethylase Activity[a] in Hepatic Microsomes of Rainbow Trout.

Week	Control	PCB	CPFA	PCB and CPFA
1	.021± .006	.657±.313[b]	.014±.013	.556±.048[c]
3	.148± .016	1.684±.105[c]	.022±.009[b]	1.242±.104[c]
5	.062 ±.018	4.068±.441[c]	.025±.007[b]	1.487±.508[c]
9	.126 ±.032	6.176±.452[c]	- -	- -
11	- -	- -	.030±.029	3.007±.569
15	.096 ±.034	7.377±3.247[b]	.055±.002	5.285±1.085[b]

[a]Values are nmoles/min/mg protein± S.D.

[b]Significantly different from control group, $p<0.05$ (AOV)

[c]Significantly different from control group, $p<0.01$

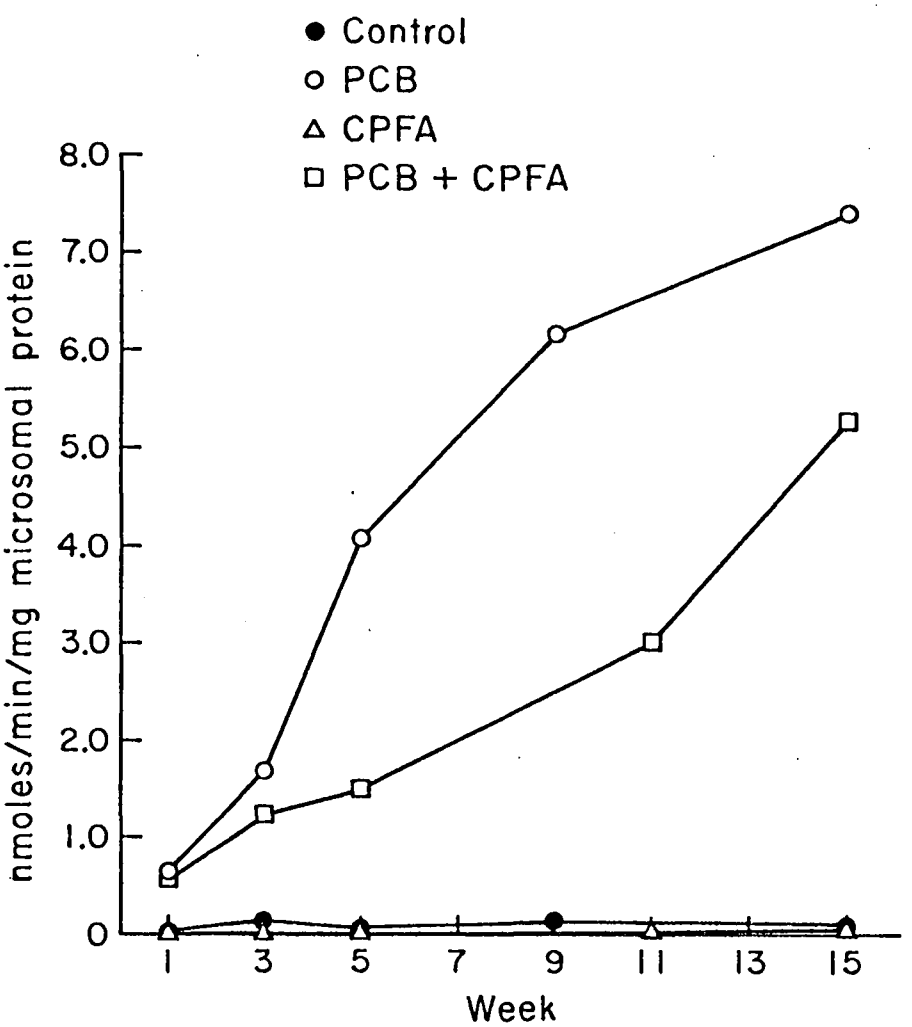

Figure 1. Effects of Dietary PCB (100 ppm) and CPFA (50 ppm) on Ethoxyresorufin O-deethylase Activity in Rainbow Trout

Table 2. Effects of Dietary PCB (100 ppm) and CPFA (50 ppm) on Ethoxycoumarin O-deethylase Activity[a] in Hepatic Microsomes of Rainbow Trout.

Week	Control	PCB	CPFA	PCB and CPFA
1	.014±.005	.058±.018[b]	.050±.029	.134±.011[c]
3	.084±.013	.308±.088[b]	- -	.230±.024[c]
5	.040±.013	.492±.018[c]	.019±.002	.161±.077
9	.086±.002	.610±.059[c]	- -	- -
11	- -	- -	.027±.013	.373±.084
15	.040±.014	.270±.079[c]	.012±.010	.318±.215

[a]Values are nmoles/min/mg protein±S.D.

