AN ABSTRACT OF THE THESIS OF

Sirinmas Intharapanith for the degree of Master of Science in Toxicology presented on December 12, 1995. Title: Effect of Xenoestrogen Exposure on The Expression of Cytochrome P450 Isoforms in Rainbow Trout Liver.

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Abstract approved: _____

Donald R. Buhler

Experimental evidence reveals that xenoestrogens such as organochlorine pesticides, pharmaceuticals, phenolic compounds and phytoestrogen exhibit reproductive effects on the health of human and wildlife populations. In rainbow trout, injection with 17β-estradiol represses expression of cytochrome P450s (CYP2K1, CYP2M1 and P450 LMC5) and reduces hepatic lauric acid hydroxylase activity. The aim of our study was to examine the effect on the regulation of rainbow trout P450s of four xenoestrogenic chemicals from the following categories: pesticides, pharmaceuticals, surfactants and phytoestrogens. Therefore, four chemicals, methoxychlor (20 mg/kg), diethylstilbestrol (15 mg/kg), 4-tert-octylphenol (25 and 50 mg/kg) and biochanin A (25 and 50 mg/kg) were injected (ip) on days 1,4 and 7 into one-year old juvenile rainbow trout using propylene glycol as vehicle. All fish were sacrificed on day 9. Plasma vitellogenin levels were measured by ELISA and used as an indicator of the estrogenic activity of the four chemicals. Plasma vitellogenin increased in all treated trout to varying degrees, ranging from high to low, in response to the test chemicals in the following order of decreasing of activities: diethylstilbestrol (15 mg/kg), 4-tert-octylphenol (50 mg/kg), 4-tert-octylphenol (25 mg/kg), biochanin A (50 mg/kg), biochanin A (25 mg/kg) and methoxychlor (20 mg/kg), respectively. As found upon treatment with estrogens, all four chemicals treated trout liver microsomes markedly repressed expression of P450's in liver microsomes from treated trout as measured by Western blots. Lauric acid hydroxylase activity also was greatly reduced in trout treated with all four chemicals. In addition, hepatic CYP1A1 was induced in trout by the two doses of biochanin A. These findings indicate that

methoxychlor, diethylstilbestrol, 4-tert-octylphenol and biochanin A are all estrogenic and act like 17β-estradiol in repressing the expression of certain cytochrome P450 isoforms in rainbow trout liver. However, biochanin A can also induce CYP1A1 in this species.

Effect of Xenoestrogen Exposure on The Expression of Cytochrome P450 Isoforms in Rainbow Trout Liver

by

Sirinmas Intharapanith

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed December 12, 1995
Commencement June 1996

Master of Science thesis of Sirinmas Intharapanith presented on December 12,1995

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Sirinmas Intharapanith, author

ACKNOWLEDGEMENT

I would like to express my appreciation to the following for their advice, friendship and patience through out the research time this thesis took to

Dr. Donald R. Buhler

Dr. Christobal L. Miranda

Dr. Jun-Lan Wang

Marilyn C. Henderson

Yea-Huey Yang.

Thanks to members of the Food Toxicology and Nutrition Laboratory for their assistance with the trout; my graduate committee: Dr. Cromack Kermit Jr., Dr. Ian Tinsley and Dr. Wilbert Gamble for their reviewing this thesis; and my good friends: Byung-Woo Ryu, Jeong-Gue Park and Srirat Kasiwong for their encouragement and friendship.

Moreover, I owe several people a debt of gratitude: my family, Department of Medical Sciences; Public Health Ministry and Royal Thai Embassy for the opportunity to study.

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INTRODUCTION

There is growing evidence that environmental pollutants that have estrogenic activity exhibit adverse effects on the health of human and wildlife populations. These effects included increased incidence of breast cancer in women, decreased sperm count in men, abnormal sexual development and impaired reproduction in alligators and gulls (Hileman, 1994). Due to the environmental regulations in the past two decades, the levels of estrogenic organochlorine pesticides and pollutants have decreased, resulting, for example, in the improvement of reproduction of highly susceptible fish-eating water birds in the Great Lakes region. However, there are still both natural and industrial chemicals present in the environment that elicit estrogenic and antiestrogenic activity. For example, several industrial chemicals, organochlorine pesticides and phenolic compounds and bioflavonoids, found in plants are known estrogens. In addition, TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), combustion products such as PAHs (polycyclic aromatic hydrocarbons) and indole-3-carbinol in cruciferol vegetables all have antiestrogenic activity. In spite of earlier evidence to the contrary, recent reports show that industrial estrogenic compounds are likely not the major causes of the increased incidence of breast cancer in women and or the reproductive problem in males. Because the relative uptake of the dietary levels of the natural and industrial antiestrogens versus that of the estrogenic chemical contaminants, the former chemicals may play a more significant role than that the estrogenic chemicals (Safe, 1994)

Cytochrome P450's are heme protein monooxygenases, part of a large family of monooxygenase or mixed-function oxidase enzymes initially involved in phase I (oxidation) biotransformation. The major roles of the P450's are in the metabolism of endogenous substrates such as arachidonic acid and steroid hormones and of exogenous substrates such as drugs and pesticides, resulting in both detoxification and toxification. The expression of different cytochrome P450 isoforms in animals may vary in relation to

various biological or physiological factors such as sex, age or animal strain and environmental factors such as temperature and chemical treatment. There are pronounced sex differences in the content and activities of hepatic microsomal cytochrome P450's in immature brook trout in response to treatment with 17β-estradiol or testosterone. Certain constitutive P450 levels in juvenile fish are repressed while others are induced by 17β-estradiol and testosterone treatment respectively (Stegeman *et al.*, 1982). There was also a report that 17β-estradiol treatment of immature rainbow trout decreased the total content of the P450's CYP2M1, CYP2K1 and P450 LMC5 and reduced lauric acid and progesterone 6β-hydroxylase (Buhler *et al.*, 1994). It appears, therefore, that natural estrogens down-regulate P450 expression in fish. However, the role of estrogenic chemicals in the regulation of P450 in fish such as rainbow trout is not known. Other estrogenic chemicals may modulate P450's that are involved in the metabolism of gonadal steroid in fish and hence causing are interruption of sexual-differentiation and reproduction in those species. The major objectives of this study are to determine the estrogenic activity of putative estrogenic chemicals in sexually immature rainbow trout by using plasma vitellogenin as a biomarker for estrogenic activity and also to determine the effect of these chemicals on the expression of cytochrome P450 isoforms in trout liver.

LITERATURE REVIEW

Cytochrome P450s are the heme protein monooxygenases that when they are reduced and complexed with carbon monoxide, give a maximum absorbance at 450 nm (Gordon and Skett, 1994). P450's are found in every biological kingdom, both prokaryotic and eukaryotic, and in every type of cell with the exceptions of red blood cells and skeletal muscle cell (Stegeman *et al.*, 1982). In eukaryotic organisms, they are membrane-bound and abundant in the endoplasmic reticulum (ER). As in mammals, teleosts have multiple forms of cytochrome P450, catalyzing the oxidation of many different substrates (Stegeman and Hahn, 1994) but the P450 activities in teleosts are lower than that of mammals (Stegeman, 1989). Depending on sex, strain and temperature, P450's in fish are also induced by their substrates or other chemicals (Kleinow *et al.*, 1987). Therefore, induction of P450's and associated mixed-function oxygenase (MFO) activities by environmental changes can be measured as a biological monitoring index. The induction of MFO enzymes can be considered as a fast response. To certain chemicals, therefore, there is a low level of MFO enzyme which can be used as a biomarker for exposure since it can be detected much earlier than liver enlargement or other pathological changes in the exposed animals (Payne *et al.*,1987).

Based on the relationship between amino acid sequences of the different P450's, not on P450 cataytic activities, the cytochrome P450's gene superfamily is divided into subfamilies (ie. those with 59% or greater sequence identity), families (ie. those with 40% or greater sequence identity) and individual P450's (Gonzalez, 1989). Providing an excellent animal model for toxicity studies, rainbow trout has been characterized to have at least 12 different hepatic and renal cytochrome P450's isoforms: five forms from liver of untreated trout, five hepatic isoforms following β-naphthoflavone (BNF) pretreatment, and two forms from the trunk kidney of sexually mature male trout (Buhler, 1995).

Purification of cytochrome P450 from hepatic microsomes of untreated rainbow trout yielded five isoforms of P450, named LMC1 to LMC5, with estimated molecular weights of 50,000 (LMC1 or CYP2M1), 54,000 (LMC2 or CYP2K1), 56,000 (LMC3), 58,000 (LMC4) and 59,000 (LMC5), respectively (Miranda *et al.*, 1989). CYP2M1 and

CYP2K1 were found to catalyze the (ω-6) and (ω-1)-hydroxylation of lauric acid, respectively (Buhler *et al.*, 1995). LMC5 demonstrated a significant progesterone 6β- and estradiol 2-hydroxylation and the oxidation of lauric acid, benzo[a]pyrene (BaP) and benzphetamine (BPT). CYP2K1 also caused substantial testosterone and lauric acid hydroxylation, BPT N-demethylation and aflatoxin B1 (AFB1) binding to DNA. Neither LMC3 nor LMC4 had any catalytic activity toward these substrates (Miranda *et al.*, 1989).

Five isozymes of hepatic microsomal P450's from BNF-treated rainbow trout also were purified and designated as LM_1, LM_2, LM_3, LM_{4a} and LM_{4b} (Williams and Buhler, 1984). Molecular weight and λ_{max} of LM_1, LM_2 and LM_3 are 50,000 and 449 nm, 54,000 and 449.5 nm and 56,500 and 447.5 nm, respectively. These P450's have little or no activity with respect to BaP. LM_{4a} and LM_{4b} are quite similar to each other when examined by molecular weight (58,000), λ_{max} of the CO-reduced difference spectrum (448 nm) and benzo[a]pyrene hydroxylase activity, differing only in the % yield obtained from purification (LM_{4a} yielded 2% and LM_{4b} yielded 5.3%). Further study showed that only LM_{4b} IgG was effective in inhibiting BaP hydroxylase activity by both LM_{4a} and LM_{4b}. It was suggested that LM_{4b} IgG bound to antigenic sites close to the active site on both isozymes which metabolized BaP (Williams and Buhler, 1984).

During the late reproductive stage, male rainbow trout trunk kidney exhibit 22-28 fold higher level of two P450 forms named KM1 and KM2 than females (Andersson and Folin, 1992). The two P450 isoforms had molecular weights 54,000 and 52,000, respectively. KM2 was also induced in juvenile rainbow trout by pretreatment with the androgen , 11-α ketotestosterone.

Vitellogenin, a precursor of the egg yolk proteins, is synthesized by the liver of oviparous (egg-laying) and nonmammalian vertebrates under the direct control of estrogen and transported to the developing ovary (Silversand *et al.*, 1993). The embryo is dependent on the yolk present in the egg for nutritional requirements. The major content of yolk are derived from lipoproteins, usually referred to vitellogenin (Silversand and Haux, 1995). Vitellogenin is composed of proteins (79%), lipids (19%), carbohydrates (0.3%), phosphorus, calcium and iron (each present at 0.3%) (Mananos *et al.*, 1994 and

Fremont and Riza, 1988). Silversand *et al.* (1995) reported the vitellogenin of 17β-estradiol-treated rainbow trout was composed of total saturated, monounsaturated and polyunsaturated 26.6, 21.1 and 52.3% of total fatty acid, respectively, by assuming that lipid bound to vitellogenin was mainly distributed to the total lipid composition of the egg. In addition, differences in the fatty acid composition of vitellogenin and liver from rainbow trout or other teleost species was explained by a specific selection of fatty acids during vitellogenesis.

Male and immature animals can also produce vitellogenin in response to estrogen (Kwon *et al.*, 1993). Although vitellogenin secretion may vary with species and route of administration, it can be used to assess the estrogenic potency of chemicals (Pelissero *et al.*, 1991, Pelissero *et al.*, 1993) and to indicate the extent of xenoestrogen exposure (Jones and King, 1995). In addition to be biomarker of estrogenic exposure, vitellogenin concentration may use as an indicator of the ovarian development stage because vitellogenesis started and increased when yolk vesicle formation occurs, ie. 3 month before ovulation, and these remains constant during ovulation (Sumpter *et al.*, 1984 and Scott and Sumpter, 1983).

Four estrogenic chemicals of interest are methoxychlor, diethylstilbestrol (DES), 4-tert-ocytlphenol and biochanin A. The structure of these chemicals are shown in Figure 1. DDT, methoxychlor and related chemicals; the cyclodiene insecticides (aldrin, dieldrin, endrin, heptachlor, chlordane, mirex and kepone); and the hexachlorocyclohexanes (lindane and toxaphane) are all classified as organochlorine insecticides (Amdur *et al.*, 1993). Methoxychlor (1,1,1-trichloro-2,2-bis (4-methoxyphenyl) ethane) has been known as DMDT, methoxy-DDT or Marlate (George, 1988). In a model ecosystem, methoxychlor was found to accumulate in fish to 60 times lesser extent than DDT. Methoxychlor has low toxicity in mammal with an oral LD_{50} in rats of 6 g/kg and a LD_{50} for mice of 2.0 ± 0.5 g/kg. The insecticide has a very pronounced selective insecticidal action, resulting from much greater efficiency of O-demethylation in the mammalian liver than in insects (Kapoor *et al.*, 1970). Therefore, methoxychlor is classified as biodegradable pesticide and a substitute for DDT (Kupfer and Bulger, 1987). However, methoxychlor is rather persistent in the environment because of its low aqueous solubility

(0.1 mg/L at 25°C) and high lipid solubility as reflected by its log octanol/water partition coefficient in the range of from 3.05-4.30. Methoxychlor is mainly degraded by microorganisms in soil or water (George, 1988). Methoxychlor can show estrogenic activity when it was incubated with rat liver microsome and NADPH, suggesting its activation by conversion to estrogenic metabolites by P450's (Ousterhout *et al.*, 1981). MDDE (1,1-dichloro-2,2-bis(4-methoxyphenyl)ethane), one of the contaminants in commercial grade methoxychlor and a metabolite formed from methoxychlor *in vivo*, is also a proestrogen. In addition, bis-OH-MDDE, bis-OH-methoxychlor, mono-OH-MDDE and mono-OH-methoxychlor are all methoxychlor metabolites that have estrogenic activity *in vitro* (Kupfer and Bulger, 1987).

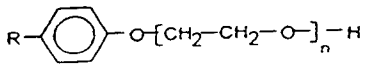
Li *et al.* (1995) demonstrated that the administration of two daily doses of methoxychlor for 4 days to female rat (150, 200 and 300 mg/kg) strongly induced hepatic microsomal cytochrome P450 2B1/2B2 (CYP2B1 and CYP2B2) and cytochrome P450 3A1/A2 (CYP3A1 and CYP3A2). In immature rats both methoxychlor low and high dose (150 and 300 mg/kg) increased CYP3A whereas only the high dose of methoxychlor (300 mg/kg) induced CYP2B1/2B2. Similar to treatment with DDT, methoxychlor induced CYP2B and CYP3A without effect on CYP1A and CYP2E1, suggesting that methoxychlor and DDT are phenobarbital-type inducers.

Liver microsomes from humans and from 3-methychloranthrene-treated and control male rats can demethylate methoxychlor, forming mono-OH and bis-OH methoxychlor. In addition to mono-OH and bis-OH methoxychlor, liver microsome from phenobarbital treated-rat and human CYP2B6 produced tris-OH and ring-hydroxylated methoxychlor. Moreover, monodemethylated (dihydroxy) compounds and didemethylated ring-hydroxylated compounds and tris-OH methoxychlor were produced upon incubation of ring-OH-methoxychlor with liver microsome from control or phenobarbital-treated rats. The proposed metabolic pathway of methoxychlor by rat liver is shown in Figure 2 (Dehal and Kupfer, 1994).

Diethylstilbestrol (DES), a synthetic non-steroidal estrogen, has been shown to be as estrogenic as the natural estrogen, 17β-estradiol. DES was widely used as a fattening agent in chickens and as a growth promoter in beef cattle and sheep until its production

was banned in 1959 and 1979, respectively, because of DES residues found in meat products. This application and resulting animal waste also caused DES contamination in the environment (Metzler and Fischer, 1981). During 1941 and 1947, DES was approved in humans for the treatment of many different gynecological conditions including miscarriages (spontaneous abortions) prevention in pregnant women. Unfortunately, there were high incidences of genitourinary abnormalities in male offspring and cancer and dysplasia of the cervix and vagina, ectopic pregnancy, spontaneous abortion and prematurity in female offspring that were exposed to DES *in utero*. In 1985, the Food and Drug Administration (FDA) reviewed and approved DES only for suppression of lactation, advanced cancer of the breast or prostate, menopausal symptoms and hypogonadism by giving estrogen replacement therapy (Edelman, 1986). However, the current indicated usage of DES is restricted to the treatment of breast and prostatic carcinomas that are inoperable and progressing (USPDI, 1994).

There are two major classes of nonionic surfactants: alcohol ethoxylate and alkylphenol ethoxylate. The important markets for alkylphenol ethoxylates are industrial uses such as uses in plastics and elastomers, textiles, agricultural chemicals and paper products (55%); institutional cleaning products (30%); and household cleaning and personal care products (15%). The branched nonyphenol ethoxylates with 9-10 ethylene oxide units are the most commonly used in cleaning products which then tend to enter in the environment. Alkylphenol ethoxylate is composed of a branched alkyl chain attached to a phenol ring and combined with ethylene oxide. The structure of alkylphenol ethoxylate is drawn as follows:



when n = number of moles of ethylene oxide per mole of alkylphenol and ranges from 1 to 100. Water solubility is increased by alkyl branching and the number of ethylene oxide molecules(n). Toxicity of the alkylphenol ethoxylates is related to their chemical structures and varies widely among animal species. Log K_{OW}, the tendency of a chemical to partition from water into a octanol (non-polar solvent), however, may not be related to toxicity (Talmage, 1994). Studies in fish addressed the relationship between the number of ethylene oxide molecule and toxicity. When tested on bluegill sunfish (*Leponis*

macrochirus), the 96-hr LC_{50} of octylphenols increased from 2.8-3.2 mg/L for C_8APE_5 (Triton X-100) to 531 mg/L for C_8APE_{30} (Triton X-305) (Macek and Krzeminski, 1975), supporting the conclusion that toxicity decrease with increasing ethylene oxide chain length. The 96-hr LC_{50} of *4*-tert-octylphenol, [4-(1,1,3,3-tetramethylbutyl)-phenol], for mysid shrimp (*Mysidopsis bahia*) ranged from 47 to 113 mg/kg (Cripe *et al.*, 1989). In rat and mouse the acute oral toxicity of *4*-tert-octylphenol were from 2,160 to 3,210 mg/kg, respectively (RTECS, 1987). Nonionic surfactants caused toxicity by interacting with proteins, resulting in changes in the shape, function and structure of the proteins. However, C_8APE_{10} (Triton X-100) has been used to solubilize membrane proteins without loss of biological activity. Although C_8APE_9 (Triton X-100) was classified as teratogen in *in vitro* tests, this compound was not teratogenic in pregnant CD-1 mice at an LD_{10} (800 mg/kg) dose, given orally on days 6-13 of pregnancy (Hardin *et al.*, 1987).

Alkylphenols are the major biodegradation products of nonionic surfactants (alkylphenol-ethoxylates) formed during sludge treatment of municipal and modified wastes under anaerobic conditions. Many of these products are weakly estrogenic to fish (Sobling and Sumpter, 1993). The biodegradation of alkylphenol-polyethoxylate during sewage treatment is shown in Figure 3 (Yoshimura *et al.*, 1986, Giger *et al.*, 1987, and Ball *et al.*, 1989). One alkylphenol, 4-nonyphenol, can also be released from plastic centrifuge tubes, showing estrogenic activity such as induced levels of cell proliferation and progesterone receptor in MCF-7 breast tumor cell and triggering mitotic activity in rat endometrium (Soto *et al.*, 1991). Vitellogenin concentrations in rainbow trout were markedly increased without observed adverse effect when fish were held in sewage effluent containing detergent degradation products (Purdom *et al.*, 1994). Based on vitellogenin synthesis by rainbow trout hepatocytes, many of these biodegradation products from detergents such as 4-tert-octylphenol, 4-tert-butylphenol and 4-nonylphenol are weak estrogenic and inhibited by tamoxifen, suggesting that this activity is mediated by estrogen receptor. These investigators also found that 4-tert-ocytlphenol has a relative estrogenic potency of 0.0000370, compared with 17β-estradiol with a potency of 1.00 (Jobling and Sumpter, 1993). They also showed that higher doses of 4-tert-octylphenol

became cytotoxic and decreased vitellogenin production. White *et al.* (1994) reported that 4-octylphenol interacted with a similar domain of the estrogen receptor as did 17β-estradiol.