[b]Significantly different from control group, $p<0.05$ (AOV)

[c]Significantly different from control group, $p<0.01$

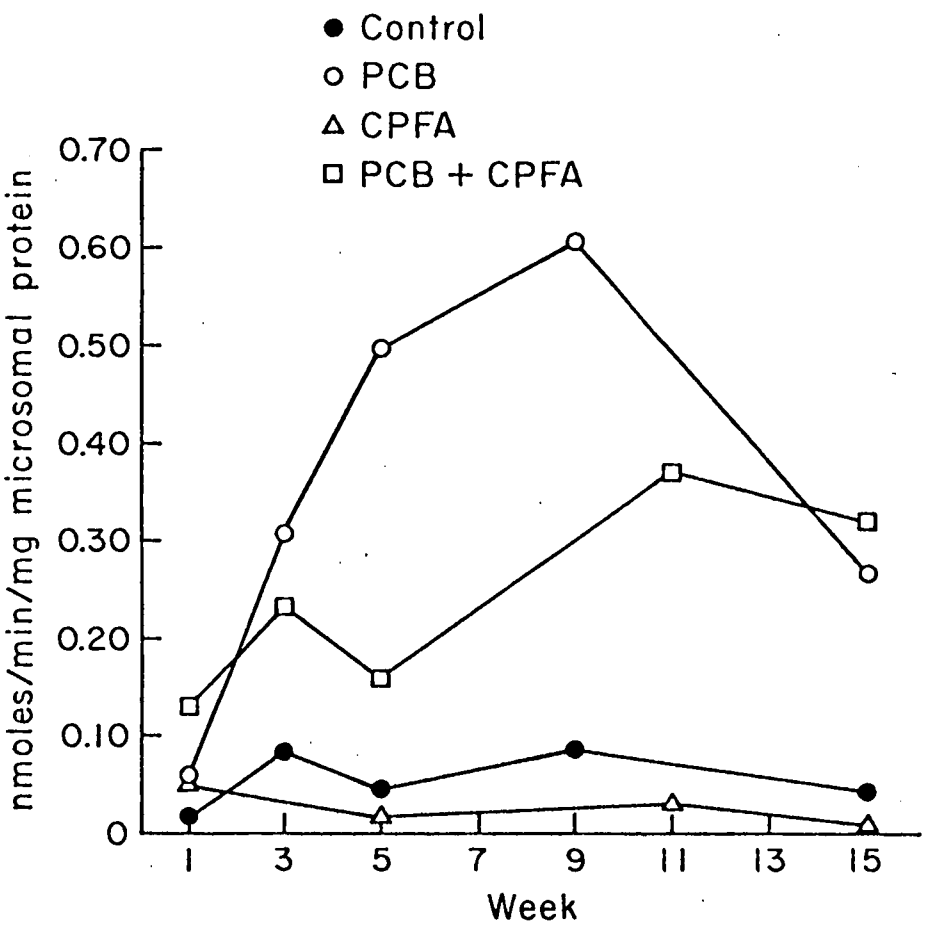

Figure 2. Effects of Dietary PCB (100 ppm) and CPFA (50 ppm) on Ethoxycoumarin O-deethylase Activity in Rainbow Trout.

Table 3. Effects of Dietary PCB (100 ppm) and CPFA (50 ppm) on the Level of Cytochrome P450[a] in Hepatic Microsomes of Rainbow Trout.

Week	Control	PCB	CPFA	PCB and CPFA
1	.181 ±.069	.180 ±.055	.331±.082	.381 ±.032[b]
3	.371 ±.023	.348 ±.040	.216±.032[c]	.259 ±.024[b]
5	.145±.041	.290 ±.048[b]	.274±.012[c]	.325 ±.055[b]
9	.401 ±.057	.657 ±.029[c]	- -	- -
11	- -	- -	.234±.014	.377 ±.042
15	.411 ±.043	.777 ±.099[c]	.246±.057[b]	.335 ±.107

[a]Values are nmoles cytochrome P450/ mg protein± S.D.