Observations have shown that Asian populations consuming large quantities of phytoestrogens derived from their soy-based diets have a lower incidence of breast and prostate cancer compared to western populations. The anticarcinogenic activity of some phytoestrogens may result from their antiestrogenic action (Sheehan, 1995). Previous studies have supported this idea and also described the infertility of animals in an grazing estrogenic clover pasture and recovering to normal after change to nonestrogenic pasturage (Moreley *et al.*, 1966). Plant also contain estrogenic chemicals or phytoestrogens such as isoflavones formononetin, daidzein, genistein and biochanin A, found in legume plant such as red clover (*Trifolium pratenase*), white alfalfa and certain cultivators of subterranean (*Trifolium subterraneum*) (Lundh *et al.*, 1988).

Biochanin A, (5,7 dihydroxy-4'-methoxyisoflavone), is a potent inhibitor of metabolic activation of benzo[a]pyrene to mutagenic diol epoxides in hamster embryo cell cultures (Cassady *et al.*,1988), and can also decrease benzo[a]pyrene metabolism by microsomes from Aroclor 1254-induced rat liver (Chae *et al.*,1991).

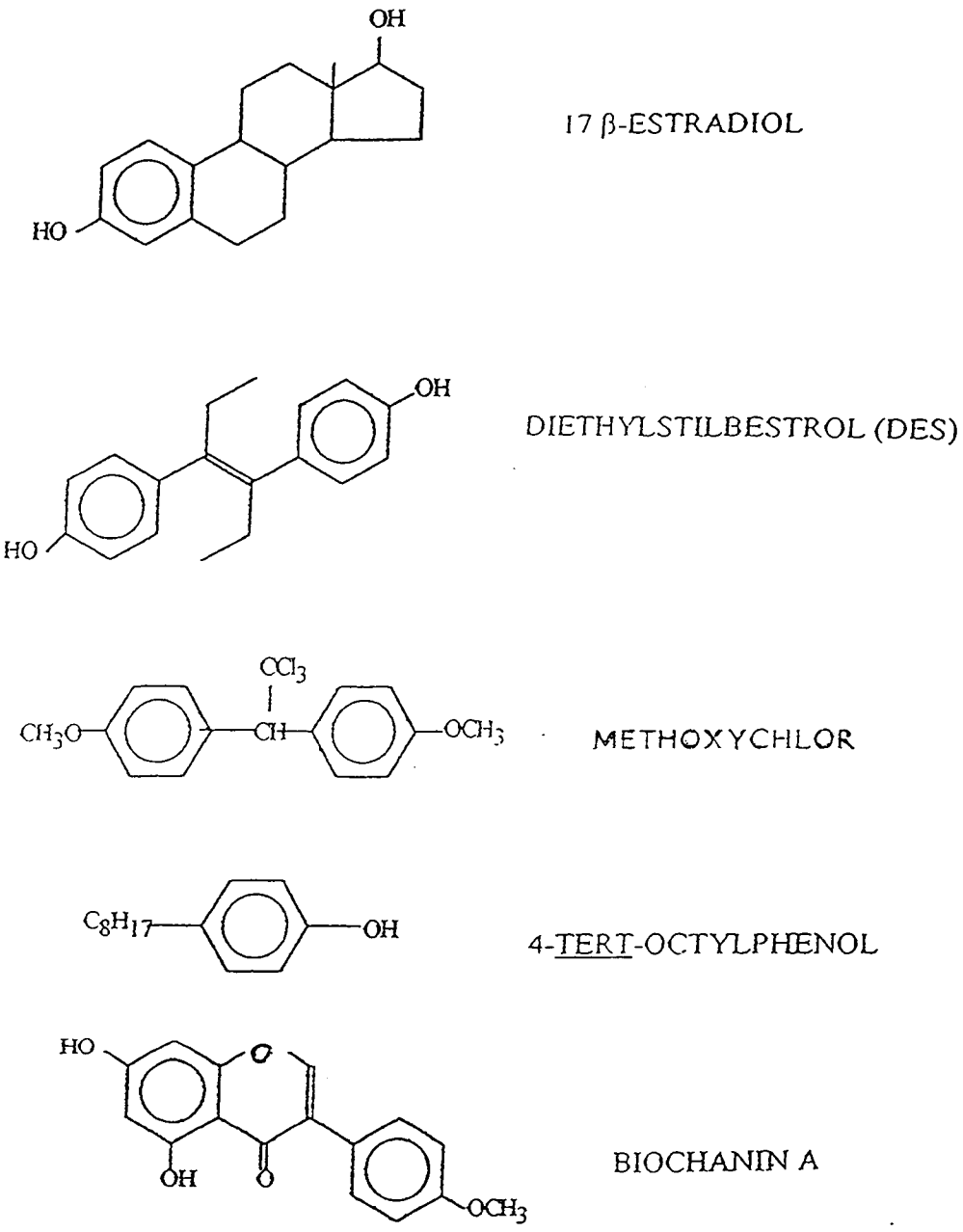


Figure 1. The structure of 17β-estradiol and four chemicals of interest; methoxychlor, diethylstilbestrol (DES), 4-tert- octylphenol and biochanin A.

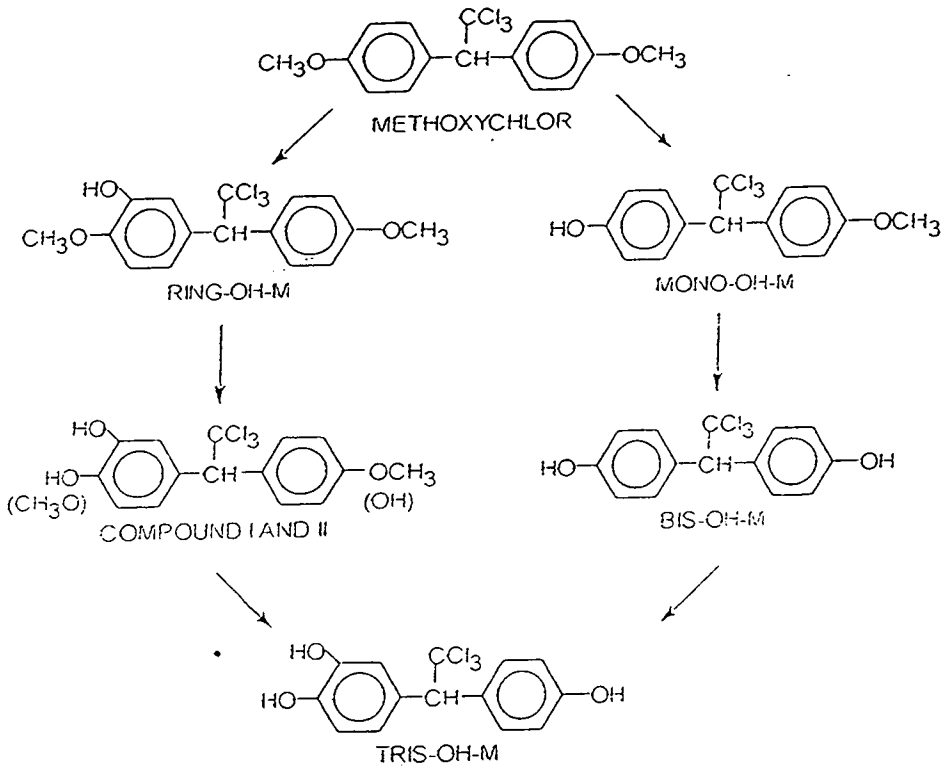


Figure 2. The proposed metabolic pathway of methoxychlor by rat liver microsome according to Dehal and Kupfer, 1994.

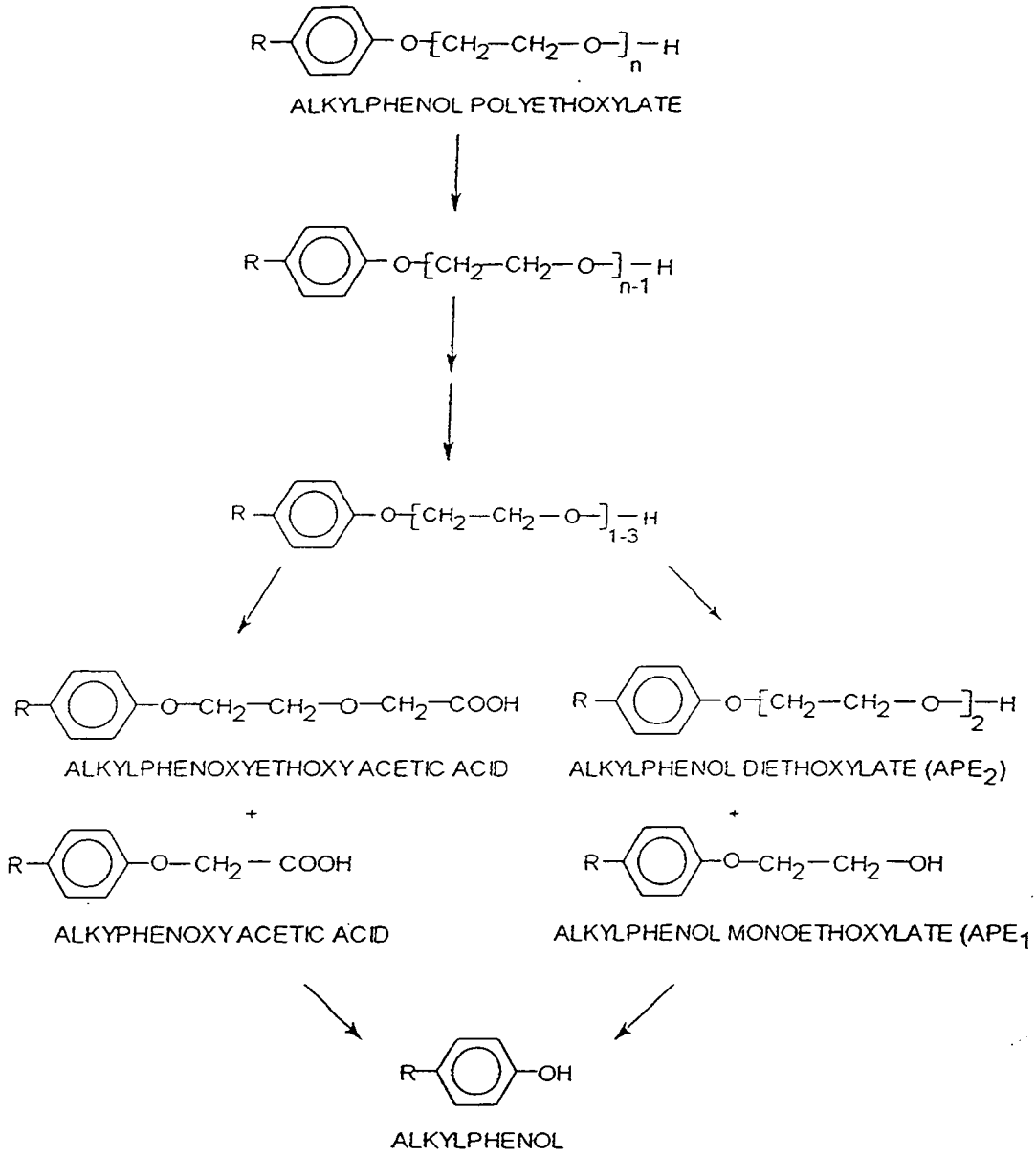


Figure 3. The biodegradation of alkylphenol-polyethoxylate during sawage treatment according to Yoshimura et al., 1986, Giger et al., 1987 and Ball et al., 1989.

MATERIALS AND METHODS

CHEMICALS

Methoxychlor, diethylstilbestrol (DES), biochanin A, Sepharose 6B (for vitellogenin purification), nicotinamide adenine dinucleotide phosphate (NADPH, reduced form), 3,3'5,5' tetramethyl benzidine, dimethyl sulfoxide (DMSO), anti-rabbit IgG biotinylate from goat, and bovine albumin (protease free for Western blotting and protein determination, essentially globulin free for ELISA) were purchased from Sigma Chemical Co. (St. Louis, MO). 4-tert-octylphenol was purchased from Aldrich Chemical Co. (St. Louis, MO); [^{14}C]-lauric acid from DuPont NEN Research Products (Boston, MA); and [^{125}I]-protein A from ICN (Irvine, CA). Strepavidine-horseradish peroxidase was from Amersham Corp. (Arlington Heights, IL). Acetonitrile was HPLC grade, obtained from EM Science (Gibbstown, NJ). Liquid scintillation counting fluid was from Research Products International Corp. (Mount Prospect, IL). Ethoxyresorufin and resorufin were obtained from Molecular Probes, (Eugene, OR). Rabbit antibodies raised against purified trout CYP2M1, CYP2K1, P450 LMC5 and CYP1A1 were prepared in our lab previously (Miranda *et al.*, 1989, Williams and Buhler, 1984). Rabbit anti-chum salmon vitellogenin IgG was the kind gift of Dr. A. Hara (Hara *et al.*, 1993). High and low affinity plate for ELISA was purchased from Dynatec (Chantilly, VA) and Costar (Cambridge, MA), respectively. The chemicals and materials used in Western blotting and SDS-PAGE were obtained from Bio-Rad (Richmond, CA). Other chemicals were all reagent grade and commercially available.

ANIMAL TREATMENTS

Two year-old rainbow trout (*Oncorhynchus mykiss*) of both sexes were injected (ip) with 17β−estradiol 5 mg/kg in propylene glycol on day 1 and 4. The blood were collected on day 10 for vitellogenin purification.

One year-old male and female rainbow trout (*Oncorhynchus mykiss*) were distributed into control and four estrogenic chemicals-treated groups. Fish were injected ip with propylene glycol (controls), methoxychlor (20 mg/kg), DES (15 mg/kg), 4-tert-octylphenol (two doses: 25 and 50 mg/kg) and biochanin A (two doses: 25 and 50 mg/kg) on day 1, 4 and 7. Eight fish from each group (4 males and 4 females) were sacrificed on day 9. Then the livers were removed and the sex determined.

PURIFICATION OF VITELLOGENIN

Purification of plasma vitellogenin was performed according to Hara *et al.* (1993). The pooled plasma was diluted with 10 volume of cold distilled water and left at 4°C for 1 hour. After centrifugation at 2,500 g for 15 min at 4°C, the pellet was dissolved in 1 ml of 0.02 M Tris-HCl buffer, pH 8.0 containing 2% NaCl and 0.1% NaN_3. The solution was applied to a 2.5 x 40 cm. gel filtration column of Sepharose 6B equilibrated with Tris-HCl buffer. After pooled plasma application, the column was washed with equilibration buffer. The fractions (160 fractions) were collected for 3 ml/fraction at a flow rate 12 ml/hour at 4°C. The protein concentration in each fraction was determined by measure the absorbance at wavelength 280 nm. And the one major peak was collected as the purified vitellogenin.

PREPARATION OF MICROSOMES

Trout livers were homogenized in 0.1 M Tris-acetate buffer (pH 7.5), 0.1 M KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT) and 0.1 mM phenylmethylsulfonylfluoride (PMSF), following sequential centrifugation at 10,000 g for 30 min and at 105,000 g for 90 min. The microsomal pellets were washed one time and resuspended in 0.1 M potassium phosphate buffer (pH 7.25) containing 1 mM EDTA, 30% glycerol, 1 mM DTT and 0.1 mM PMSF for storage -80°C until used. Protein concentration were determined by the method of Lowry *et al.* (1951) using bovine albumin as standard. Total microsomal

cytochrome P450 were determined from the CO-reduced difference spectrum by the method of Estabrook *et al.* (1972) on a Cary 219 spectrophotometer.

GEL ELECTROPHORESIS

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Separating and stacking gel contained 7.5 and 4% acrylamide, respectively. Microsomes were prepared in 2X loading buffer, modified from Sambrook *et al.* (1989) at a concentration of 2 μg/μl and 15 μl applied of sample then plasma samples were diluted in 2X loading buffer at a concentration of 4 μg/μl and applied 10 μl. The gels were run at 150 volt until the stacking dye reached the bottom of the gels. Kaleidoscope prestained standards used for molecular weight (MW) determinations were bovine serum albumin (71,000), carbonic anhydrase (41,800), soybean trypsin inhibitor (30,600), lysozyme (17,800) and aprotinin (6,900). The gels were fixed and stained in 0.15% Coomassie brilliant blue-30% methanol-10% acetic acid overnight at room temperature and destained in 40% methanol- 10% acetic acid.

WESTERN BLOT ANALYSIS

Microsomes and plasma were separated on a 7.5% SDS-polyacrylamide gel,electrophoretically transferred to nitrocellulose. The blots were incubated in 2% BSA in Tris buffer saline with Tween 20 (TBS-Tween) 1 hour, followed with rabbit anti-trout CYP2K1, CYP2M1, P450 LMC5 or CYP1A1 IgG or rabbit anti-chum salmon vitellogenin IgG for 1 hour. After washing in TBS-Tween buffer four times, the blots were incubated with [^{125}I]-protein A for 1 hour. Visualization and immunoquantification were performed by scanning the autoradiography on a Molecular Dynamics phosphorimager, scanning system with associated software. After immunoquantification, the membrane was incubated in 50°C for 1 hour with stripping buffer, washed twice with TBS-Tween, incubated 1 hour at room temperature with 10 g of non-fat dried milk, washed 4 times with TBS-Tween and then probe with another antibody. The amount of

each P450 isoform present in the trout liver samples was calculated utilizing the purified form as standard.

LAURIC ACID HYDROXYLASE ACTIVITY

Lauric acid hydroxylase activity was measured according to Buhler *et al.* (1995). The incubation mixtures contained 50 mM Tris-acetate buffer (pH 7.4), 200 μM [1-^{14} C]-lauric acid, 500 μg microsomal protein and 1 mM NADPH. After a 1 hour incubation at 30°C, the reaction was stopped by the addition of 100 μl 10% (v/v) H_2SO_4 and extracted twice with 4 ml of diethylether. The extract was dried under a stream of N_2, dissolved in CH_3CN 100 μl and analyzed by HPLC. Sample were analyzed on a Beckman HPLC equipped with a 421 controller, two 100A pumps and a 5 μm Zorbax ODS column (0.46 x 25 cm) (Mac-Mod Analytical Inc., Chadds Ford, PA) using CH_3CN and water each containing 0.2% acetic acid, as the mobile phases, a flow rate of 1.5 ml/min, and detection by a Packard Flo-One-A-100 radioactivity flow monitor. The mobile phase concentration was 25% CH_3CN for 35 min followed by a 2 min linear gradient to 80% CH_3CN to elute the parent compound. At 4 min, the CH_3CN was returned to 25% over 2 min. The column was allowed to equilibrate 23 min before the next injection. The hydroxylated lauric acid metabolites formed by the trout microsomes were found to be eluted in the order: (ω-1), (ω-2), (ω-3), (ω+ω-4), (ω-5), (ω-6) hydroxylauric acid and unoxidized parent lauric acid with the retention time 29.5, 31.5, 33.8, 37.4, 38.3, 40.3 and 45.2 min., respectively.

ETHOXYRESORUFIN O-DEETHYLASE (EROD) ACTIVITY

EROD activity was modified from Burke and Mayer (1974) and Prough *et al.* (1978). The assay samples, prepared in a fluorometer cuvette and performed on a Perkin-Elmer 650-10s fluorescence spectrophotometer, contained in a final volume of 1 ml 0.1 M Tris-acetate buffer (pH 7.8), 50 μg microsomal protein and 1 mM 7-ethoxyresorufin. The reaction was started by adding NADPH 0.1 mM into the cuvette. 7-Ethoxyresorufin was

deethylated to resorufin, increasing fluorescence and recorded at an excitation wavelength of 510 nm and an emission wavelength of 586 nm for 2 min. After adding 50 picomole of resorufin as an internal standard, the fluorescence was read. EROD activity was calculated as resorufin production/min/mg protein of microsome.

VITELLOGENIN ANALYSIS

Vitellogenin in plasma was determined by competitive enzyme linked immunosorbent assay (ELISA), according to Donohoe (1995). High affinity 96-well plates were coated in 100 µl/well with purified rainbow trout vitellogenin standard in 0.05 M sodium carbonate buffer (pH 9.6) 250 ng/ml and incubated overnight at 4°C. Standard vitellogenin (25-800 ng/ml) or plasma sample, diluted with phosphate buffer-saline-0.05% Tween 20 (PBST) containing 1% BSA and rabbit anti-chum salmon vitellogenin IgG were added in low affinity 96-well plates and stored overnight at 4°C. After washing high affinity 96-well plates with PBST four time, non-specific binding was blocked with 1% BSA in PBST 1 hr, at 37°C and washed. The samples were transferred to the high affinity 96-well plates and stored overnight at 4°C. After washing the plates with PBST, anti-rabbit IgG was added and incubated for 2 hour at 37°C and the plate then washed with PBST. Strepavidine-horseradish peroxidase was added, the plate incubated for 2 hour at 37°C. and then again washed with PBST. The color was developed by adding the chromogen 3,3',5,5'- tetramethylbenzidine dissolved in DMSO, 0.01% H_2O_2 (30%) and 0.01 M sodium acetate buffer, pH 6.0. After 20 min incubation at room temperature, the reaction was stopped by adding 10% H_2SO_4. Absorbance was measured at 450 nm on a Biotek microplate reader model EL 309.

STATISTICAL ANALYSIS

Reported values are the means and standard error from 7 observations. Means were compared by analysis of variance (ANOVA) and Student's t test (two-tailed), and differences were considered statistically significant at $P < 0.05$. All analyses were carried out using the statistical package (Statgraphics version 5.0) provided by Oregon State University.

RESULTS

VITELLOGENIN PURIFICATION

We were able to precipitate vitellogenin successfully from plasma by adding 10 volumes of cold water at 4°C for 1 hour. After centrifugation, the pellet was resuspended in Tris-HCl buffer, pH 8.0 and passed through the Sepharose 6B column at a flow rate 12 ml/hr. Each 3 ml fractions was collected and the absorbance at 280 nm then measured. Fraction numbers 47-55 showed the high absorbance as shown in Figure 4. These fractions were pooled and determined to be purified vitellogenin as confirmed by probing with rabbit anti-chum salmon vitellogenin IgG in Western blotting. Protein concentration were determined by the method of Lowry *et al.* (1951). The Coomassie blue stained SDS-PAGE pattern of purified vitellogenin is shown in Figure 14.

ELECTROPHORESIS PATTERNS OF MICROSOMES AND PLASMA

The Coomassie blue stained SDS-PAGE patterns of microsome and plasma from control and treated-trout are shown in Figures 5 to 9 and 10 to 14, respectively. There were a high variability between individual trout in both the microsomes and plasma from treated and control trout. Liver microsomes from trout treated with DES, two doses of 4-tert-octylphenol and biochanin A (Figures 5 to 9), showed six strong Coomassie blue detectable bands with a relative mobility corresponding to a molecular mass about 55, 71, 150, 164, 172 and 190 kDa, respectively.

In plasma, we found two pronounced bands with a molecular mobility corresponding to a molecular mass about 71 and 161 kDa in all both male and female fish treated with DES, Figure 10. The same two bands were seen with the two doses of 4-tert-octylphenol (Figure 12) but they were fainter than in the DES-treated fish, differing in intensity and inter-individual variability within the same treatment group. The high dose of 4-tert-octylphenol gave more intense bands than the low dose of the same chemical. The two bands may relate to the vitellogenin because the greater the intensity of these two

bands, the higher were to measure plasma vitellogenin concentration. When plasma from each male and female from fish in a group were run on SDS-PAGE with purified vitellogenin (Figure 14), only one band with a relative molecular mass 71 kDa was similar to purified vitellogenin.

WESTERN BLOT ANALYSIS

Western blot of liver microsomes from trout treated with four chemicals probed with anti-chum salmon vitellogenin IgG (Figure 20, E) showed that only one band can be recognized by the anti-chum salmon vitellogenin IgG in DES and two doses of 4-tert-octylphenol treated trout. The result confirmed that this band was the subunit of vitellogenin.

Several bands could be detected by anti-chum salmon vitellogenin IgG in Western blotting of plasma from trout treated with DES and two doses of 4-tert-ocytlphenol (Figure 25). These bands had a molecular mobility corresponding to a molecular mass lower than that seen in SDS-PAGE, detected by anti-chum salmon vitellogenin IgG in DES and two doses of 4-tert-octylphenol treated-fish.

EFFECTS OF CHEMICAL TREATMENTS ON CYTOCHROME P450

The total hepatic microsomal P450 levels after pretreatment with the four estrogenic chemicals is shown in Figure 15 and Table 1, as determined by measurement of the CO-reduced difference spectra. There were no significant difference in total P450 concentrations that could be related to chemical exposure. Pretreatment with the four chemicals caused only a slight change in the total P450 content with no significant differences except for a small increase with a 50 mg/kg dose of biochanin A and small decreases in the other groups.

Determination of the different trout P450 isoforms was performed and quantified by Western blotting and scanning with a phosphorimager. Pretreatment of immature rainbow trout with 17β-estradiol caused a significant reduction in the translation level of

three P450 isoforms [CYP2M1, CYP2K1 and LMC5] (Buhler *et al.*, 1994). Unlike the natural estrogen, 17β-estradiol, treatment with the putative estrogenic chemicals showed much smaller differences on the expression of the P450 isoforms. Nevertheless, fish pretreated with methoxychlor, DES and both high doses (50 mg/kg) of 4-tert-octylphenol and biochanin A gave a significant decrease of CYP2M1 protein expression. There also were repression of CYP2K1 protein expression in the methoxychlor, 4-tert-octylphenol (25 mg/kg) and biochanin A (50 mg/kg) treatment groups. Trout LMC5 was suppressed significantly in fish treated with methoxychlor, DES, both doses of 4-tert-octylphenol (25 and 50 mg/kg) and the high dose of biochanin A (50 mg/kg). Interestingly, two doses of biochanin A (25 and 50 mg/kg) induced CYP1A1 as shown by Western blotting, Figures 19 and 20. These latter result were confirmed by the increase in EROD activity in the biochanin A exposed fish (Figure 26 and Table 3).

EFFECTS OF CHEMICAL TREATMENTS OF LAURIC ACID METABOLISM

Cytochrome P450 monooxygenase activities can be examined with different substrates. For example, aflatoxin B1 is substrate for CYP2K1 (Williams and Buhler, 1983), and benzo[a]pyrene is a substrate for CYP1A1 in rainbow trout liver (Andersson and Forlin, 1992). Buhler *et al.* (1995) demonstrated that in a reconstituted system with P450 LMC1 (CYP2M1) and P450 LMC2 (CYP2K1) from rainbow trout liver catalyzes, respectively, the (ω-6) and (ω-1)-hydroxylation of lauric acid, an endogenous substrate for P450. Pretreatment of immature rainbow trout with 17β-estradiol caused a significant reduction in total lauric acid hydroxylation (Buhler *et al.*, 1994). We confirmed these earlier results showing that the total conversion to hydroxylated lauric acid metabolites was decreased significantly for all treatment group, compared to controls group, (Figures 21,22 and Table 2). In addition, there was reduced formation of (ω-6) and (ω-1) hydroxylaurate for all treatments (Figures 21 and 22). These results are consistent with the results obtained from the measurements of hepatic microsomal P450 (Figure 15 and Table 1) which showed that both CYP2M1 and CYP2K1 were reduced as reflected by Western blotting. Because no inter-individual and male and female differences were

detected in the lauric acid metabolism patterns, only one HPLC profile is shown from each treatment. The HPLC profile of [1-^{14}C]-lauric acid metabolites formed by liver microsome from each treatment are shown in Figure 22.

EFFECTS OF CHEMICAL TREATMENTS ON PLASMA VITELLOGENIN AND EROD ACTIVITY

There were no differences in the plasma total protein concentration among the control and four treatment groups (Table 3). All four estrogenic chemicals induced plasma vitellogenin level with responses ranging from high to low; DES, 4-tert-octylphenol (50 mg/kg), biochanin A (50 mg/kg), 4-tert-ocytlphenol (25 mg/kg), biochanin A (25 mg/kg) and methoxychlor, respectively (Figure 24 and Table 3). There was no difference in induction by chemicals between male and female immature fish. While there was no band in control and methoxychlor (20 mg/kg) treated-trout. We saw this result markedly when ran each treatment in the same SDS-PAGE. The Western blot of plasma (Figure 23) probed with rabbit anti-chum salmon vitelellogenin IgG, gave results consistent with those obtained from the ELISA analysis.

7-Ethoxyresorufin O-deethylase is a model substrate for CYP1A1 activity (Andersson and Forlin, 1992). Hepatic microsomes from trout can deethylate 7-ethoxyresorufin in the presence of NADPH and oxygen to resorufin, eliciting by increase the fluorescence from 510 nm to 586 nm (Burke and Mayer, 1974). Because of the result from Western blotting showed the induction of CYP1A1 by pretreatment with biochanin A (25 and 50 mg/kg), we attempted to confirm these observations by measuring the EROD activity in the liver microsomes from the biochanin A treated fish. EROD activity clearly demonstrated that resorufin production (picomole) per min per mg protein of microsome was increased significantly in the fish preexposed to two doses of biochanin A as compared to control and the other treatments (Figure 26 and Table 3).

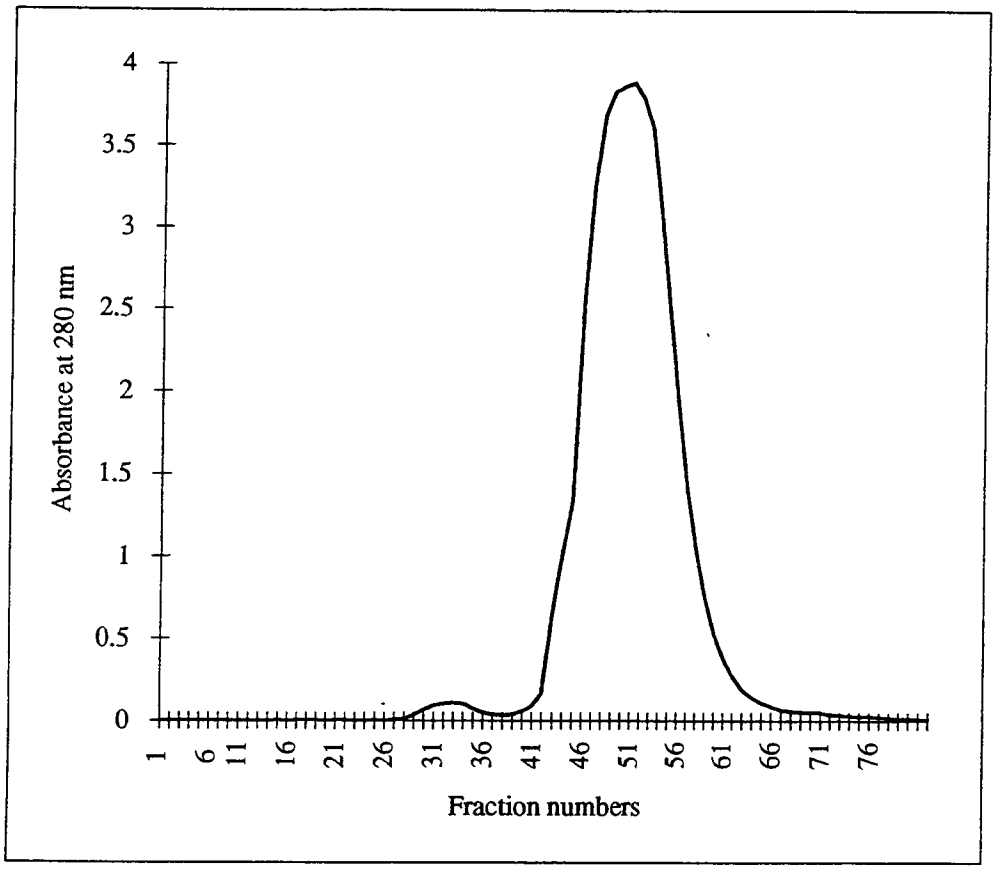


Figure 4. Gel filtration on a 2.5 x 40 cm column of Sepharose 6B of plasma from two year-old rainbow trout of both sexes after injection (ip) with 17β-estradiol 5 mg/kg on day 1 and 4. the column was developed in 0.02 M Tris-HCl buffer (pH 8.0) containing 2% NaCl and 0.1% NaN_3 at a flow rate of 12 ml/hour and 3 ml fractions were collected.

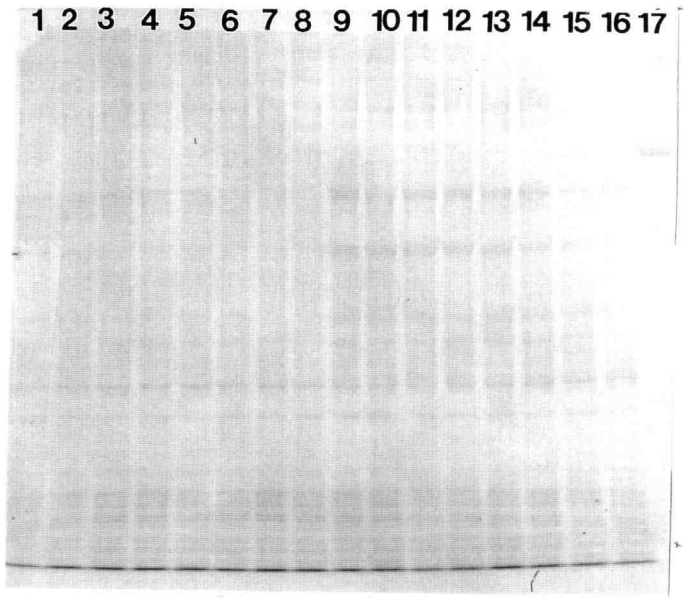


Figure 5. SDS-PAGE of hepatic microsomes (30 μg per lane) from trout treated with one chemical. The gel was performed according to Laemmli (1970) with separating and stacking gel contained 7.5 and 4% acrylamide, respectively. After running at 150 volt until the stacking dye reached to the bottom of the gel, the gel was fixed, stained in 0.15% Coomassie brilliant blue-30% methanol-10% acetic acid and subsequently destained in 40% methanol-10% acetic acid. Lanes 1 to 4, control males; lanes 5 to 8, control females; lanes 9 to 12, DES (15 mg/kg) treated males; lanes 13 to 16, DES (15 mg/kg) treated females; and lane 17, Kaleidoscope Prestained Standards: myosin (202 kDa), β-galactosidase (133 kDa), bovine serum albumin (71 kDa), carbonic anhydrase (41.8 kDa), and soybean trypsin inhibitor (30.6 kDa), respectively.

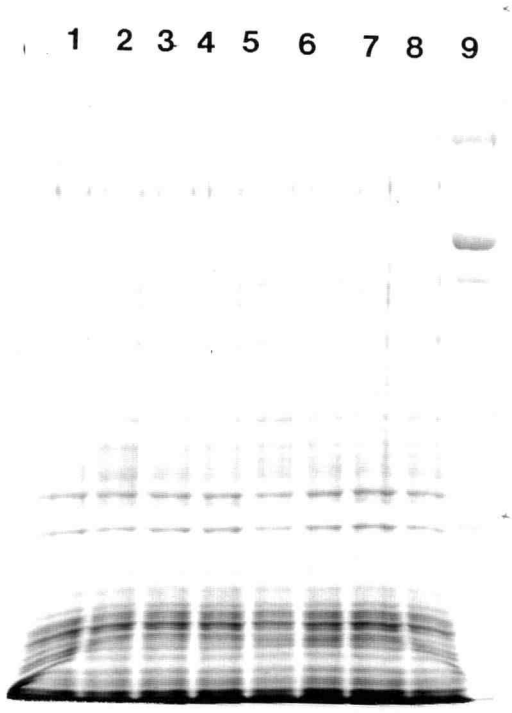


Figure 6. SDS-PAGE of hepatic microsomes (30 μg per lane) from trout treated with one chemical. The gel was performed according to Laemmli (1970) with separating and stacking gel contained 7.5 and 4% acrylamide, respectively. After running at 150 volt until the stacking dye reached to the bottom of the gel, the gel was fixed, stained in 0.15% Coomassie brilliant blue-30% methanol-10% acetic acid and subsequently destained in 40% methanol-10% acetic acid. Lanes 1 to 4, methoxychlor (20 mg/kg) treated males; lanes 5 to 8, methoxychlor (20 mg/kg) treated females; and lane 9, Kaleidoscope Prestained Standards: myosin (202 kDa), β-galactosidase (133 kDa), bovine serum albumin (71 kDa), carbonic anhydrase (41.8 kDa), and soybean trypsin inhibitor (30.6 kDa), respectively.

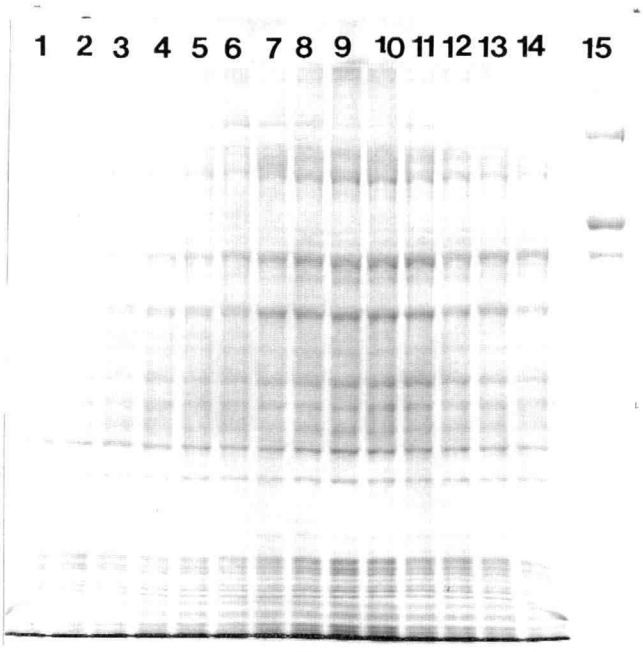


Figure 7. SDS-PAGE of hepatic microsomes (30 μg per lane) from trout treated with one chemical. The gel was performed according to Laemmli (1970) with separating and stacking gel contained 7.5 and 4% acrylamide, respectively. After running at 150 volt until the stacking dye reached to the bottom of the gel, the gel was fixed, stained in 0.15% Coomassie brilliant blue-30% methanol-10% acetic acid and subsequently destained in 40% methanol-10% acetic acid. Lanes 1 to 3, 4-tert-octylphenol (25 mg/kg) treated males; lanes 4 to 6, 4-tert-octylphenol (25 mg/kg) treated females; lanes 7 to 10, 4-tert-octylphenol (50 mg/kg) treated males; lanes 11 to 14, 4-tert-octylphenol (50 mg/kg) treated females; and lane 15, Kaleidoscope Prestained Standards: myosin (202 kDa), β-galactosidase (133 kDa), bovine serum albumin (71 kDa), carbonic anhydrase (41.8 kDa), and soybean trypsin inhibitor (30.6 kDa), respectively.