[b]Significantly different from control group, $p<0.05$ (AOV)

[c]Significantly different from control group, $p<0.01$

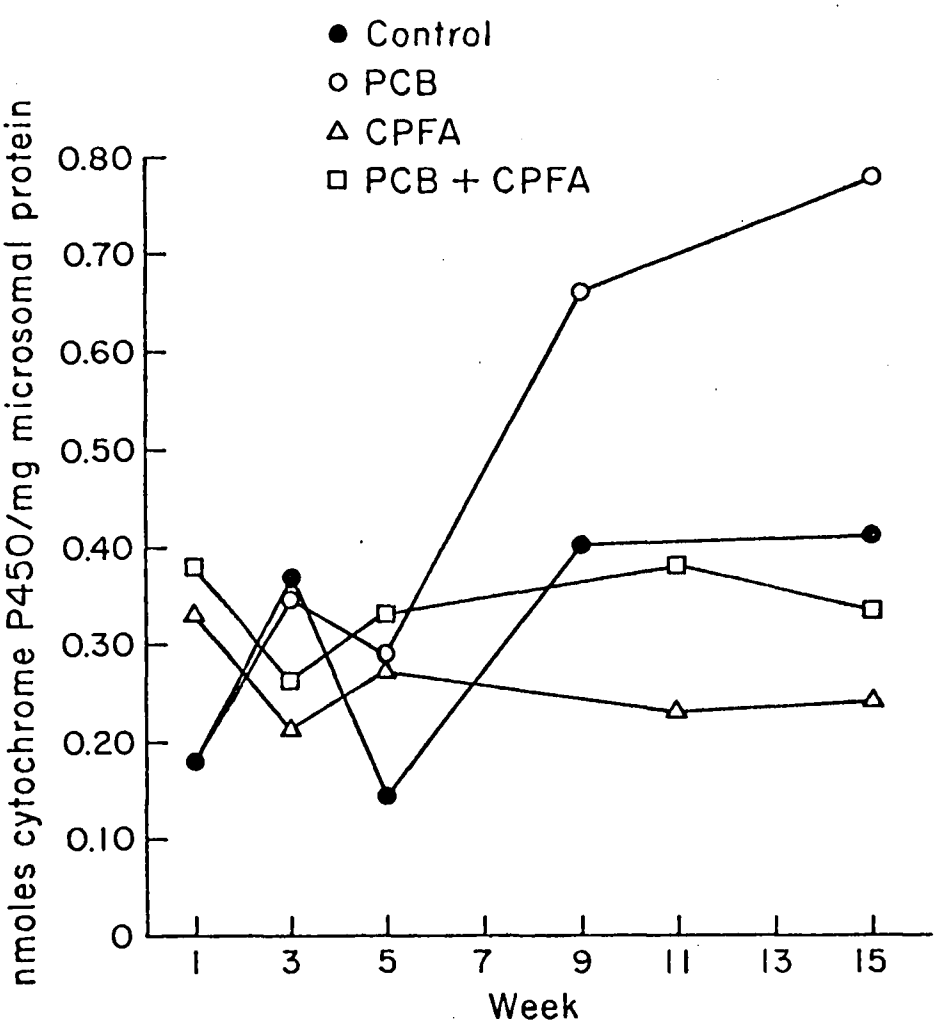

Figure 3. Effects of Dietary PCB (100 ppm) and CPFA (50 ppm) on the Level of Cytochrome P450 in Rainbow Trout.

Table 4. Effects of Dietary PCB (100 ppm) and CPFA (50 ppm) on Benzo(a)pyrene Monooxygenase Activity[a] in Hepatic Microsomes of Rainbow Trout.

Week	Control	PCB	CPFA	PCB and CPFA
1	0	.078±.029[b]	0	.057±.043[c]
3	.023±.027	.216±.041[c]	.002±.004	.184±.024[c]
5	.004±.006	.968±.041[c]	.007±.013	.333±.248
9	.024±.023	1.136±.069[c]	- -	- -
11	- -	- -	.025±.021	.724±.174
15	.015±.004	.655±.272[b]	.014±.001	.498±.257[b]

[a]Values are nmoles[^{3}H]benzo(a)pyrene oxidized/min/mg protein±S.D.

[b]Significantly different from control group, $p<0.05$ (AOV)

[c]Significantly different from control group, $p<0.01$

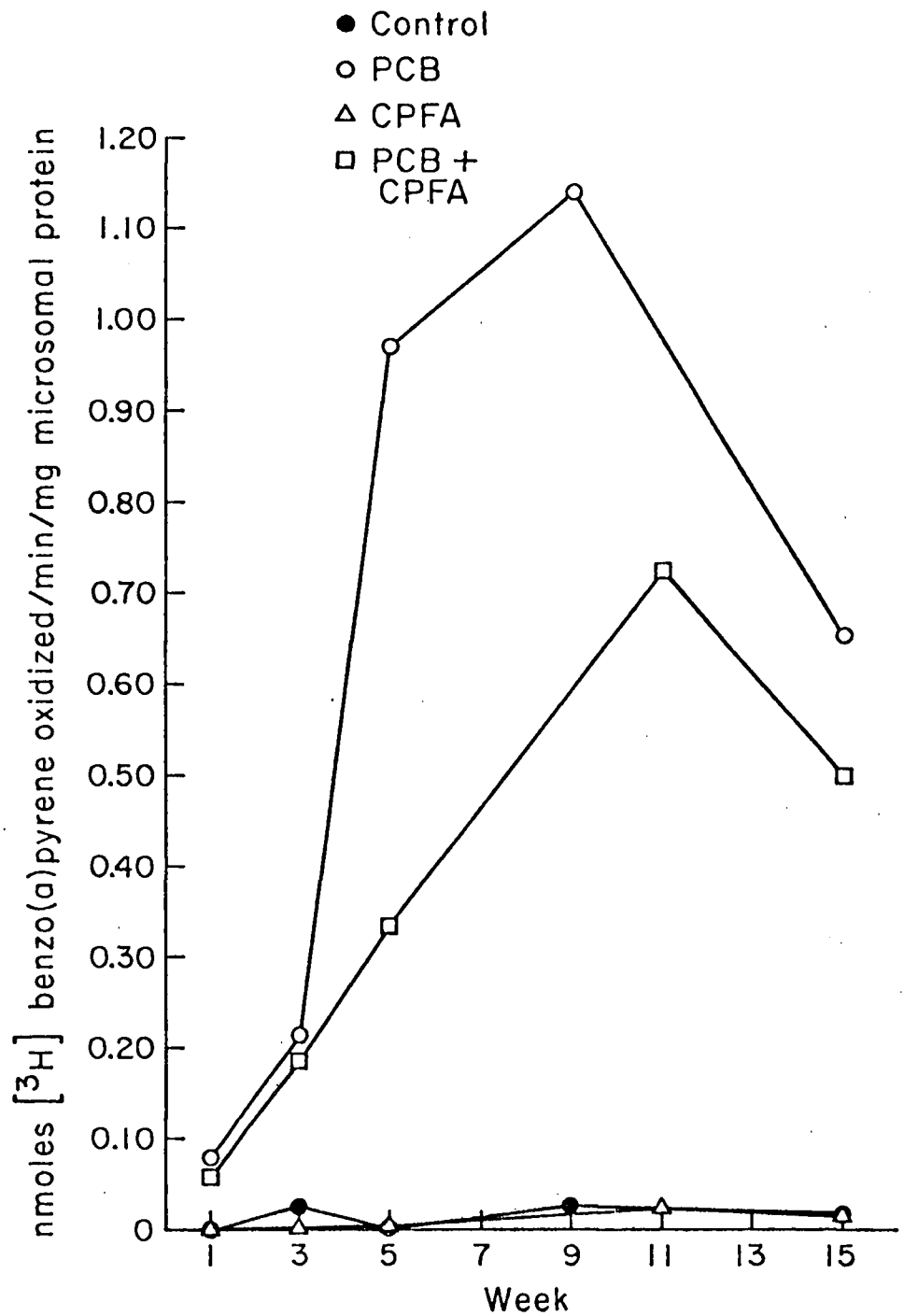

Figure 4. Effects of Dietary PCB (100 ppm) and CPFA (50 ppm) on Benzo(a)pyrene Monooxygenase Activity in Rainbow Trout.

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APPENDICES

APPENDIX I

Ethoxyresorufin O-deethylase Assay

Emission: 586 nm
EM slit width: 1.0 mm

Excitation: 510 nm
EX slit width: 0.5 mm

1. Put into small test tube: 2 mls 0.12 M sodium-potassium phosphate buffer, pH 7.8, variable microsomes (0.04-4 mg), and variable water to make 0.4 ml

2. Incubate 3-5 min in 30°C water bath

3. Pour into cuvette

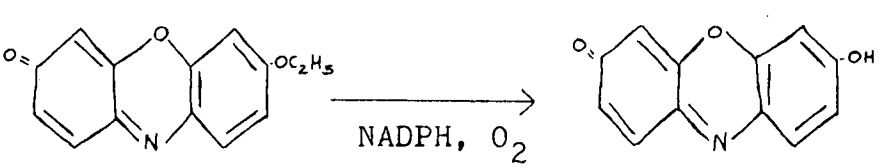
4. With a microliter syringe, add: 20 ul ethoxyresorufin (50 uM in MeOH) and 10 ul NADPH (50 mM)

5. Final volume is 2.43 ml

6. Read change in fluorescence for 2-3 min; record every 30 sec

   (Set sensitivity of the fluorimeter with 0.15 ml resorufin (0.01 mM))