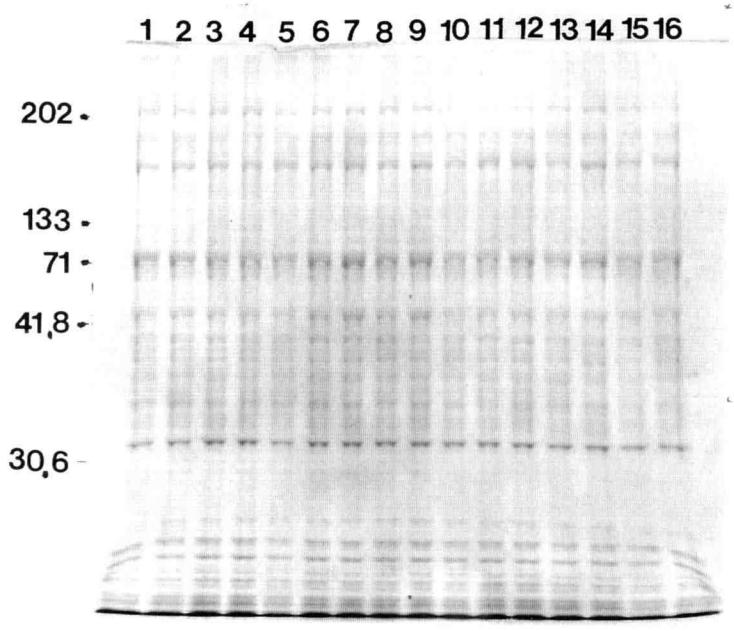


Figure 8. SDS-PAGE of hepatic microsomes (30 μg per lane) from trout treated with one chemical. The gel was performed according to Laemmli (1970) with separating and stacking gel contained 7.5 and 4% acrylamide, respectively. After running at 150 volt until the stacking dye reached to the bottom of the gel, the gel was fixed, stained in 0.15% Coomassie brilliant blue-30% methanol-10% acetic acid and subsequently destained in 40% methanol-10% acetic acid. Lanes 1 to 4, biochanin A (25 mg/kg) treated males; lanes 5 to 8, biochanin A (25 mg/kg) treated females; lanes 9 to 12, biochanin A (50 mg/kg) treated males; and lanes 13 to 16, biochanin A (50 mg/kg) treated females

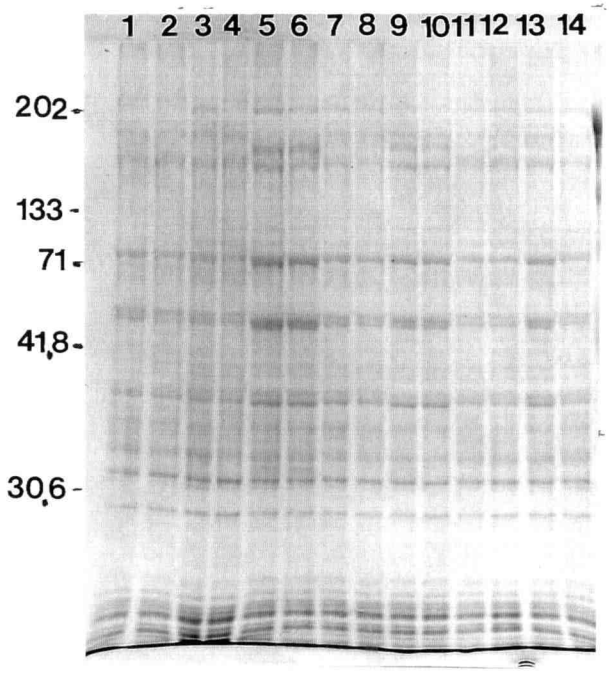


Figure 9. SDS-PAGE of hepatic microsomes (30 μg per lane) from trout treated with four chemicals. The gel was performed according to Laemmli (1970) with separating and stacking gel contained 7.5 and 4% acrylamide, respectively. After running at 150 volt until the stacking dye reached to the bottom of the gel, the gel was fixed, stained in 0.15% Coomassie brilliant blue-30% methanol-10% acetic acid and subsequently destained in 40% methanol-10% acetic acid. Lanes 1 to 2, control male and female; lanes 3 to 4, methoxychlor (20 mg/kg) treated male and female; lanes 5 to 6, DES (15 mg/kg) treated male and female; lanes 7 to 8, 4-tert-octylphenol (25 mg/kg) treated male and female; lanes 9 to 10, 4-tert-octylphenol (50 mg/kg); lanes 11 to 12, biochanin A (25 mg/kg) treated male and female; and lanes 13 to 14, biochanin A (50 mg/kg) treated male and female, respectively.

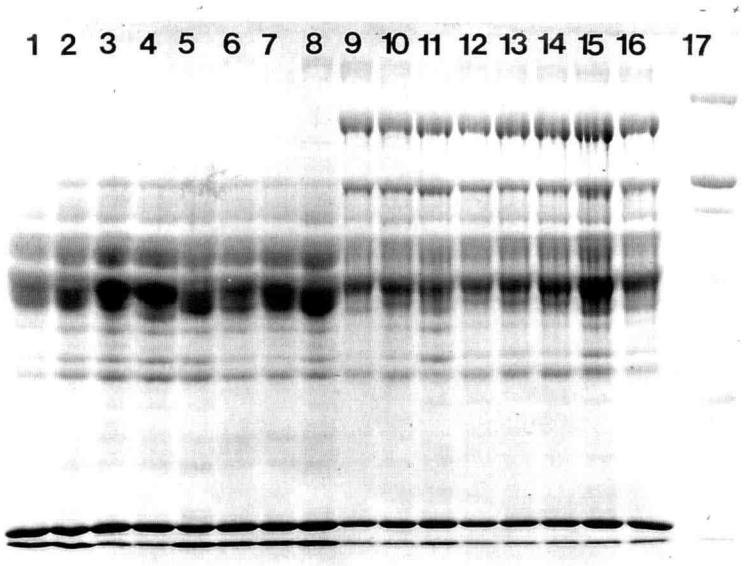


Figure 10. SDS-PAGE of plasma (40 μg per lane) from trout treated with one chemical. The gel was performed according to Laemmli (1970) with separating and stacking gel contained 7.5 and 4% acrylamide, respectively. After running at 150 volt until the stacking dye reached to the bottom of the gel, the gel was fixed, stained in 0.15% Coomassie brilliant blue-30% methanol-10% acetic acid and subsequently destained in 40% methanol-10% acetic acid. Lanes 1 to 4, control males; lanes 5 to 8, control females; lanes 9 to 12, DES (15 mg/kg) treated males; lanes 13 to 16, DES (15 mg/kg) treated females; and lane 17, Kaleidoscope Prestained Standards: myosin (202 kDa), β-galactosidase (133 kDa), bovine serum albumin (71 kDa), carbonic anhydrase (41.8 kDa), and soybean trypsin inhibitor (30.6 kDa), respectively.

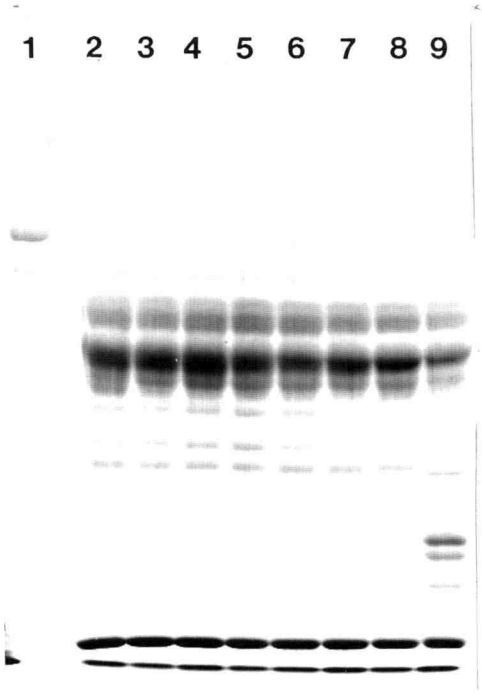


Figure 11. SDS-PAGE of plasma (40 μg per lane) from trout treated with one chemical. The gel was performed according to Laemmli (1970) with separating and stacking gel contained 7.5 and 4% acrylamide, respectively. After running at 150 volt until the stacking dye reached to the bottom of the gel, the gel was fixed, stained in 0.15% Coomassie brilliant blue-30% methanol-10% acetic acid and subsequently destained in 40% methanol-10% acetic acid. Lanes 1 to 4, methoxychlor (20 mg/kg) treated males; lanes 5 to 8, methoxychlor (20 mg/kg) treated females; and lane 9, Kaleidoscope Prestained Standards: myosin (202 kDa), β-galactosidase (133 kDa), bovine serum albumin (71 kDa), carbonic anhydrase (41.8 kDa), and soybean trypsin inhibitor (30.6 kDa), respectively.

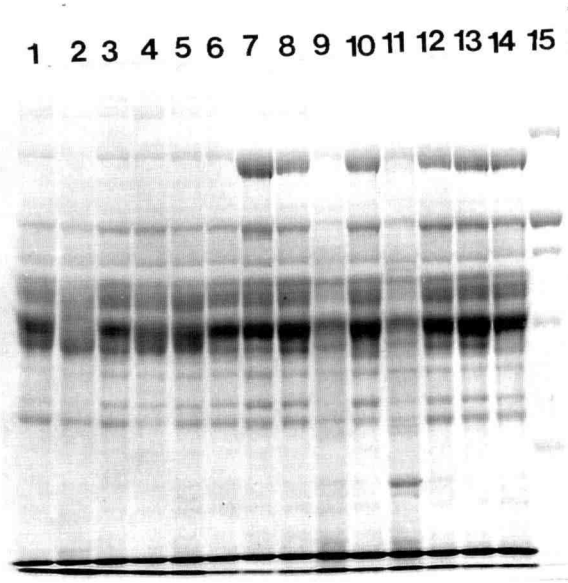


Figure 12. SDS-PAGE of plasma (40 μg per lane) from trout treated with one chemical. The gel was performed according to Laemmli (1970) with separating and stacking gel contained 7.5 and 4% acrylamide, respectively. After running at 150 volt until the stacking dye reached to the bottom of the gel, the gel was fixed, stained in 0.15% Coomassie brilliant blue-30% methanol-10% acetic acid and subsequently destained in 40% methanol-10% acetic acid. Lanes 1 to 3, 4-tert-octylphenol (25 mg/kg) treated males; lanes 4 to 6, 4-tert-octylphenol (25 mg/kg) treated females; lanes 7 to 10, 4-tert-octylphenol (50 mg/kg) treated males; lanes 11 to 14, 4-tert-octylphenol (50 mg/kg) treated females; and lane 15, Kaleidoscope Prestained Standards: myosin (202 kDa), β-galactosidase (133 kDa), bovine serum albumin (71 kDa), carbonic anhydrase (41.8 kDa), and soybean trypsin inhibitor (30.6 kDa), respectively.

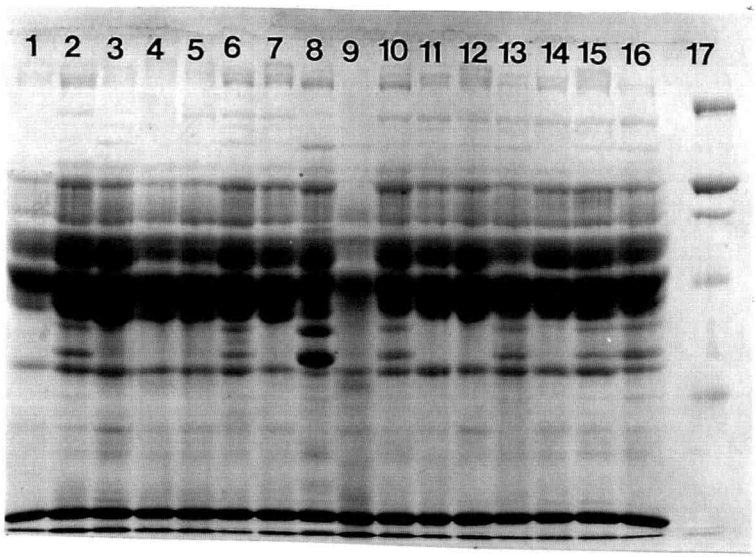


Figure 13. SDS-PAGE of plasma (40 μg per lane) from trout treated with one chemical. The gel was performed according to Laemmli (1970) with separating and stacking gel contained 7.5 and 4% acrylamide, respectively. After running at 150 volt until the stacking dye reached to the bottom of the gel, the gel was fixed, stained in 0.15% Coomassie brilliant blue-30% methanol-10% acetic acid and subsequently destained in 40% methanol-10% acetic acid. Lanes 1 to 4, biochanin A (25 mg/kg) treated males; lanes 5 to 8, biochanin A (25 mg/kg) treated females; lanes 9 to 12, biochanin A (50 mg/kg) treated males; lanes 13 to 16, biochanin A (50 mg/kg) treated females; and lane 17, Kaleidoscope Prestained Standards: myosin (202 kDa), β-galactosidase (133 kDa), bovine serum albumin (71 kDa), carbonic anhydrase (41.8 kDa), and soybean trypsin inhibitor (30.6 kDa), respectively.

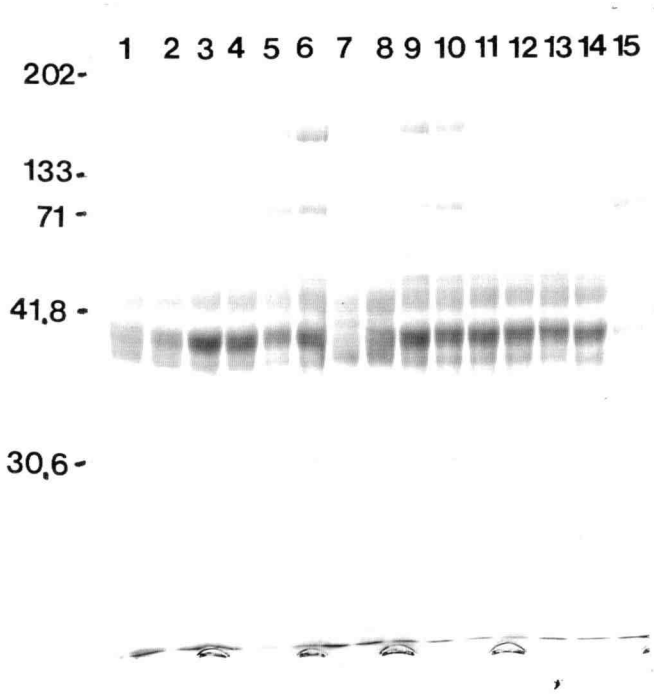


Figure 14. SDS-PAGE of plasma (40 μg per lane) from trout treated with four chemicals. The gel was performed according to Laemmli (1970) with separating and stacking gel contained 7.5 and 4% acrylamide, respectively. After running at 150 volt until the stacking dye reached to the bottom of the gel, the gel was fixed, stained in 0.15% Coomassie brilliant blue-30% methanol-10% acetic acid and subsequently destained in 40% methanol-10% acetic acid. Lanes 1 to 2, control male and female; lanes 3 to 4, methoxychlor (20 mg/kg) treated male and female; lanes 5 to 6, DES (15 mg/kg) treated male and female; lanes 7 to 8, 4-tert-octylphenol (25 mg/kg) treated male and female; lanes 9 to 10, 4-tert-octylphenol (50 mg/kg) treated male and female, lanes 11 to 12, biochanin A (25 mg/kg) treated male and female, lanes 13 to 14, biochanin A (50 mg/kg) treated male and female; and lane 15, purified vitellogenin 10 μg, respectively.

Table 1. Effects of chemical treatments on hepatic cytochrome P450 levels.

Treatment	Total P450 (pmoles/mg protein)	CYP2M1 (pmoles/mg protein)	CYP2K1 (pmoles/mg protein)	LMC5 (pmoles/mg protein)	CYP1A1 (pmoles/mg protein)
control	157 ± 16	41.3 ± 3.1 [b]	50.0 ± 4.9 [b]	134 ± 15	ND
methoxychlor 20 mg/kg	117 ± 24	34.9 ± 2.4 [a]	32.3 ± 2.0 [a]	55.6 ± 4.3 [a]	ND
DES 15 mg/kg	134 ± 9	33.2 ± 1.7 [a]	40.9 ± 2.6	83.9 ± 7.7 [a]	ND
4-tert-octylphenol 25 mg/kg	127 ± 13	36.4 ± 0.8	67.1 ± 5.0 [a]	96.2 ± 8.0 [a]	ND
4-tert-octylphenol 50 mg/kg	96 ± 11	31.5 ± 1.3 [a]	50.2 ± 2.5	88.7 ± 5.5 [a]	ND
biochanin A 25 mg/kg	120 ± 28	43.6 ± 2.9 [b]	49.1 ± 4.1	123 ± 13 [b]	17.5 ± 1.4 [a]
biochanin A 50 mg/kg	164 ± 41	27.0 ± 1.9 [a]	37.3 ± 2.5 [a]	96.7 ± 7.2 [a]	17.8 ± 0.4 [a]

Values are mean ± S.E. for 8 animals
ND: not detected
[a] = $P < 0.05$, compared with control.
[b] = $P < 0.05$, compared between males and females within group.

Figure 15. Effects of chemical treatments on hepatic cytochrome P450 levels in immature rainbow trout. The data shown are mean of eight animals per treatment. Visualization and immunoquantification were performed by scanning the autoradiography on a Molecular Dynamics phosphorimager scanning system with associated software. There were significantly decreases of CYP2M1 expression in the livers of trout treated with methoxychlor (20 mg/kg), DES (15 mg/kg), 4-tert-octylphenol (50 mg/kg) and biochanin A (50 mg/kg), compared to control. CYP2K1 was decreased significantly in methoxychlor (20 mg/kg), 4-tert-octylphenol (50 mg/kg)and biochanin A (50 mg/kg). In methoxychlor (20 mg/kg), DES (15 mg/kg), 4-tert-octylphenol (25 and 50 mg/kg) and biochanin A (50 mg/kg). P450 LMC5 was decreased significantly. And only biochanin A (25 and 50 mg/kg) treatment induced CYP1A1.

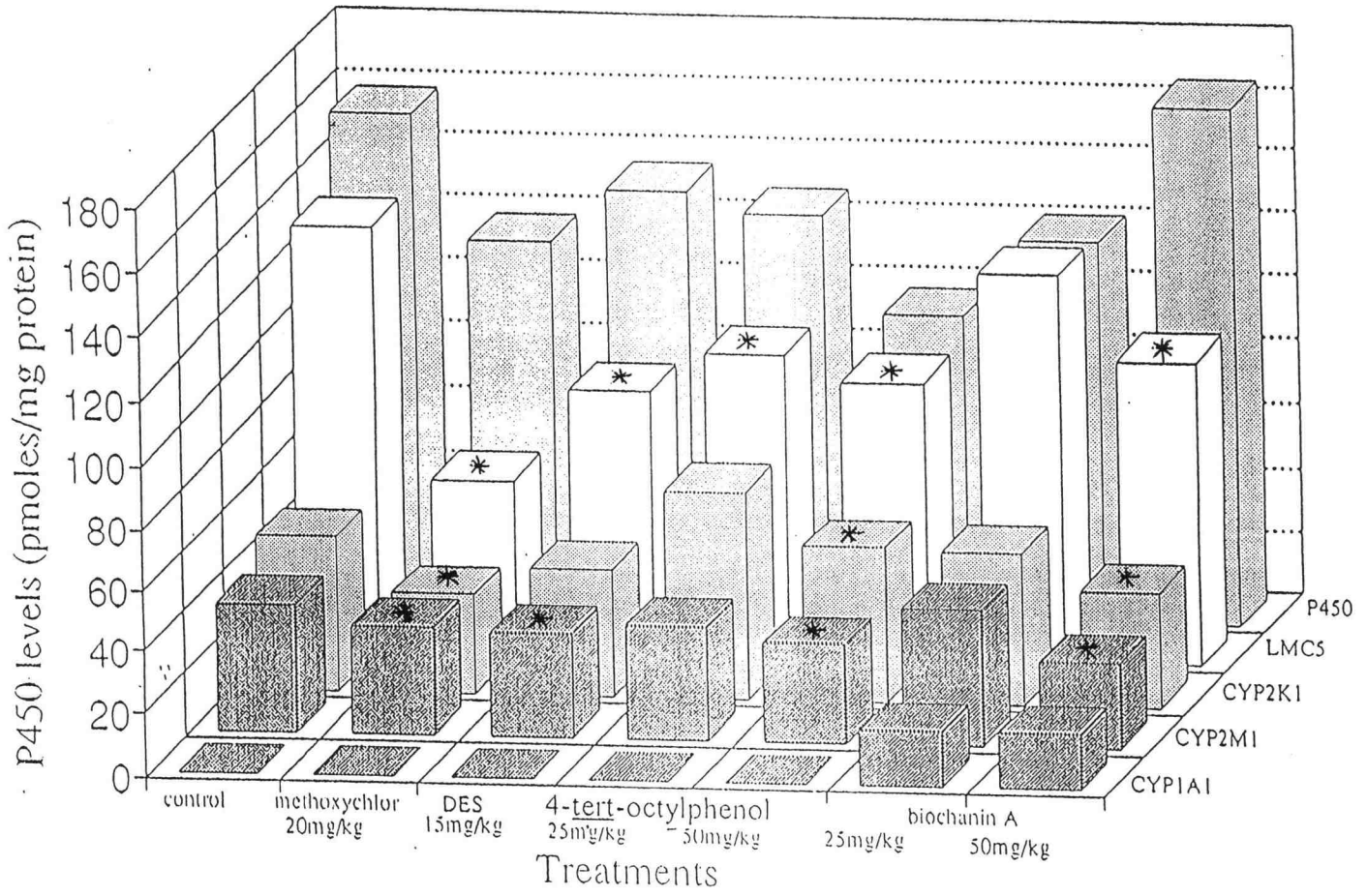


Figure 15

* = P < 0.05, compared with control

Figure 16. Western blot analysis of microsomal protein isolated from the livers of control trout and trout pretreated with four chemicals, using rabbit anti-trout CYP2M1 antibody as a probe after stripping the membrane, previously probed with rabbit anti-trout CYP2K1. Visualization and immunoquantification were performed by scanning the autoradiography on a Molecular Dynamics phosphorimager scanning system associated software. (A). Control trout: lanes 1 to 4, male; lanes 5 to 8, female. DES (15 mg/kg) treated-trout: lanes 9 to 12, males; lanes 13 to 16, female; lane 17, purified CYP2M1 (1 picomole); and lane 18, purified CYP2K1 (1 picomole). (B). Methoxychlor (20 mg/kg) treated -trout: lanes 1 to 4, male; lane 5 to 8, female; lane 9 purified purified CYP2M1 (1 picomole); and lane 18, purified CYP2K1 (1 picomole). (C). 4-tert-octylphenol (25 mg/kg) treated-trout: lanes 1 to 3, male; and lanes 4 to 6, female. 4-tert-octylphenol (50 mg/kg) treated-trout: lanes 7 to 10, male; lanes 11 to 14, female; lane 15, purified CYP2M1 (1 picomole); and lane 16, purified CYP2K1 (1 picomole). (D). Biochanin A (25 mg/kg) treated-trout: lanes 1 to 4, male; lanes 5 to 8, female. Biochanin A (50 mg/kg) treated-trout: lanes 9 to 12, male; lanes 13 to 16, female; lane 17, purified CYP2M1 (1 picomole); and lane 18, purified CYP2K1 (1 picomole). Each lane was loaded with 15 μg protein. The faint upper band was CYP2K1, while the stronger lower one was CYP2M1.