7. Calculation: $\frac{.0193 \times FU/min}{mg\ prot}$ = nmoles/min/mg prot



7-ethoxyresorufin resorufin

APPENDIX II

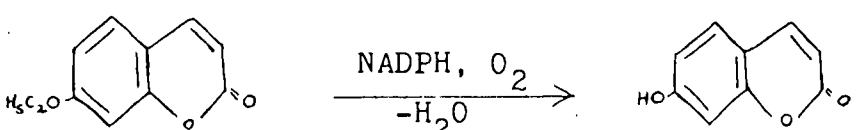
Ethoxycoumarin O-deethylase Assay

Emission: 450 nm
EM slit width: 0.5 mm

Excitation: 380 nm
EX slit width: 0.5 mm

1. Put into small test tube: 1 ml 0.1 M Tris/HCl buffer, pH 7.6, 1 ml microsomes (2-4 mg), and 0.5 ml water
2. Incubate 3-5 min in 30°C water bath
3. Pour into cuvette
4. With a microliter syringe, add: 5 ul ethoxycoumarin (0.1 mM in DMSO) and 10 ul NADPH (12.6 mM)
5. Final volume is 2.515 ml
6. Read change in fluorescence for 2-3 min; record every 30 sec

   (Set sensitivity of the fluorimeter with 5 ul umbelliferone (1 mM))
7. Calculation: $\frac{.0625 \times FU/min}{mg\ prot}$ = nmoles/min/mg prot



7-ethoxycoumarin → umbelliferone

APPENDIX III

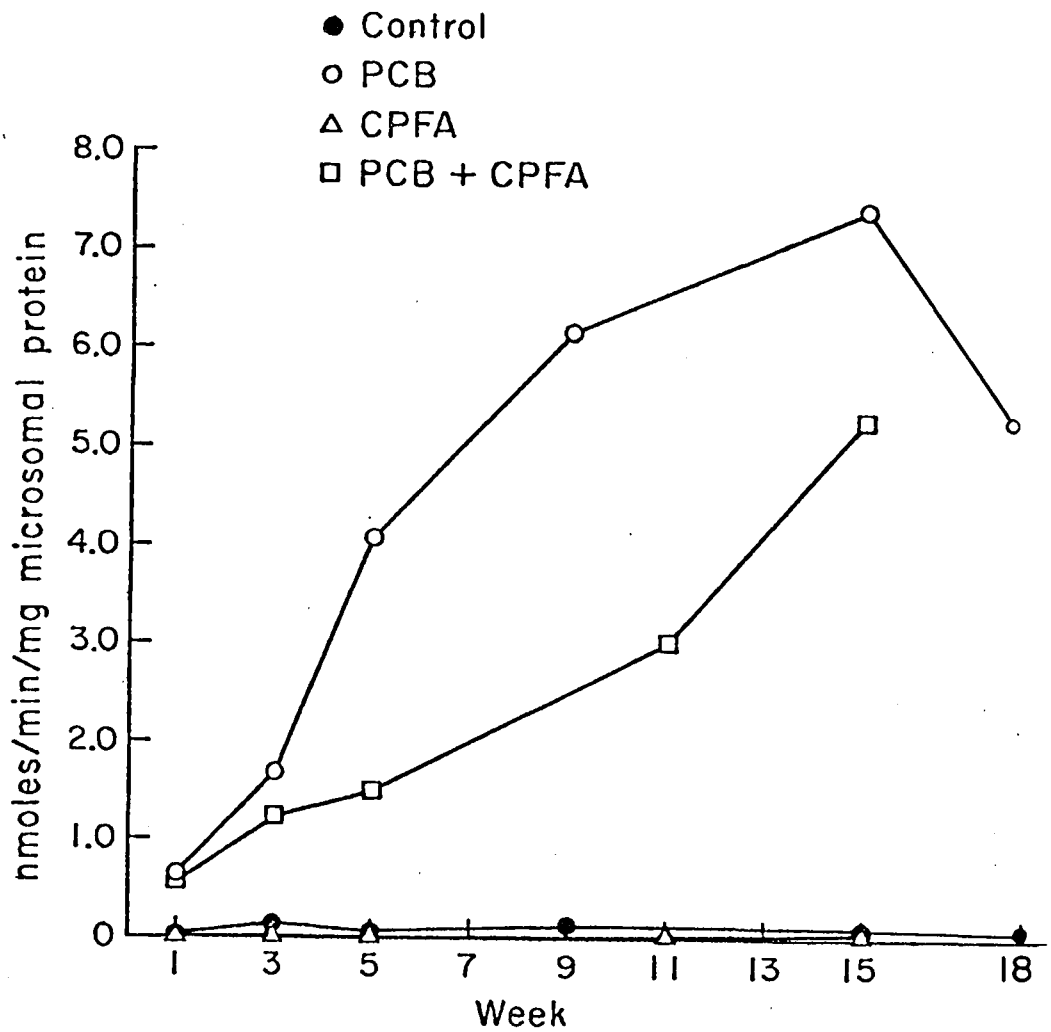


Figure 1. Effects of Dietary PCB (100 ppm) and CPFA (50 ppm) on Ethoxyresorufin O-deethylase Activity in Rainbow Trout.