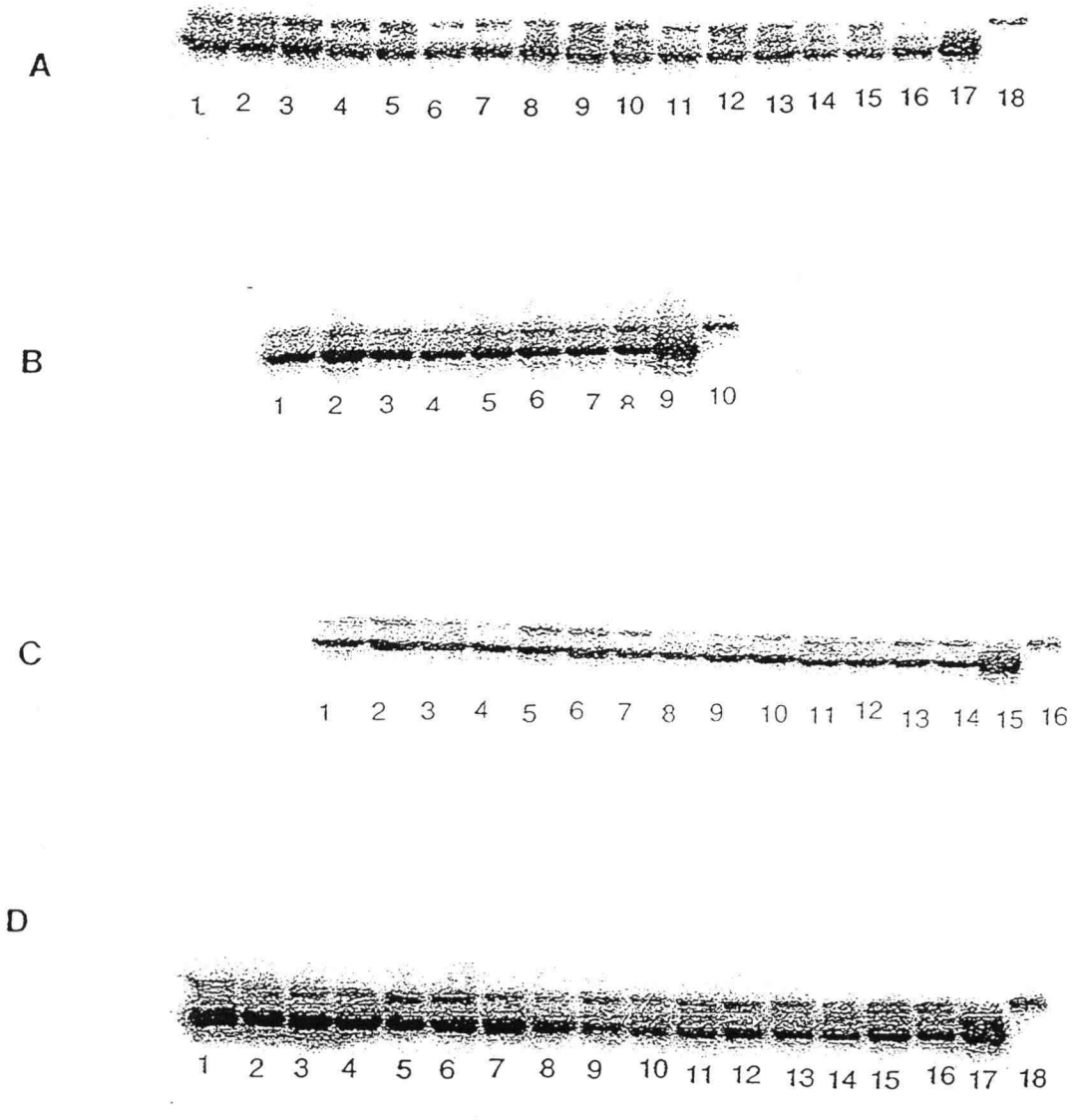

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
B
1 2 3 4 5 6 7 8 9 10
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
D
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 16

Figure 17. Western blot analysis of microsomal protein isolated from the livers of control trout and trout pretreated with four chemicals, using rabbit anti-trout CYP2K1 antibody as a probe. Visualization and immunoquantification were performed by scanning the autoradiography on a Molecular Dynamics phosphorimager scanning system associated software. (A). Control trout: lanes 1 to 4, male; lanes 5 to 8, female. DES (15 mg/kg) treated-trout: lanes 9 to 12, males; lanes 13 to 16, female; lane 17, purified CYP2M1 (1 picomole); and lane 18, purified CYP2K1 (1 picomole). (B). Methoxychlor (20 mg/kg) treated -trout: lanes 1 to 4, male; lane 5 to 8, female; lane 9 purified purified CYP2M1 (1 picomole); and lane 18, purified CYP2K1 (1 picomole). (C). 4-tert-octylphenol (25 mg/kg) treated-trout: lanes 1 to 3, male; and lanes 4 to 6, female. 4-tert-octylphenol (50 mg/kg) treated-trout: lanes 7 to 10, male; lanes 11 to 14, female; lane 15, purified CYP2M1 (1 picomole); and lane 16, purified CYP2K1 (1 picomole). (D). Biochanin A (25 mg/kg) treated-trout: lanes 1 to 4, male; lanes 5 to 8, female. Biochanin A (50 mg/kg) treated-trout: lanes 9 to 12, male; lanes 13 to 16, female; lane 17, purified CYP2M1 (1 picomole); and lane 18, purified CYP2K1 (1 picomole). Each lane was loaded with 15 μg protein. The upper band was CYP2K1, the two middle bands were possible unknown P450 isozymes, while the lower one was CYP2M1.

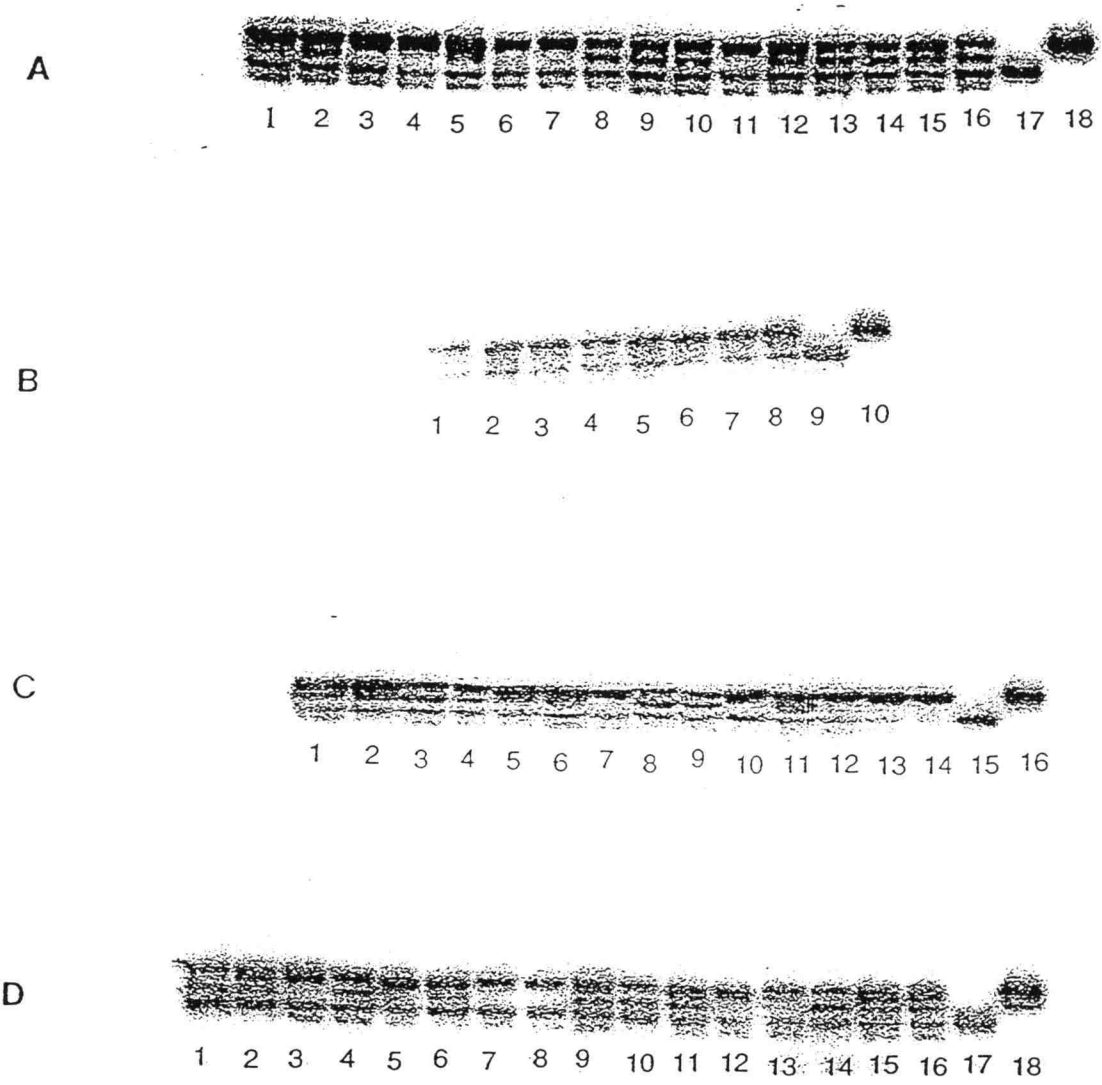

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
B
1 2 3 4 5 6 7 8 9 10
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
D
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 17

Figure 18. Western blot analysis of microsomal protein isolated from the livers of control trout and trout pretreated with four chemicals, using rabbit anti-trout P450 LMC5 antibody as a probe after stripping the membrane, previously probed with rabbit anti-trout CYP2M1 and CYP2K, respectively. Visualization and immunoquantification were performed by scanning the autoradiography on a Molecular Dynamics phosphorimager scanning system associated software. (A). Control trout: lanes 1 to 4, male; lanes 5 to 8, female. DES (15 mg/kg) treated-trout: lanes 9 to 12, males; lanes 13 to 16, female; lane 17, purified CYP2M1 (1 picomole); lane 18, purified CYP2K1 (1 picomole); and lane 19, purified P450 LMC5 (1 picomole). (B). Methoxychlor (20 mg/kg) treated -trout: lanes 1 to 4, male; lane 5 to 8, female; lane 9 purified purified CYP2M1 (1 picomole); lane 10, purified CYP2K1 (1 picomole); and lane 11, purified P450 LMC5 (1 picomole). (C). 4-tert-octylphenol (25 mg/kg) treated-trout: lanes 1 to 3, male; and lanes 4 to 6, female. 4-tert-octylphenol (50 mg/kg) treated-trout: lanes 7 to 10, male; lanes 11 to 14, female; lane 15, purified CYP2M1 (1 picomole); lane 16, purified CYP2K1 (1 picomole), and lane 17, purified P450 LMC5 (1 picomole). (D). Biochanin A (25 mg/kg) treated-trout: lanes 1 to 4, male; lanes 5 to 8, female. Biochanin A (50 mg/kg) treated-trout: lanes 9 to 12, male; lanes 13 to 16, female; lane 17, purified CYP2M1 (1 picomole); lane 18, purified CYP2K1 (1 picomole); and lane 19, purified P450 LMC5 (1 picomole). Each lane was loaded with 15 μg protein.

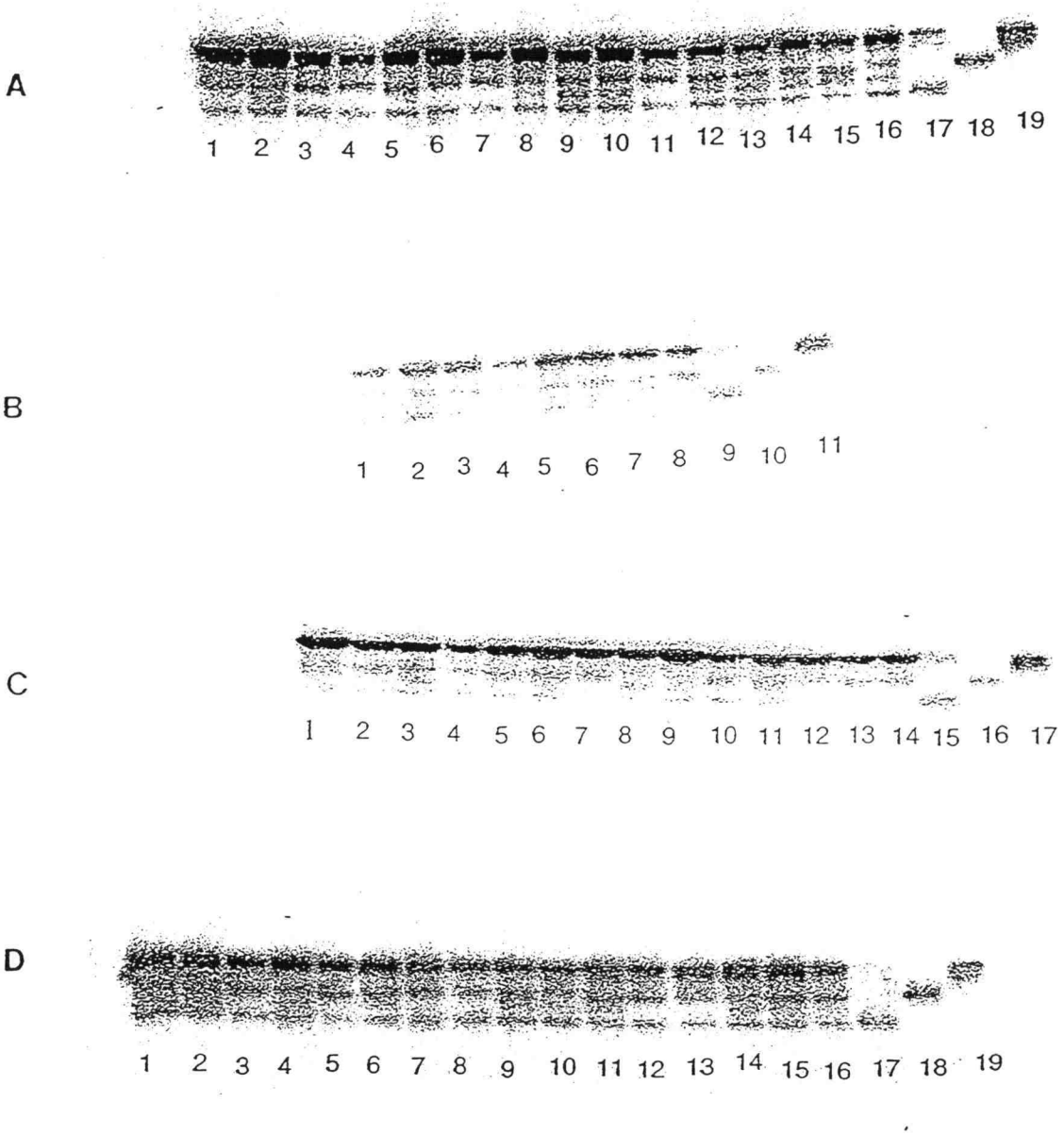

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
B
1 2 3 4 5 6 7 8 9 10 11
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
D
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Figure 18

Figure 19. Western blot analysis of microsomal protein isolated from the livers of control trout and trout pretreated with four chemicals, using rabbit anti-trout CYP1A1 antibody as a probe after stripping the membrane, previously probed with rabbit anti-trout CYP2M1, CYP2K1 and P450 LMC5, respectively. Visualization and immunoquantification were performed by scanning the autoradiography on a Molecular Dynamics phosphorimager scanning system associated software. (A). Control trout: lanes 1 to 4, male; lanes 5 to 8, female. DES (15 mg/kg) treated-trout: lanes 9 to 12, males; lanes 13 to 16, female; lane 17, purified CYP2M1 (1 picomole); lane 18, purified CYP2K1 (1 picomole); lane 19, purified P450 LMC5 (1 picomole); and lane 20, purified CYP1A1 (1 picomole). (B). Methoxychlor (20 mg/kg) treated - trout: lanes 1 to 4, male; lane 5 to 8, female; lane 9 purified purified CYP2M1 (1 picomole); lane 10, purified CYP2K1 (1 picomole); lane 11, purified P450 LMC5 (1 picomole); and lane 12, purified CYP1A1 (1 picomole). (C). 4-tert-octylphenol (25 mg/kg) treated-trout: lanes 1 to 3, male; and lanes 4 to 6, female. 4-tert-octylphenol (50 mg/kg) treated-trout: lanes 7 to 10, male; lanes 11 to 14, female; lane 15, purified CYP2M1 (1 picomole); lane 16, purified CYP2K1 (1 picomole), lane 17, purified P450 LMC5 (1 picomole); and lane 18, purified CYP1A1 (1 picomole). (D). Biochanin A (25 mg/kg) treated-trout: lanes 1 to 4, male; lanes 5 to 8, female. Biochanin A (50 mg/kg) treated-trout: lanes 9 to 12, male; lanes 13 to 16, female; lane 17, purified CYP2M1 (1 picomole); lane 18, purified CYP2K1 (1 picomole); lane 19, purified P450 LMC5 (1 picomole); and lane 20, CYP1A1 (1 picomole). Each lane was loaded with 15 μg protein.