Note: PCB-fed trout were put back on control diet at week 15. Activity remained significantly above control, $p<0.05$. Values at week 18 were $.038 \pm .051$ for control and 5.091 ± 2.907 for PCB fish.

APPENDIX IV

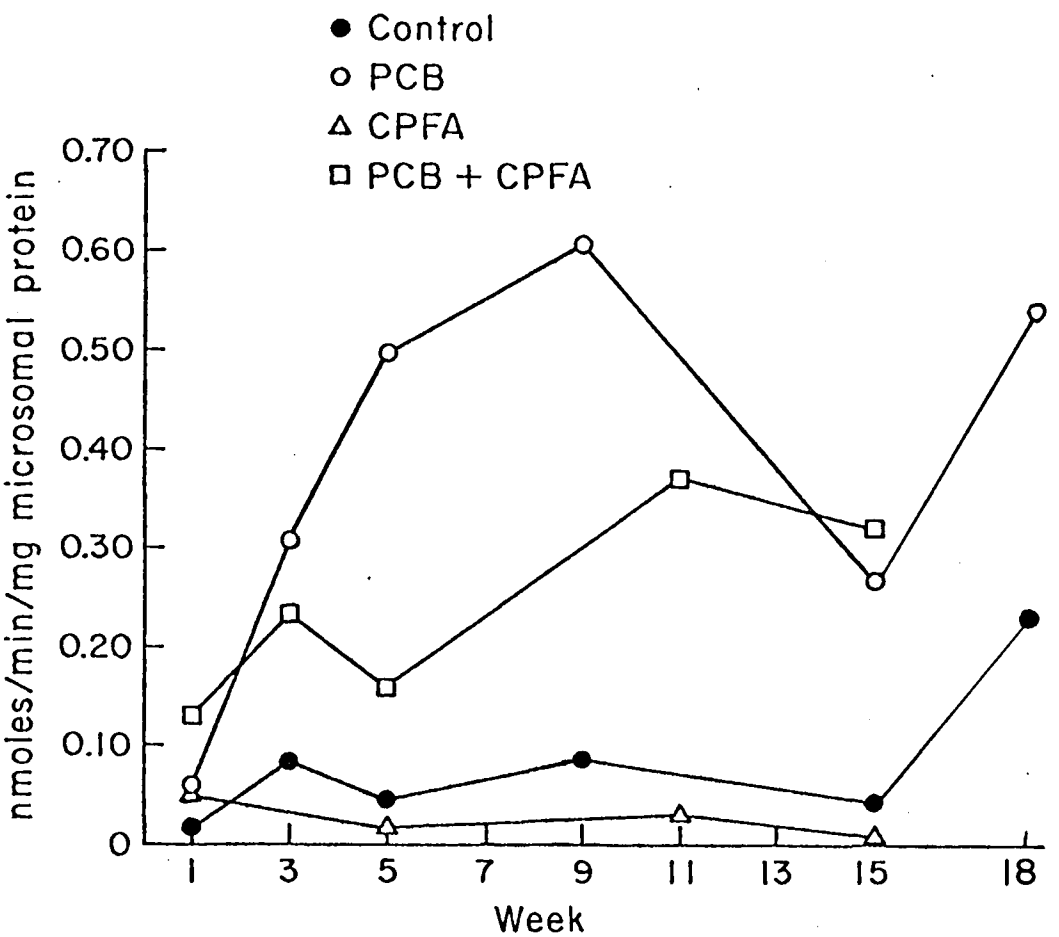


Figure 2. Effects of Dietary PCB (100 ppm) and CPFA (50 ppm) on Ethoxycoumarin O-deethylase Activity in Rainbow Trout.

Note: PCB-fed trout were put back on control diet at week 15. Activity remained significantly above control, $p<0.01$. Values at week 18 were $.244 \pm .064$ for control and $.581 \pm .038$ for PCB fish.

APPENDIX V

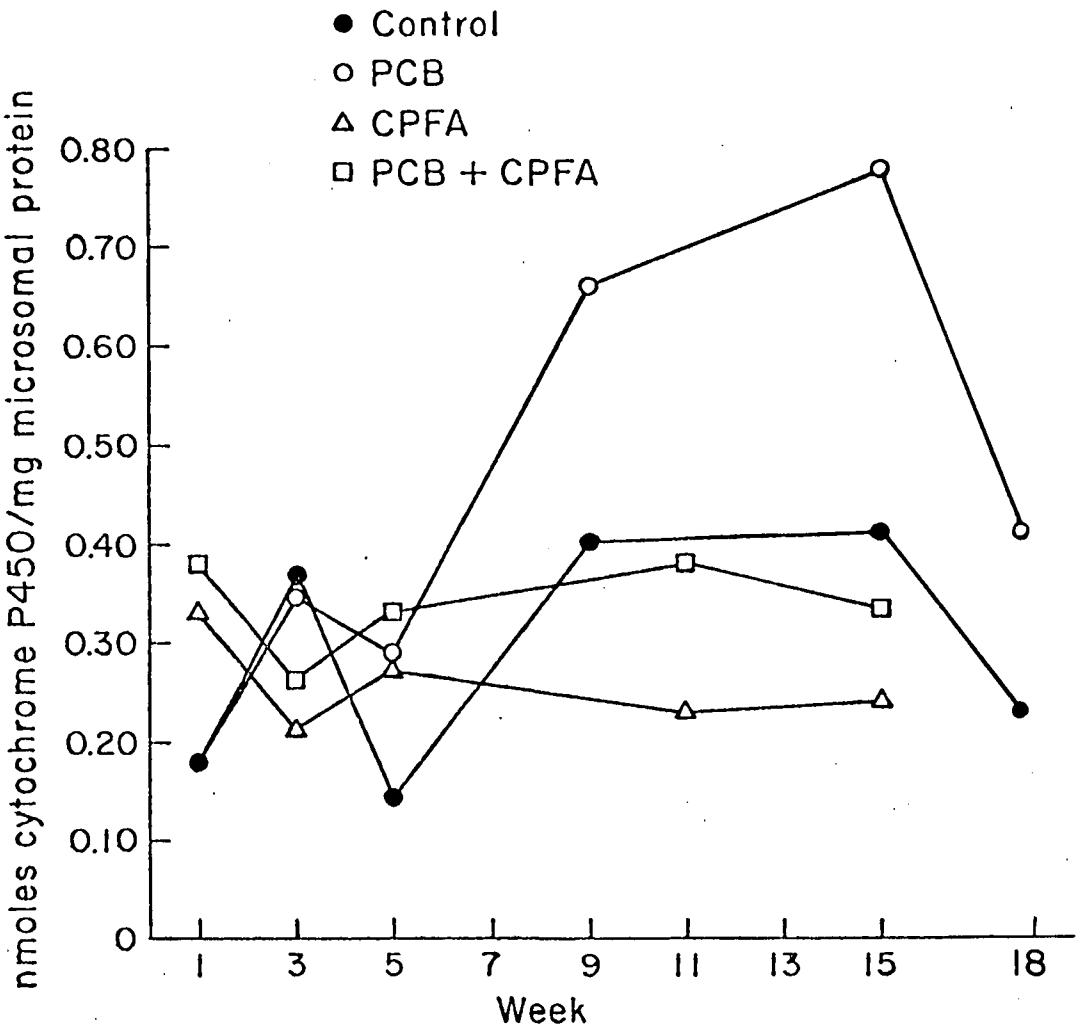


Figure 3. Effects of Dietary PCB (100 ppm) and CPFA (50 ppm) on the Level of Cytochrome P450 in Rainbow Trout.

Note: PCB-fed trout were put back on the control diet at week 15. Activity remained significantly above control, $p<0.01$. Values at week 18 were $.226 \pm .011$ for control and $.409 \pm .016$ for PCB fish.

APPENDIX VI

Figure 4. Effects of Dietary PCB (100 ppm) and CPFA (50 ppm) on Benzo(a)pyrene Monooxygenase Activity in Rainbow Trout.

Note: PCB-fed trout were put back on control diet at week 15. Activity remained significantly above control, $p<0.01$. Values at week 18 were $.012 \pm .005$ for control and $.684 \pm .153$ for PCB fish.