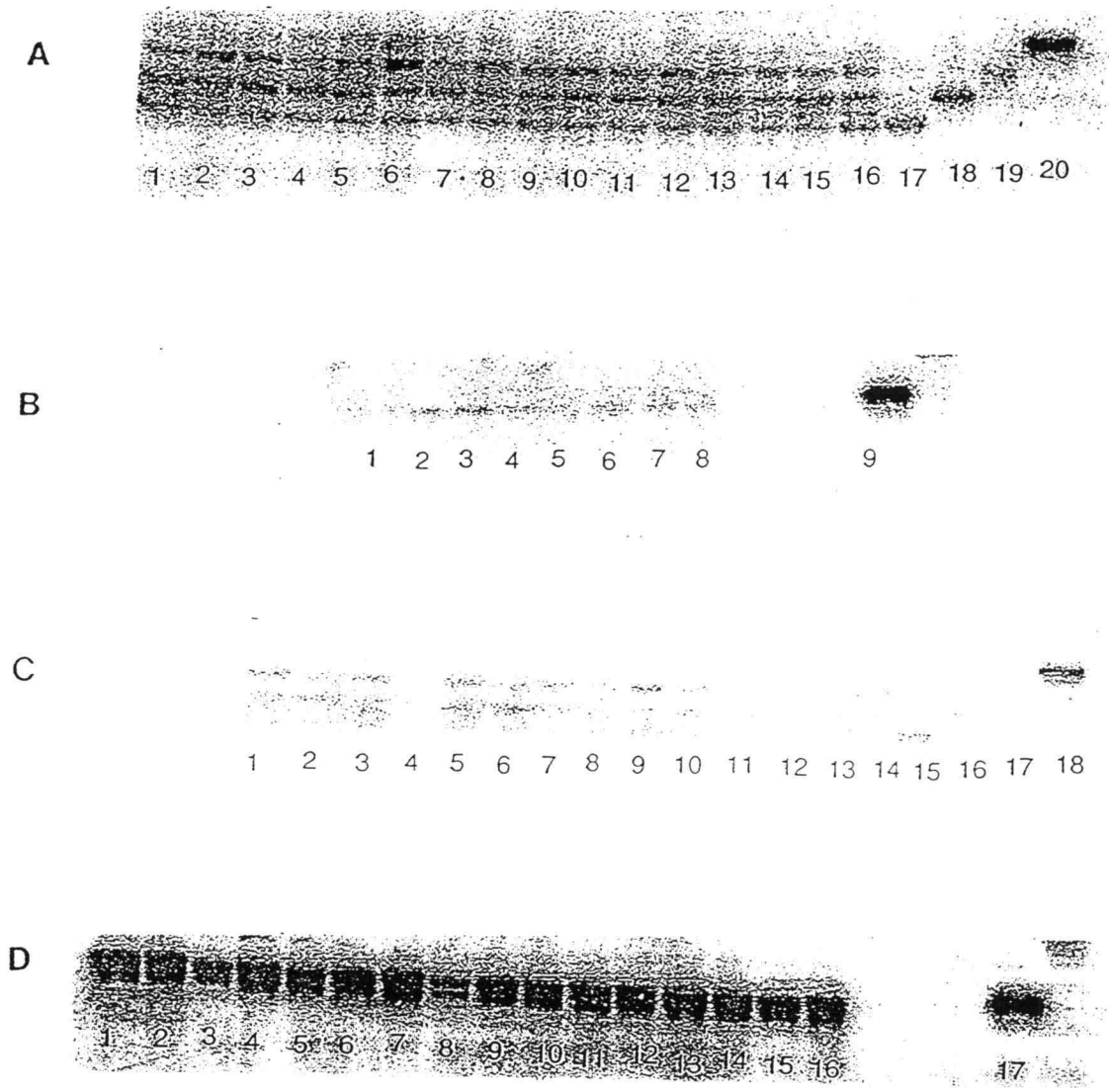

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
B
1 2 3 4 5 6 7 8 9
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
D
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Figure 19

Figure 20. Western blot analysis of microsomal protein isolated from the livers of control trout and trout pretreated with four chemicals, using rabbit anti-trout CYP2M1, CYP2K1, P450 LMC5, CYP1A1 antibody and anti-chum salmon vitellogenin IgG as a probe and shown in (A), (B), (C), (D), and (E), respectively. Visualization and immunoquantification were performed by scanning the autoradiography on a Molecular Dynamics phosphorimager scanning system with associated software. Each figure contained lanes 1 and 2, control male and female; lanes 3 and 4, methoxychlor (20 mg/kg) treated male and female; lanes 5 and 6, DES (15 mg/kg) treated male and female; lanes 7 and 8, 4-tert-octylphenol (25 mg/kg) treated male and female; lanes 9 and 10, 4-tert-ocytlphenol (50 mg/kg) treated male and female; lanes 11 and 12, biochanin A (25 mg/kg) treated male and female; lanes 13 and 14, biochanin A (50 mg/kg) treated male and female and lane 15 for each Figure, purified CYP2M1, CYP2K1, P450 LMC5, CYP1A1 and vitellogenin, respectively. Each lane was loaded with 15 μg protein.

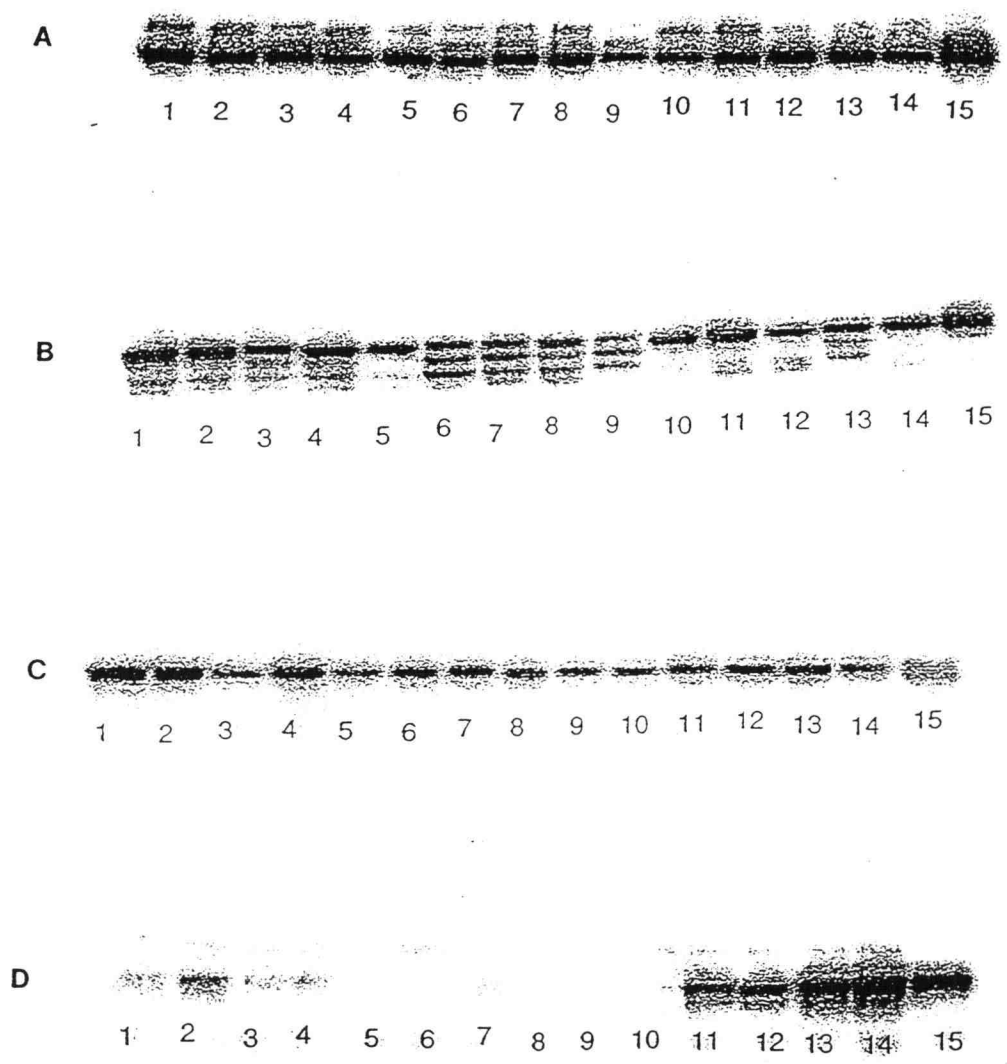

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
D
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 20

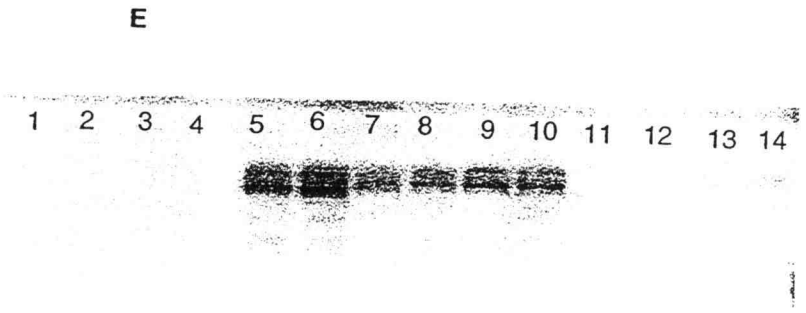


Figure 20 (continue)

Table 2. Effects of chemical treatments on lauric acid metabolism.

Treatment	% Total radioactivity				
	(ω-1)- hydroxy lauric acid	(ω-2)-hydroxy lauric acid	(ω-3)- hydroxy lauric acid	(ω-6)-hydroxy lauric acid	Total lauric acid metabolites
control	17.1 ± 1.8	2.22 ± 0.46	1.42 ± 1.09	3.56 ± 0.47	24.8 ± 2.7
methoxychlor 20 mg/kg	11.2 ± 1.1 [a]	1.37 ± 0.24	0.89 ± 0.47	3.17 ± 0.41	16.1 ± 1.0 [a]
DES 15 mg/kg	4.78 ± 0.91 [a]	0.98 ± 0.39 [a]	1.88 ± 0.42	1.43 ± 0.50 [a]	9.1 ± 1.0 [a]
4-tert-octylphenol 25 mg/kg	3.58 ± 1.11 [a]	0.91 ± 0.33	0.97 ± 0.59	1.12 ± 0.29 [a]	9.0 ± 1.1 [a]
4-tert-octylphenol 50 mg/kg	4.15 ± 1.03 [a]	1.46 [a,b]	0.46 ± 0.21 [a]	1.11 ± 1.17 [a]	6.9 ± 1.01 [a]
biochanin A 25 mg/kg	2.86 ± 1.43 [a]	ND	ND	0.92 ±0.26 [a]	4.5 ±1.5 [a]
biochanin A 50 mg/kg	1.91 ± 0.47 [a]	0.25 ± 0.08 [a]	1.01 [b]	0.88 ± 0.32 [a]	3.5 ± 0.3 [a]

Values are mean ± S.E. for 4 animals.
[a] = $P < 0.05$, compared with control.
[b] = only one sample showed this metabolite

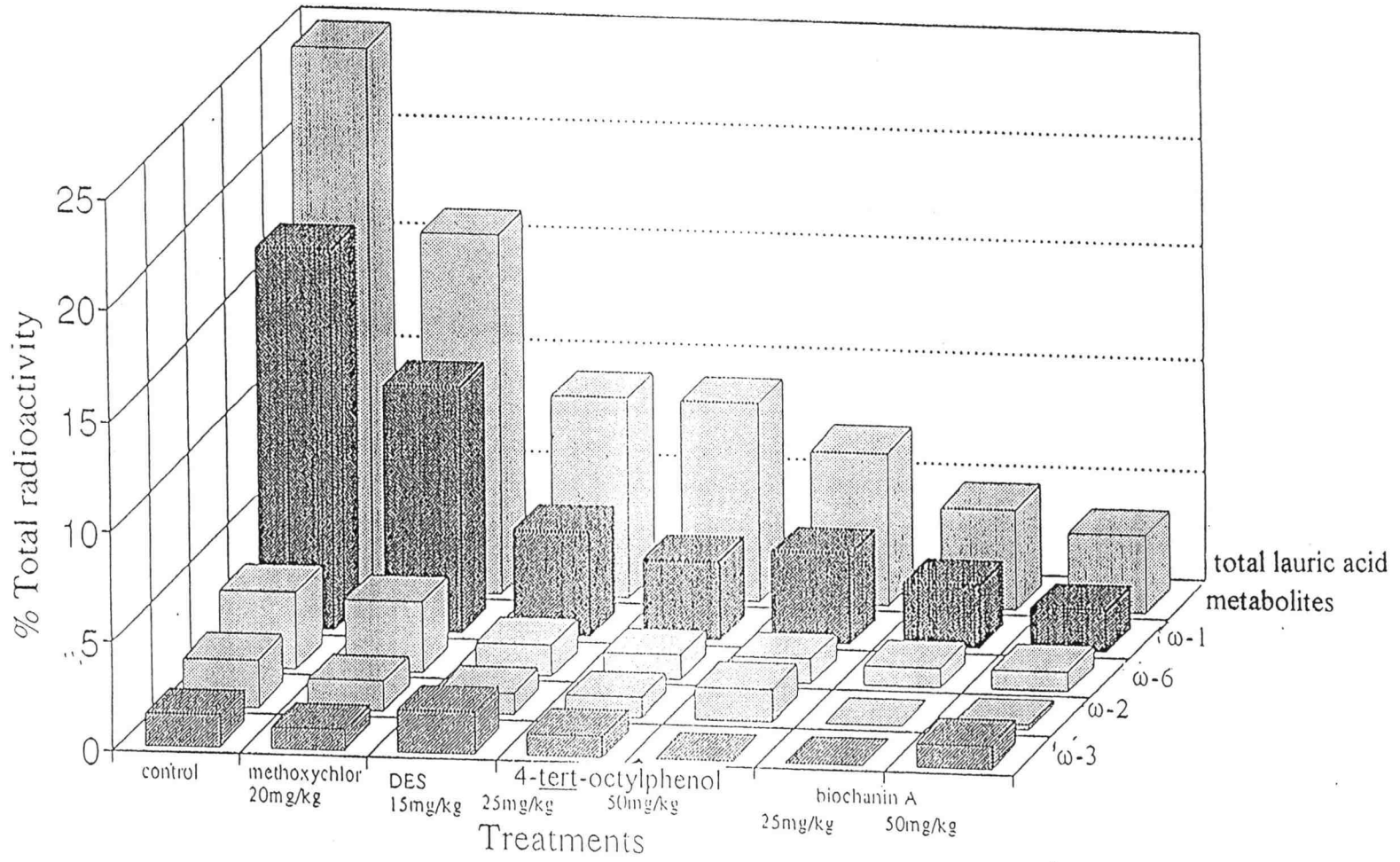


Figure 21. Effects of chemical pretreatment of trout on hepatic microsomal lauric acid metabolism.

Figure 22. HPLC profile of [1-^{14}C] lauric acid metabolites formed by liver microsomes from each treatment (4 animals per treatment) containing 500 μg protein, were incubated with 50 mM Tris-acetate buffer (pH 7.4), 200 μM [1-^{14}C]-lauric acid and 1 mM NADPH at 30° c for 1 hour. After stopping the reaction and extraction with diethyether, the dried extract was dissolved in CH_3CN and water each containing 0.2% acetic acid as the mobile phase with flow rate of 1.5 min/ml and detection by a Packard Flo-One-A-100 radioactivity flow monitor. The mobile phase concentration was 25% CH_3CN for 35 min followed by 2 min linear gradient to 80% CH_3CN to elute the parent compound. At 4 min, the CH_3CN was returned to 25% over 2 min. The column was allowed to equilibrate 23 min before the next injection. Because no inter-individual and male and female differences were detected in lauric acid metabolism, only one HPLC profile is shown from each treatment. (A) Control; (B). Methoxychlor (20 mg/kg); (C). DES (15 mg/kg); (D). 4-tert-octylphenol (25 mg/kg); (E). 4-tert-octylphenol (50 mg/kg); (F). Biochanin A (25 mg/kg); (G); Biochanin A (50 mg/kg).

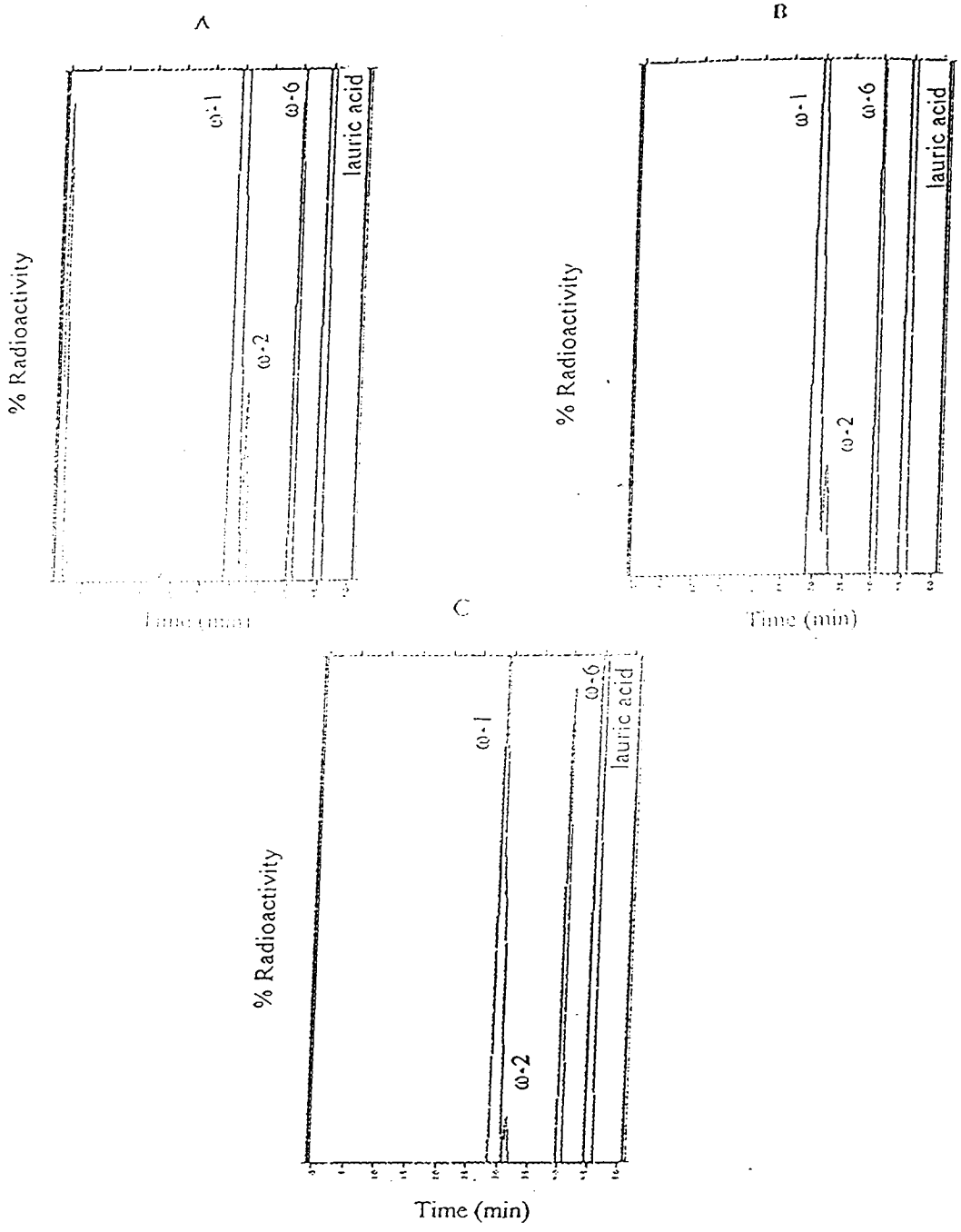

A
% Radioactivity
ω-1
ω-2
ω-6
lauric acid
Time (min)
B
% Radioactivity
ω-1
ω-2
ω-6
lauric acid
Time (min)
C
% Radioactivity
ω-1
ω-2
ω-6
lauric acid
Time (min)

Figure 22

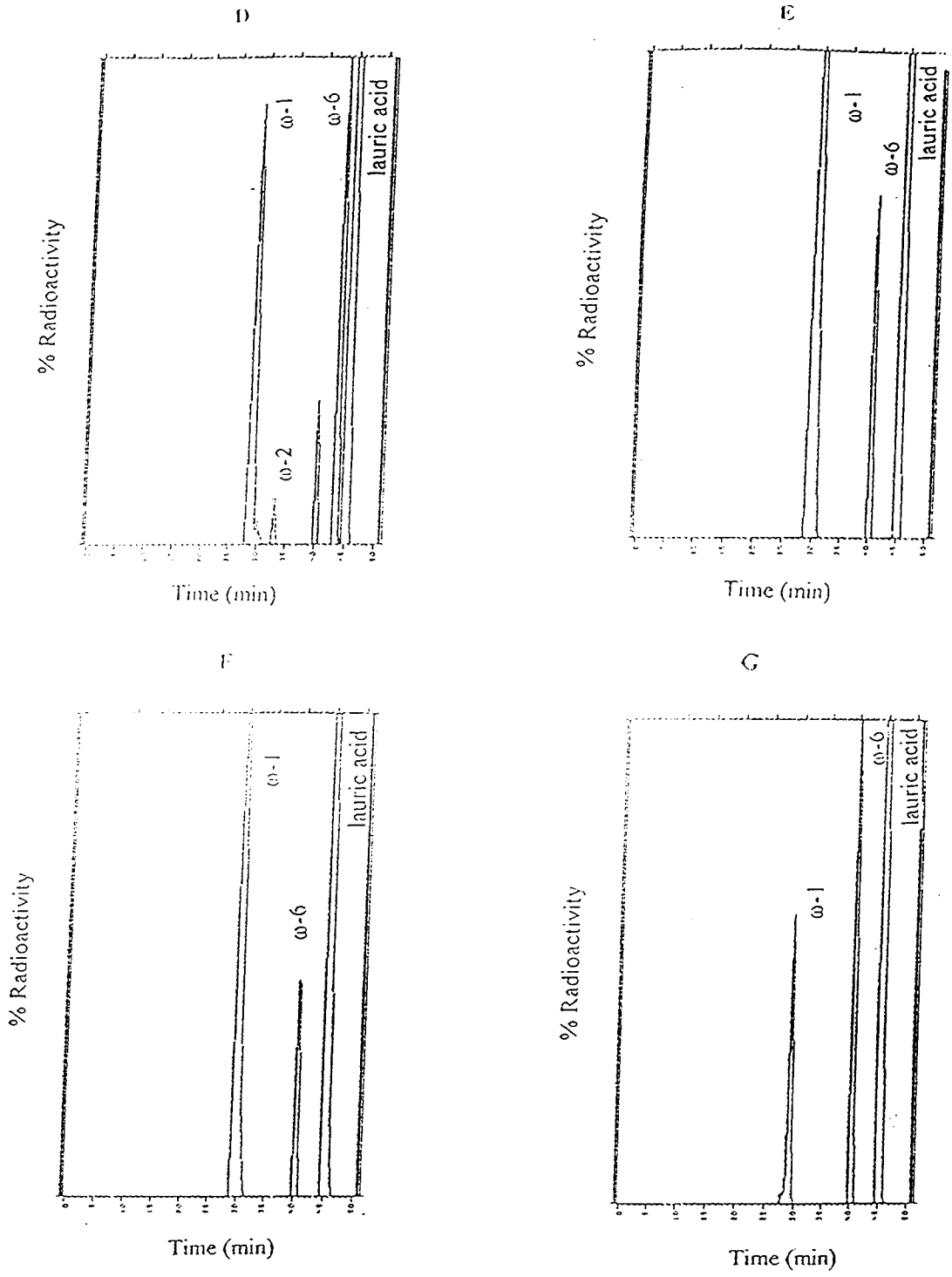


Figure 22 (continued)

Table 3. Effects of chemical treatments on plasma protein, vitellogenin levels and hepatic EROD activity.

Treatment	Plasma protein (mg/ml)	Plasma vitellogenin (µg/ml)	Hepatic EROD activity (pmoles/min/mg protein)
control	36.30 ± 2.37	2.96 ± 0.70	6.93 ± 2.87
methoxychlor 20 mg/kg	36.69 ± 1.06	134 ± 81 [a]	5.26 ± 0.72
DES 15 mg/kg	43.90 ± 3.00	28,076 ± 1,561 [a]	3.30 ± 0.56
4-tert-octylphenol 25 mg/kg	38.08 ± 1.79	3,887 ± 861 [a]	4.62 ± 1.34
4-tert-octylphenol 50 mg/kg	37.38 ± 1.55	23,121 ± 3,108 [a]	6.24 ± 0.76
biochanin A 25 mg/kg	39.50 ± 1.69	596 ± 153 [a]	444 ± 43 [a]
biochanin A 50 mg/kg	34.18 ± 1.26	1,401 ± 218 [a]	550 ± 31 [a,b]

Values are mean ± S.E. for 8 animals.
[a] = $P < 0.05$, compared with control
[b] = $P < 0.05$, compared between males and females within group

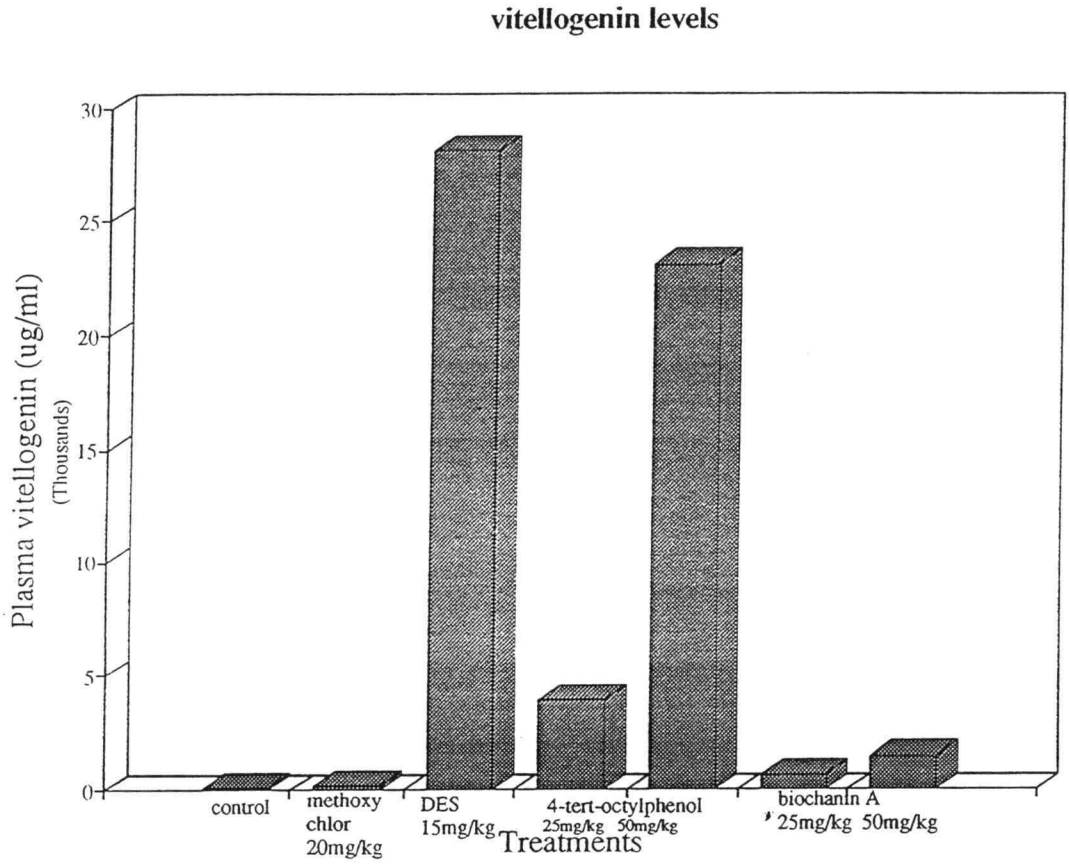


Figure 23. Effect of chemical treatments on plasma vitellogenin level. The plasma vitellogenin levels of trout pretreated with four chemicals were significantly higher than control, measured by ELISA.

Figure 24. Western blot analysis of plasma from all control trout and all trout pretreated with four chemicals, using rabbit anti-chum salmon vitellogenin IgG as a probe. Visualization and immunoquantification were performed by scanning the autoradiography on a Molecular Dynamics phosphorimager scanning system with associated software. (A). Lane 1 to 4, male; lane 5 to 8, female of control; and lane 9, purified vitellogenin (10 μg). (B). Lane 1 to 4, male; lane 5 to 8, female of methoxychlor (20 mg/kg); and lane 9, purified vitellogenin (10 μg). (C). Lane 1 to 4, male; lane 5 to 8, female of DES (15 mg/kg); and lane 9, purified vitellogenin (10 μg). (D). Lane 1 to 3, male; lane 4 to 6, female of 4-tert-octylphenol (25 mg/kg); and lane 9, purified vitellogenin (10 μg). (E). Lane 1 to 4, male; lane 5 to 8, female of 4-tert-octylphenol (50 mg/kg); and lane 9, purified vitellogenin (10 μg). (F). Lane 1 to 4, male; lane 5 to 8, female of biochanin A (25 mg/kg); and lane 9, purified vitellogenin (10 μg). (G). Lane 1 to 4, male; lane 5 to 8, female of biochanin A (50 mg/kg); and lane 9, purified vitellogenin (10 μg).

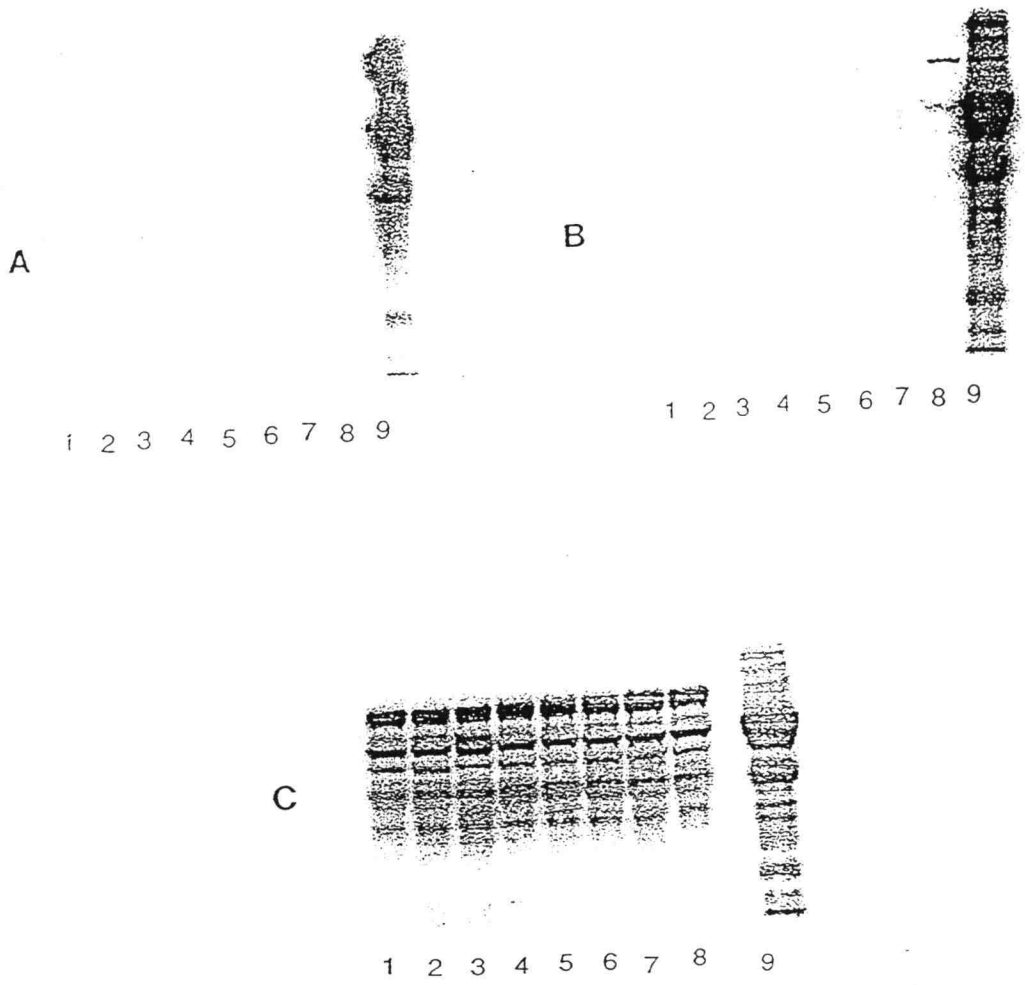


Figure 24

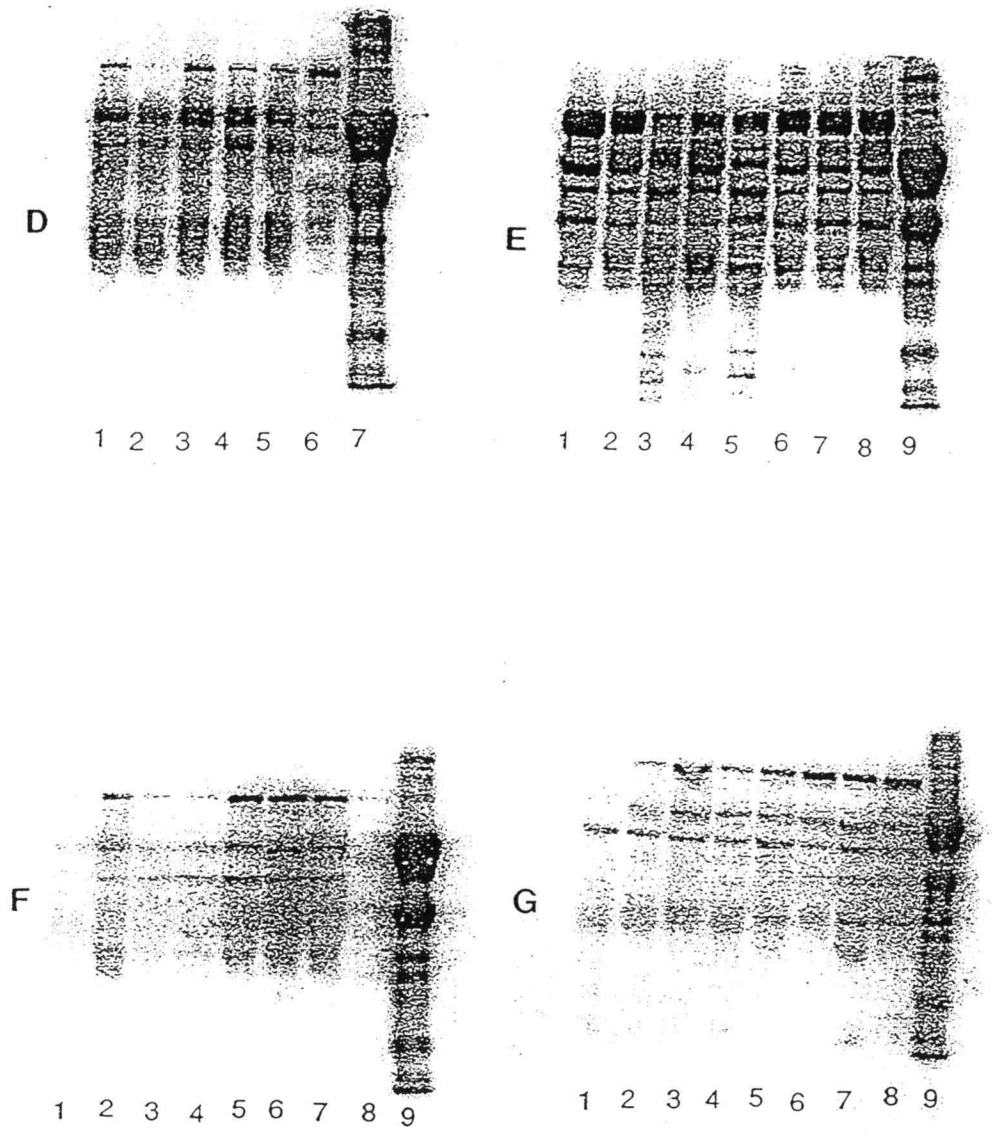


Figure 24 (continued)

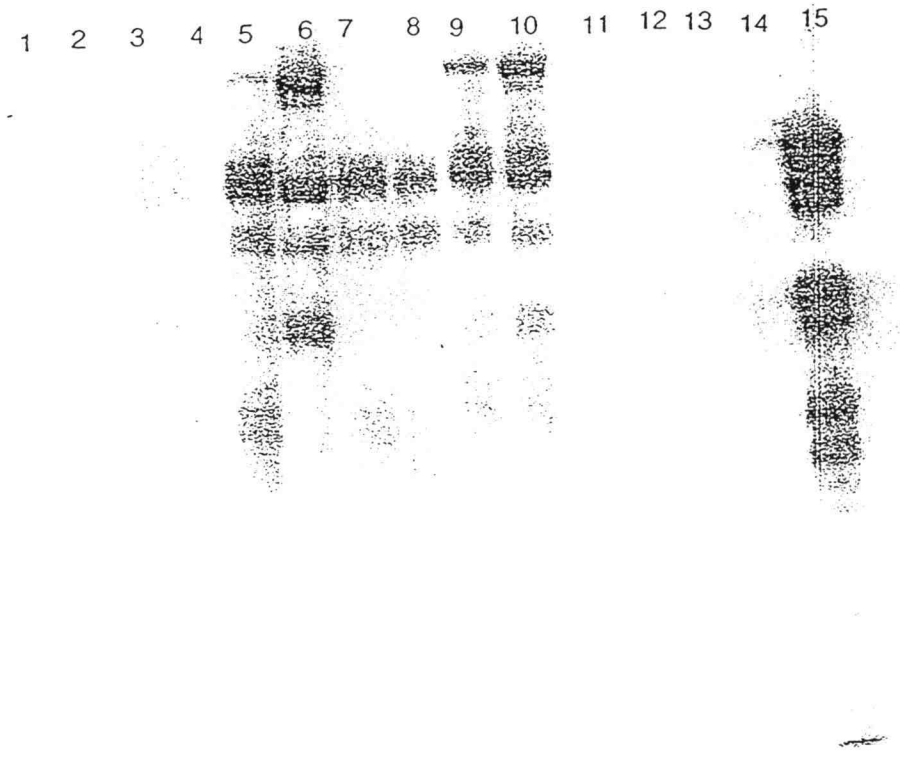


Figure 25. Western blot analysis of plasma from control trout and trout pretreated with four chemicals, using rabbit anti-chum salmon vitellogenin IgG as a probe. Visualization was performed by scanning the autoradiography on a Molecular Dynamics phosphorimager scanning system with associated software. Lanes 1 to 2, control male and female; lanes 3 to 4, methoxychlor (20 mg/kg) treated male and female; lanes 5 to 6, DES (15 mg/kg) treated male and female; lanes 7 to 8, 4-tert-octylphenol (25 mg/kg) treated male and female; lanes 9 to 10, 4-tert-octylphenol (50 mg/kg) treated male and female; lanes 11 to 12, biochanin A (25 mg/kg) treated male and female; lanes 13 to 14, biochanin A (50 mg/kg) treated male and female; and lane 15, purified vitellogenin 10 μg, respectively. Each lane was loaded with 20 μg protein.

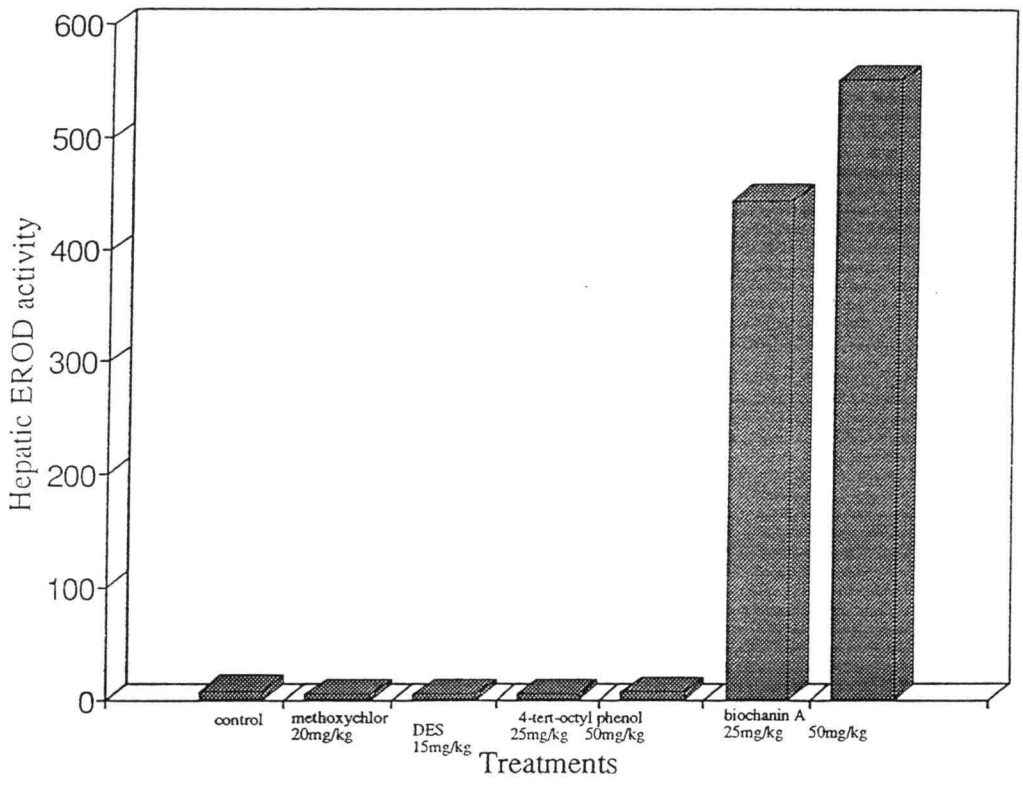


Figure 26. Effect of chemical treatments on hepatic EROD activity (pmoles/min/mg protein). The EROD activity in two doses of biochanin A (25 and 50 mg/kg) were significantly higher than control and other treatments.