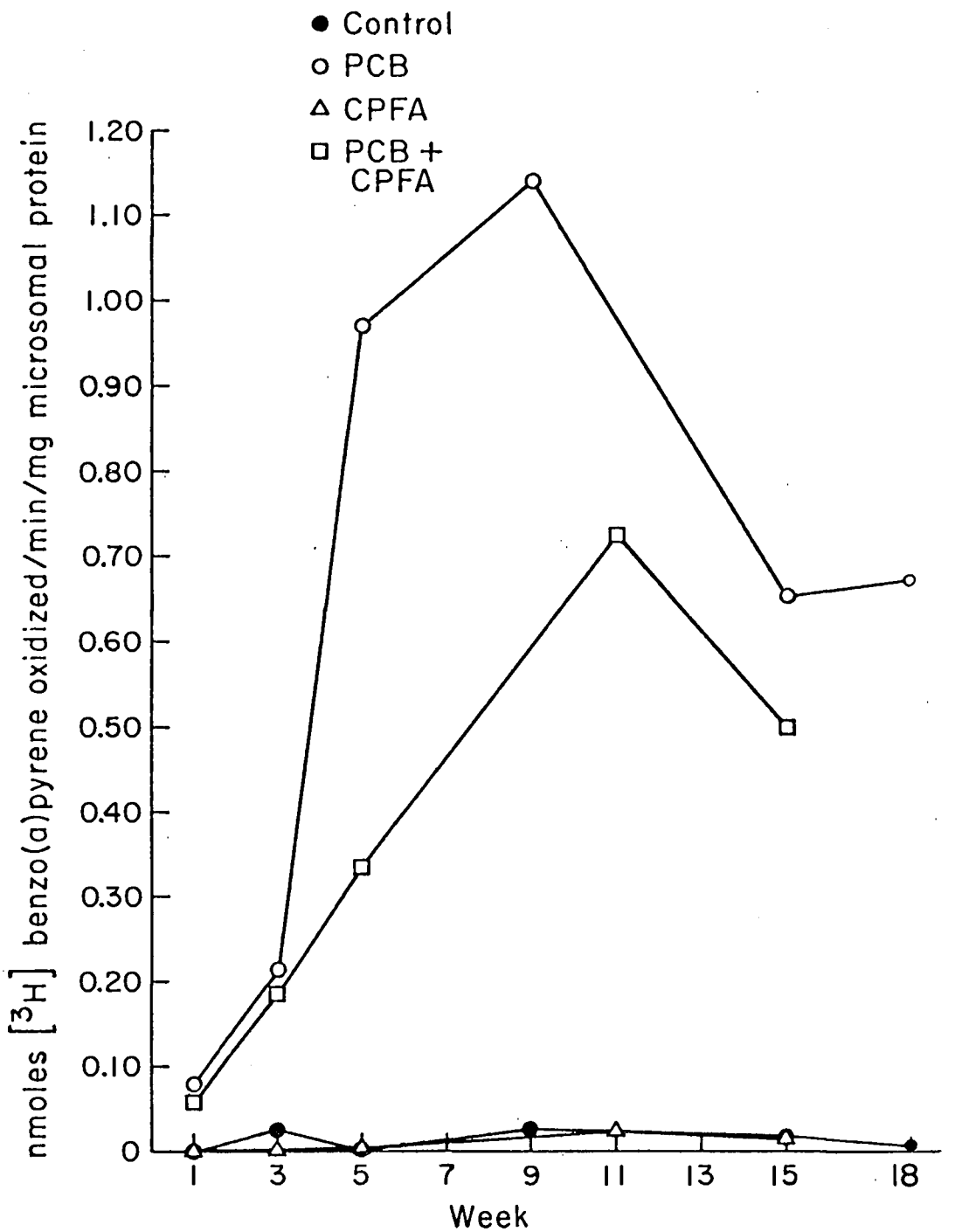

Control
PCB
CPFA
PCB + CPFA
nmoles [3H] benzo(a)pyrene oxidized/min/mg microsomal protein
1.20
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0
1
3
5
7
9
11
13
15
18
Week

Figure 4.

APPENDIX VII

Table 5. Liver Somatic Index[a] of Fish Fed PCB (100 ppm) and CPFA (50 ppm).

Week	Control	PCB	CPFA	PCB and CPFA
1	1.62±.48	1.57±.30	1.49±.22	1.36±.20
3	1.26±.20	1.22±.23	1.12±.15	1.14±.18
5	1.50±.17	1.18±.16	1.37±.40	1.56±.39
9	1.22±.29	1.33±.22	- -	- -
11	- -	- -	1.18±.23	1.41±.17
15	1.10±.12	1.41±.19	1.41±.20	1.60±.24
18	1.18±.19	1.32±.19		

[a]Values are LSI±S.D.

Liver somatic index (LSI): $\frac{\text{liver weight}}{\text{body weight}} \times 100$