DISCUSSION

We first tried to precipitate the vitellogenin from plasma treated with 17β-estradiol by adding 20 ml of 20 mM EDTA to 5 ml of plasma, adjusting to pH 7.7 with NaOH, mixing, adding 1.6 ml of 0.5 M $MgCl_2$ and gently inverting the flask (Wiley *et al.*, 1979). However, the plasma would not be precipitated by this treatment apparently because teleost vitellogenin is less phosphorylated than other vertebrates vitellogenin. Precipitation of highly phosphorylated vitellogein with EDTA:Mg^{2+} may occur only when there is high concentrations of vitellogenin (Silversand *et al.*, 1993) or the total plasma protein is above 70-80 mg/ml (Norberg and Haux, 1985). However, our pooled plasma protein content from 17β-estradiol treated trout was 40 mg/ml and was thus not precipitated.

Our SDS-PAGE pattern for purified vitellogenin (Figure 14) is not similar to that of Silversand *et al.* (1993) which showed one major protein with a relative mobility corresponding to a molecular mass below 200 kDa. Under reducing condition, salmonid vitellogenin was resolved into a 165 kDa band and several minor 70-150 kDa bands on SDS-PAGE (Hara *et al.*, 1993). The SDS-PAGE pattern of our purified vitellogenin, however, showed several bands with a relative mobility corresponding to a molecular mass about 71, below 71 and below 41.8 kDa (Figure 14). These protein bands are subunit of vitellogenin because they were recognized by anti-chum salmon vitellogenin IgG on a Western blot (Figure 25). The anti-chum salmon vitellogenin IgG also recognized an 161 kDa in plasma from protein in DES and 4-tert-octylphenol-treated trout after Western blotting (Figure 25), a size similar to that seen with salmon vitellogenin (Hare *et al.*, 1993). This band was not detected in control or the other treatment groups. The 161 kDa protein band was not present in our purified vitellogenin (Figure 14), presumably, because of proteolysis of the main vitellogenin protein during purification. Proteolytic cleavage of vitellogenin, however, does not destroy the antigenic determinants of vitellogenin (Covens *et al.*, 1987).

In the microsomal protein patterns after separation by SDS-PAGE (Figure 9), there are six proteins induced in liver microsome of trout by DES and 4-tert-octylphenol with a molecular mobility corresponding to a molecule mass about 55, 71, 150, 164, 172 and 190 kDa, respectively. None of the induced protein has molecular weights that corresponded to those of cytochrome P450's (MW 50-60 kDa). The other proteins induced by DES and 4-tert-octylphenol could be other subunits of vitellogenin This possibility could be confirmed by immunoblotting of the liver microsomes with anti-chum salmon vitellogenin IgG (Figure 20,E). Further work is needed to establish the identity of the microsomal protein induced by DES and 4-tert-octylphenol.

The exposure doses selected for the four test chemicals was based on the following studies. Cummings (1993) has found that methoxychlor 75-100 mg/kg/day (by gavage) can replace estrogen in ovarectiomized rat. Thus, we initially injected rainbow trout with methoxychlor at doses of 60 and 200 mg/kg on days 1,4,7,10,13 and 16. However, more than 50% and 100% of the animals died before sacrificed on day 18 at doses 60 and 200 mg/kg, respectively. Methoxychlor may produce several hepatic, kidney and heart changes such as necrosis and fatty acid degeneration similar to giving single oral dose (2 to 8 g/kg) to rat (Lillie *et al.*, 1947). Therefore, we decreased the methoxychlor dose to 20 mg/kg and injected to fish on day 1, 4, 7 and sacrificed on day 10.

The dose of DES used in the present study was the same as that used by Zangar *et al.* (1992). He demonstrated that when neonatal male rats were injected with DES (15 mg/kg) on days 1, 3 and 5 of age and then exposed to aflatoxin B1 at five months age long-term increases in hepatic glutathione S-transferase levels and decreases in DNA adduction levels resulted as compared to rats exposed to aflatoxin B1 alone.

The estrogenic activity of 4-tert-octylphenol and other alkylphenols is associated with the position of alkyl side chain on the benzene ring whereby no estrogenic activity is seen in *ortho* or *meta* alkyl analogs (Jobling and Sumpter, 1993). Soto *et al.* (1992) showed that the alkylphenols were 3-times more potent than the estrogenic pesticides o'p'-DDT and kepone. In addition, we observed that the plasma vitellogenin levels of trout pretreated with 4-tert-octylphenol (25 and 50 mg/kg) were higher than methoxychlor (20 mg/kg).

The exposure doses selected for biochanin A was based on the study of Pelissero *et al.* (1991). Injection (ip) of Siberian sturgeon four times on alternate day with 50 mg/kg of biochanin A induced plasma vitellogenin to level as high as 98 μg/ml.

In order to determine the relative potency of the four test chemicals to induce vitellogenin, the plasma from control and treated fish was examined for total vitellogenin content by ELISA. The use of ELISA in quantifying the vitellogenin content of plasma is valid because the anti-chum salmon vitellogenin IgG can recognize all the subunits of vitellogenin (70-165 kDa) on a Western blot and does not recognized any protein in plasma from control trout (Figure 24). Based on ELISA, the plasma vitellogenin of the following groups was measured as following ranging from high to low: DES (15 mg/kg) 28.1 mg/ml, 4-tert-octylphenol (50 mg/kg) 23.1 mg/ml, 4-tert-octylphenol (25 mg/kg) 3.89 mg/ml, biochanin A (50 mg/kg) 1.4 mg/ml, biochanin A (25 mg/kg) 0.6 mg/ml and methoxychlor (20 mg/kg) 0.13 mg/ml, and control 0.003 mg/ml, respectively (Figure 23 and Table 3). These results showed that DES (15 mg/ml) and 4-tert-octylphenol (50 mg/ml) used in this study were most estrogenic whereas methoxychlor (20 mg/kg) and biochanin A (25 and 50 mg/kg) were mildly estrogenic in trout.

In addition to species, strain, age and diet, environmental changes can affect the reproductive system through a direct action on sex steroids by the stimulation of hormone release from the hypothalamus, pituitary and gonad (Whitehead *et al.*, 1978). In mammal the expression of P450 in liver is based on growth hormone pattern, the pattern of growth hormone release in fish is not known (Waxman *et al.*, 1991). Photoperiod is the dominant environmental factor that regulates the salmonid family reproductive cycles (de Vlaming, 1972). Scott and Sumpter (1983) and Forlin and Haux (1990) showed that in the plasma of female spring-spawning strains of rainbow trout, 17β-estradiol, testosterone and vitellogenin increased markedly before ovulation and decreased to basal level after ovulation. The plasma testosterone level in males of the same strain were high at the beginning of spermiation. Furthermore, total amounts of P450 and associated enzyme activities elicited difference in male and female related to 17β-estradiol and testosterone. Thus the contents and activities of P450 such as aryl hydrocarbon hydroxylase (AHH) and ethoxymorphine-N-demethylase (END) were increased in males and markedly decreased

in female. These studies suggested that changes in the level and type of sex steroids associated with the reproductive cycles can exhibit the sex related differences in P450 activities. Cytochrome P450 level and activities (i.e. lauric acid hydroxylase and aflatoxin B1-DNA binding) in kidney of mature rainbow trout were lower in sexually mature females than in males (Williams *et al.*, 1986). However, the cytochrome P450 responses to testosterone treatment data in fish were not consistent with either P450 suppression or induction (Buhler *et al.*, 1994, Vodicnik and Lech 1933, Pajor *et al.*, 1990). In contrast to the testosterone treatment data, Forlin and Hausson (1982) and Vodicnik and Lech (1983) found that in juvenile rainbow trout treated with 17β-estradiol, hepatic P450 activities such as benzo[a]pyrene hydroxylase and EROD activity were decreased significantly. The plasma levels of 17β-estradiol and the liver weight to body weight ratios were increased. It was concluded that some P450 isoforms were changed due to sex steroids. Recent study has shown that pretreatment with 17β-estradiol caused a significant reduction in the translational expression of hepatic P450 isoforms [CYP2M1, CYP2K1 and LMC5]. Also CYP2K1 mRNA was down-regulated by 17β-estradiol treatment, suggesting that transcriptional levels of other P450 isoforms may be similarly regulated (Buhler *et al.*, 1994).

Similar to the findings of Buhler *et al.* (1994), juvenile rainbow trout in the present study that were treated with four estrogenic chemicals resulted in a suppression in the expression of P450 isoforms [CYP2M1, CYP2K1 and LMC5]. However, there was no significant difference of total P450 levels observed in our study (Table 1). This may reflect the combination result of an induction of some unknown P450's (six bands in SDS-PAGE of hepatic microsome, Figure 9) together with the suppression of three P450 isoforms (CYP2M1, CYP2K1 and P450 LMC5) in different ratios, as a result of the chemical treatments. And the reduction of the three P450 isoforms in trout treated with four estrogenic chemicals suggested that the expression of CYP2K1 and other two isoform (CYP2M1 and P450 LMC5) may be regulated at transcriptional levels and mediated via the estrogen receptor, since all of the chemicals resulted in vitellogenesis. However, all four chemicals act as estrogens in suppression of the expression of hepatic cytochrome P450's (CYP2M1, CYP2K1 and P450 LMC5) without a parallel relationship

between estrogenic activity and cytochrome P450 isoform expression. For example, DES had higher estrogenic potency, as reflected by relative vitellogenin levels, but didn't suppress CYP2M1, CYP2K1 and P450 LMC5 to the greatest degree when compared to the other chemicals (Tables 1 and 3). This observation suggested that the xenoestrogen may not be the only regulatory factor involved in expression of hepatic cytochrome P450. Based on the prominent activities of CYP2M1 and CYP2K1 in hydroxylation of lauric acid at ω-6 and ω-1, respectively, the suppression of two isoform of cytochrome P450 by chemical exposure caused a significant reduction of lauric acid metabolism (Tables 1 and 2). Alteration of these two cytochrome P450 isoforms may alter the metabolism of endogenous fatty acids including arachidonic acid and steroid biosynthesis, resulting in biological changes. P450 LMC5 in trout also significantly decreased after xenoestrogen treatment (Tables 1 and 2). The major role of this isoform is the 6β-hydroxylation of testosterone and progesterone. The importance of progesterone in teleost reproduction is served as an intermediate in the biosynthesis of 17α-hydroxy-20 β-dihydroprogesterone (DHP), testosterone and 17β-estradiol. 17α-Hydroxy-20β-dihydroprogesterone is synthesized by the ovary or testes in response for gonadotrophin (GTH) during sexual maturation (Kime, 1987 and Ueda *et al.*, 1983). Scott *et al.* (1983) showed that GTH, DHP was elevated from 8 days before to 28 days after ovulation, 17β-estradiol reached basal level 4 days before ovulation and testosterone peaked 8 days before ovulation and then fall down. Consequently, alteration of this biosynthesis pathway may alter reproductive system in trout.

It has been shown that mature female trout have lower CYP1A1 expression, and AHH and EROD activities than do mature male trout. There is no sex difference in P450 expression in juvenile fish (Stegemann and Hahn, 1994). Pajor *et al.* (1990) and Gray *et al.* (1991) also showed the suppression of CYP1A1 by17β-estradiol. Elskus *et al.* (1992) also reported that mature female fish pretreated with polychlorinated biphenyl (PCB) decreased mRNA for CYP1A1, indicating that the response could be pretranslation effect. In our study, all four chemicals were estrogenic, resulting in plasma vitellogenin induction. Methoxychlor, DES and 4-tert-octylphenol, however, had no effect on CYP1A1 expression whereas biochanin A induced CYP1A1, as reflected by Western blotting and

EROD activity measurement. EROD activity was increased markedly in hepatic microsomes from trout treated with two doses of biochanin A, as compared to controls and with the other chemical-treatment groups (Figure 25 and Table 3). Biochanin A may thus be capable of interacting with the estrogen receptor to induce vitellogenin as well as interacting with the Ah receptor to elicit CYP1A1 induction.

In contrast to our study, Chae *et al.* (1991) and Cassady *et al.* (1988) reported that biochanin A can inhibit benzo[a]pyrene metabolism by rat hepatic microsomes after treatment with Aroclor 1254 and in hamster cell culture. Yanagihara *et al.* (1993) found that biochanin A at low dose (< 20 μg/ml) and high dose (> 40 μg/ml) were cytostatic and cytotoxic to human stomach cancer cell lines, respectively. Biochanin A also has an inhibitory effect on the initiation and promotion step of benzo[a]pyrene carcinogenesis in female Swiss-Webster mice (Lee *et al*, 1992). It is possible that in fish biochanin A acts similar to β-naphthoflavone (BNF), polyaromatic hydrocarbon (PAH) and polychlorinated biphenyls (PCB) to induce CYP1A1 via the Ah receptor.

Although methoxychlor induces P450's in mammals (Li *et al.*, 1995), we didn't find any induction of P450 isoforms in rainbow trout after exposure to methoxychlor, probably because of the absence of phenobarbital-type induction in fish (Stegeman, 1993). In contrast, three isoforms of P450 (CYP2M1, CYP2K1 and P450 LMC5) were repressed by methoxychlor exposure. There is evidence that metabolites of methoxychlor are estrogenic chemicals than the parent pesticide. Hence, metabolites of methoxychlor from rat microsomes, mono-OH and bis-OH methoxychlor, have been shown to have estrogenic activity. In addition, bis-OH methoxychlor is a pure estrogen agonist in the MCF-7 breast cancer cell line (Li *et al.*, 1995). It is possible that a fast elimination of methoxychlor, similar to that seen in the rat (Lamartiniere *et al.*, 1982), resulted in low level of plasma vitellogenin from methoxychlor-treated trout, compared to the other treated-group.

In 21-day old rainbow trout, initiated with subcarcinogenic dose (0.025 ppm, 30 min.) of aflatoxin B1 and subjected to dietary 17β-estradiol treatment demonstrated the promotion of hepatic tumor, increase DNA synthesis and mortality and growth reduction significantly suggesting the proliferative effect of 17β-estradiol (Nunez *et al.*, 1989). These findings were unlike those of Zangar *et al.* (1992) that injection of DES in neonatal

male rat and then observed upon exposure to aflatoxin B1, decreased DNA adduction levels. The effect of estrogen on aflatoxin B1 suggested the importance of exposure time. Aflatoxin B1, a potent hepatocarcinogen, and exogenous substrate for CYP2K1 (Williams and Buhler, 1983) , is identified as a human carcinogen (Bailey *et al.*, 1987)). Aflatoxin B1 requires metabolic activation to form the ultimate carcinogen, aflatoxin B1-8,9-epoxide, species before interacting with DNA, and is called an indirect acting carcinogen (Smart, 1994). It is possible that liver tumor caused by aflatoxin B1 may be promoted by xenoestrogens with promotion effect although there is decrease of CYP2K1, responding to aflatoxin B1 bioactivation. Preexposure of trout to a xenoestrogen may provide protection against liver tumor caused by aflatoxin B1 due to reduction of the expression of the bioactivating enzyme (CYP2K1). Bailey *et al.* (1987) also reported that rainbow trout treated with BNF or PCB before and during exposure to aflatoxin B1 reduced liver tumors by increasing conversion to aflatoxin M_1, a non-carcinogenic metabolite of aflatoxin B1 and decreasing DNA adduct formation. Because biochanin A acts like BNF and PCB to induce CYP1A1, preexposure to biochanin A may decrease DNA adduction caused by aflatoxin B1 more than by the other three chemicals. However, fish CYP1A1 is responsible for the activation of benzo[a]pyrene to (+)-anti-benzo[a]pyrene-7,8 -diol-9,10 epoxide that can bind covalently to DNA (Stegeman and Hahn, 1994). Therefore, fish treated with biochanin A may increase benzo[a]pyrene toxicity.

CONCLUSION

The juvenile rainbow trout was used as an in vivo model to examine the estrogenic effects of four xenoestrogenic chemicals from the following categories: pesticides (methoxychlor), pharmaceuticals (diethylstilbestrol), surfactants (4-tert-octylphenol) and phytoestrogens (biochanin A). Based on plasma vitellogenin, an indicator of estrogenic activity, the estrogenic potency of the chemicals ranged from high to low: DES (15 mg/kg), 4-tert-octylphenol (50 mg/kg), 4-tert-octylphenol (25 mg/kg), biochanin A (50 mg/kg), biochanin A (25 mg/kg) and methoxychlor (20 mg/kg), respectively. Similar to the 17β-estradiol treatment, the four chemicals suppressed the expression of P450 isoforms such as : CYP2M1, CYP2K1 and P450 LMC5 in trout liver microsomes. There was no parallel relationship between estrogenic activity and cytochrome P450 isoform expression. The result suggested that the xenoestrogen may not be the only regulatory factor involved in expression of hepatic cytochrome P450.

Moreover, the estrogenic chemical treatments decreased lauric acid metabolism especially at the (ω-6) and (ω-1) positions catalyzed by CYP2M1 and CYP2K1, respectively. Alteration of these P450 two isoforms may alter the metabolism of endogenous fatty acids including arachidonic acid and exogenous substrates such as aflatoxin B1, resulting in biological changes. For example, CYP2K1 is the activating enzyme for aflatoxin B1 in rainbow trout. Therefore, preexposure to xenoestrogens may decrease aflatoxin B1 toxicity in this species. P450 LMC5 was also significantly decreased after xenoestrogen treatment. The prominent activity of this isoform is 6β-hydroxylation of testosterone and progesterone in the liver. Hepatic metabolism of progesterone by P450 leads to enhanced elimination, resulting in decreased available of the steroid for the biosynthesis of 17α-hydroxy-20β-dihydroprogesterone (DHP), testosterone and 17β-estradiol that are involved in reproductive system. Alteration of the biosynthesis of these chemicals may affect on reproductive system in rainbow trout.

There were some proteins in liver microsome induced by DES (15 mg/kg) and 4-tert-octylphenol (50 mg/kg) as judged by staining intensity on SDS-PAGE. One of the protein bands could be a subunit of vitellogenin with a molecular mass about 164 kDa,

which was recognized by anti-salmon vitellogenin IgG on a Westren blot. This finding suggests that vitellogenin is translocated into the endoplasmic retuculum after synthesis or is trapped in the microsomes during the preparation of subcellular fractions of trout liver. The actual site of vitellogenin synthesis and translocation in rainbow trout liver need to be established in future studies.

Interestingly, one xenoestrogen in this study, biochanin A strongly induced CYP1A1 as judged by Western blot analysis and EROD activity. On the other hand, biochanin A suppressed the three P450 isoforms and induced plasma vitellogenin similar to 17β-estradiol and the other three chemicals. Biochanin A may be a potent inhibitor of aflatoxin B1-induced carcinogenesis by repressing the activating enzyme CYP2K1 and inducing the aflatoxin B1 detoxifying enzyme, CYP1A1. However, the mechanism involved in the suppression of the three P450 isoforms and induction of CYP1A1 by biochanin A are not known. Thus, the focus of future research will be to investigate not only how biochain A decreases CYP2M1, CYP2K1 and P450 LMC5, but also how biochanin A increases CYP1A1 and plasma vitellogenin in rainbow trout. The additional studies are also needed to examine the hypothesis that biochanin A is a potent inhibitor of aflatoxin B1-induced carcinogenesis in trout.

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